Biological Wastes As Plant Growth Substrate Amendments.

Evaluation Of :

i) **Their Efficacy As A Means Of Control Of Soil-borne Fungal Diseases**

ii) **Possible Consequential Risks To Human Health**

By

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Abstract

Globally, agriculture is being intensified with mechanisation and increased use of synthetic fertilisers and pesticides. There has been a scaling up of production to satisfy the demands of supermarket distribution. Problems associated with intensification of production, trade globalisation and a larger market demand for greater volumes of fresh produce, include consumers' concern about pesticide residues and leaching of nutrients and pesticides into the environment, as well as increases in the transmission of human food-poisoning pathogens on raw vegetables and in fruit juices.

The first part of this research was concerned with the evaluation of a biological control strategy for soilborne pathogens, these are difficult to eliminate and the chemicals of which the most effective fumigants e.g. methyl bromide, are being withdrawn from use. Chitin-containing crustaceans shellfish waste was investigated as a selective growth substrate amendment in the field, in glasshouse and in storage trials against *Sclerotinia* disease of *Helianthus tuberosus, Phytophthora fragariae* disease of *Fragaria vesca* and *Fusarium* disease of *Dianthus*. Results showed that addition of the shellfish waste stimulated substrate microbial populations and lytic activity and induced plant defense proteins, namely chitinases and cellulases. Protective effects were seen in all crop models but the results indicate that further trials are required to confirm long-term efficacy.

The second part of the research investigated the persistence of enteric bacteria in raw salad vegetables using model food poisoning isolates. In clinical investigations plants are sampled for bacterial contamination but no attempt is made to differentiate between epiphytes and endophytes. Results here indicate that the model isolates persist endophytically thereby escaping conventional chlorine washes and they may also induce host defenses, which results in their suppression and in negative results in conventional plate count screening. Finally a discussion of criteria that should be considered for a HACCP plan for safe raw salad vegetable production is presented.

Preface to thesis structure

This thesis is written in the form of journal publications. The instructions to authors of the relevant journals have been followed, as appropriate. The chapters are in the format of manuscripts for submission, submitted or published, the journal format is indicated in the preface to each chapter.

Section A consists of three chapters, the first of which is an introduction to the area of study and includes an outline of the aims and objectives. This is followed by two chapters, which review related literature. The first review has been published in *Radiation Research.*

Section B contains four chapters dealing with the investigations carried out on the use of crushed crustacean shells as a growth substrate amendment. Of the four chapters, three chapters report collaborative research, which is indicated in the chapter prefaces. One chapter was published in *Applied Soil Ecology.*

Two chapters make up Section C and these deal with the persistence of enteric bacteria in plants. The first chapter was written in conjunction with co-workers in St James Hospital/Trinity College, Dublin and has been published after peer review, in *Acta Horticulturae.*

The final section includes a general discussion , followed by a chapter with recommendations for HACCP criteria for production of raw or minimally processed plant produce.

Each chapter includes a bibliography citing the relevant literature

Dedication

In honor of my parents

and

In memory of my eldest brother Gerard
(July 6th 1962-August 6th 2000)
Beidh tú beo in ár gcroí go deo

Work like you don't need the money.

Love like you've never been hurt.

Dance like nobody's watching.

Sing like nobody's listening.

Live like it's Heaven on Earth.

Author Unknown

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- To Maurs for everything!
- To my siblings, Gerard, Paula, Pat and Tony, their partners and families.
- To my parents, for their love and support
- To Louis, my partner .....so many things .....so little space!

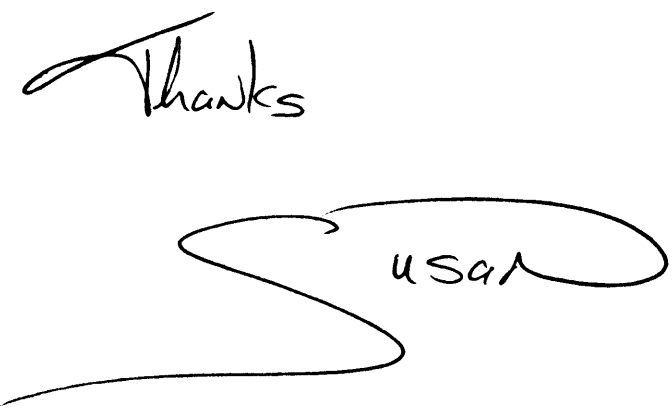


Table of Contents

Chapter One

Introduction and Objectives

Background to current concerns about pest and disease control

Over the last 150 years agriculture has been intensified greatly with mechanisation and the introduction of synthetic fertilisers and pesticides. Plant breeding and improved agronomic methods have allowed cultivation in formerly non-productive areas. In parallel with these developments there has a scaling up of production to satisfy the demands of supermarket distribution. Problems associated with these developments include consumer concern about pesticide residues (Weger *et al.*, 1993, Katan, 2000) and leaching of nutrients and pesticides into the surrounding environment (Kirchmann and Thorvaldsson, 2000). Many common soil pesticides such as methyl bromide are being phased out of use (Gamliel *et al.*, 2000). Pesticide resistance coupled with reports of mammalian toxicity has meant that the arsenal of methods for pest and disease suppression is diminishing. Build up and biomagnification of residues in birds and animals, extrapolated to humans, has caused concern both in the public domain and the scientific world. Reports of the degree of accumulation of pesticide residues through the food chain vary, depending on the pesticide and species chains being studied (Bard 1999, Borga *et al.*, 2001), however, a very general consensus would be that biomagnification can and does occur and a trend would seem to be that the tissues and organs most commonly affected are those involved in reproduction (Jones *et al.*, 1994, Varnagy 1996, Beard *et al.*, 1997, Albanis *et al.*, 1997). Advocates from the general public and 'green' movements are leading the demand for the reduction of pesticide usage and urging investigation into alternative methods of pest and disease control.

Currently, fungicides are still a vital force in the control of phytopathogens where they complement plant breeding for disease resistance (Gullino *et al.*, 2000). However, resistance to pesticides is causing problems for farmers. Benzimidazole resistance was first reported in *Rhynchosporium secalis* (causal agent of leaf spot on winter barley) in the 1980s and despite anti-resistance strategies e.g. use in mixtures with other fungicides, it was reported again in the 1990s (Taggart *et al.*, 1999). Another example of emerging resistance is that of *Botrytis* to benzimidazoles and phenylcarbamates at a low frequency, it was recorded in French vineyards. The strategy currently recommended is to spray infrequently, subject to forecasting, in an effort to limit resistance build up and to deploy fungicides in strategic combinations (Gullino *et al.*, 2000).

Emerging strategies for disease control

Given the above concerns regarding pesticides, there has been renewed interest in traditional methods and into research for alternative methods of disease control. Soil Solarisation has been used widely in Greece for a number of years (Tjamos *et al.*, 2000). It was found that solarisation of soil was effective in controlling *Fusarium* and *Clavibacter*. The widespread use of this method is limited, due to the requirement for cover of the land by polyethylene for up to 6 weeks and a dependence on a hot climate. Coupled to this there is the added expense of purchasing extra equipment for covering and uncovering the land with polyethylene plastic. However, recent research has reduced the time of land coverage down to 2 weeks if impermeable plastic sheeting is used. When an endophytic *Bacillus* biological control agent was also used, significantly better

disease control was achieved than with metham sodium (a chemical substitute for methyl bromide) and also increased yields were reported.

Methods being evaluated include physical methods such as steaming or microwaving of substrates. Though costly, these methods are being investigated due to the imminent withdrawal of methyl bromide (Katan, 2000).

Cultural methods applied for control include rotation of crops, altered cropping sequence, changing of irrigation patterns and water sources, altering the dates for planting and planting density. Soil flooding for a period of a few weeks can be effective in ridding areas of certain fungi, insects and nematodes (Katan, *loc. cit.*).

As well as research into synthetic antimicrobial chemicals, other strategies of chemical control such as elicitation of the systemic acquired resistance (SAR) in plants and development of natural antimicrobial compounds are being evaluated. Plant resistance mechanisms e.g. SAR can be specific for plant cultivars and pathogen strains. The gene-for-gene resistance response can lead to a cascade of reactions leading to localised host cell death - the Hypersensitive Response (HR). This cascade can be induced or elicited by secondary messengers eliminating the specificity of the recognition phenomenon necessary in nature for the activation of host defenses. SAR has been the subject of extensive research. This gives a broad range of protection against pests and diseases and provides an immune like state in the plant. Salicylic acid (SA) is produced in the plant and plays an important role as a secondary messenger in the SAR mechanism. Further classes of chemicals that induce salicylic acid have been studied. Among these is acibenzolar-S-methyl (commercialised as BION) which affords protection from bacterial, viral and fungal attack (Guillino *et al.*, 2000). Its mode of action is to stimulate pathogenesis-related

(PR) protein synthesis and so it has no direct effect on the pathogen. Bion replaces the SA signal and hence prior infection is not necessary. Three to seven days is recommended for induction of 'immunity'. It has been shown to be effective on a range of crops including cereals, vegetables, fruits and flowers (Romero *et al.*, 2001, Brisset *et al.*, 2000, Terry & Joyce, 2000, Tosi & Zazzerini 2000, Ishii *et al.*, 1999). Resistance is not expected due to the indirect mode of action.

Biological Control – inoculation with biocontrol microorganisms

Other alternative methods of pest and disease control being investigated are based on biological control. The use of fungi as biological control agents goes back many years. An example of this was the work done by Risbeth (1951) who applied a spore suspension of the saprophytic *Peniophora gigantea* to the stumps of recently felled trees to prevent infection by the pathogen *Hetrobasidion (Fomes) annosum*. The *P. gigantea* colonises the stump and spreads through the roots where it successfully out-competes the pathogen *H. annosum* thus preventing the infection of the roots of adjacent standing trees by the pathogen. Another example of a well-studied biocontrol agent is the fungus *Trichoderma*. Its varied properties (including hyperparasitism and production of antimicrobial substances) and applications were reviewed by Henis (1984).

Bacteria are also used as biological control agents and a widely used example is the use of *Agrobacterium radiobacter* as a dip for seedlings or cutting s that would otherwise be susceptible to the gall-causing agent *Agrobacterium tumefaciens* (Jones, 1989). The non-pathogenic strain K84 produces a bacteriocin called agrocin 84, which is active against *A. tumefaciens*. However, gene transfer occurs naturally and *A. tumefaciens* acquired resistance to agrocin 84. The K84 strain was

genetically modified to give the new strain K1026 which lacks the ability to transfer the resistance gene to the pathogen (Agrios, 1997). *Pseudomonas fluorescens* has also been shown to produce antibiotic substances that are effective against *Rhizoctonia solani* damping off (Howell and Stipanovic, 1979). Many more fungi and bacteria have been studied and found protective against specified diseases. Lists of the agents and the diseases they protect against are available from the USDA website (2001)

Many studies attribute, at least in part, the biocontrol ability of the microbes to chitinase production. Selection of biocontrol agents has been carried out by including chitin in the media (Kobayashi and El Barrad, 1996). Chitinolytic bacteria, *Xanthomonas* and *Serratia* were tested in growth chamber studies and found to suppress summer patch disease in Kentucky blue grass cv. Baron by up to 70% (Kobayashi *et al* 1995). Chitinase was seen to be an important factor in the biocontrol activity of *Trichoderma* species (Chet and Inbar, 1994). In addition to this, plant chitinases are also important in the role of plant defense (see SAR above) and are elicited by infection (Benhamou, 1995)

Biological control – use of soil amendment to promote soil antagonists

Studies on growth substrate amendments report the use of various manures (cow, chicken and swine), bonemeal and soybean meal (Viteri and Schmidt, 1996, Lazarovits, 2001) to improve soil fertility. While cow manure has been used for centuries as a fertiliser, its properties as a selective amendment to enhance certain antagonists such as *Trichoderma* and *Bacillus cereus* have been recently reported (Tsror *et al.*, 2001). The latter found that cattle manure in drills along with a *Trichoderma* or non-virulent *Rhizoctonia* inoculum prevented black scurf in potato without significantly affecting yield. It has long been thought that chitin could

enhance biological control activity if added as an amendment to soil (Sneh 1971, Tu *et al* 1992). The mechanism of action is not clear and has been the subject of much investigation. Mitchell and Alexander (1962) reported an increase in the soil microflora of chitinase producers and other antagonistic populations such as actinomycetes, following chitin amendment. Whether these actually attack the pathogens and/or produce secondary metabolites or volatiles that act as anti-fungal agents is as yet unclear (Papavizas and Davey 1961, Henis 1994, Sneh 1971). Another hypothesis is that chitin, as well as its breakdown products such as chitosan, act as elicitors of the plants defence mechanism (Ren and West, 1992, Evans, 1993; Gagnon and Ibrahim, 1997; Pearce *et al.*, 1998). Crushed crustacean shells (CCS) are a source of chitin (up to 30%) (Noomhorm et al., 1998). This resource was chosen as an environmentally friendly and organic chitin source. The use of crustacean shellfish waste, (Sugimoto *et al.*, 1998), is based on observations of biological control properties against soil fungi (*Fusarium solani* f. *phaseoli*) described by Mitchell and Alexander (1962).

Health concerns regarding microbial contamination of raw salad vegetables

Intensification of production methods including those associated with trade globalisation and a larger market demand for greater volumes of fresh produce in recent years, has seen an increase in the transmission of human food-poisoning pathogens on raw vegetables and juices- See Fig. 1(Beuchat, 1996; Little *et al.*, 1997, Mahon et al., 1997; Trauxe *et al.*, 1997). Organic food production is a growth sector which also may increase microbial contamination of raw salad vegetables. Twenty five percent of the US population have purchased organic foodstuffs at least once (Thompson, 1998). Organic production increased from a quarter of a million hectares to almost 2 million hectares between the years 1990 and 1997 in Europe alone. (Lampkin, 1999). Most Western countries are steering agriculture in this direction by use of subsidies, not only to counteract overproduction but also because

Fig. 1. US fresh produce outbreaks: 1990-1998. From Rangarajan *et al* 2000, reproduced with the kind permission of E. Bihn M.S., Good Agricultural Practices (GAPS) Project Co-Ordinator, Ithaca & Geneva Offices, Cornell University, USA

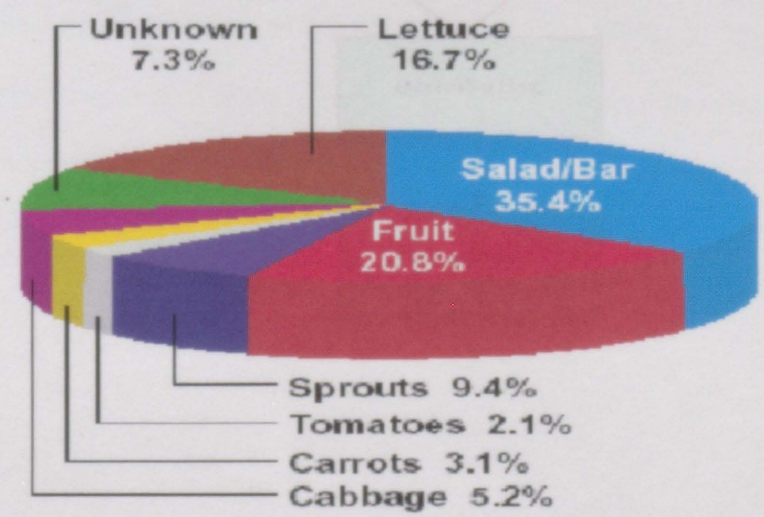
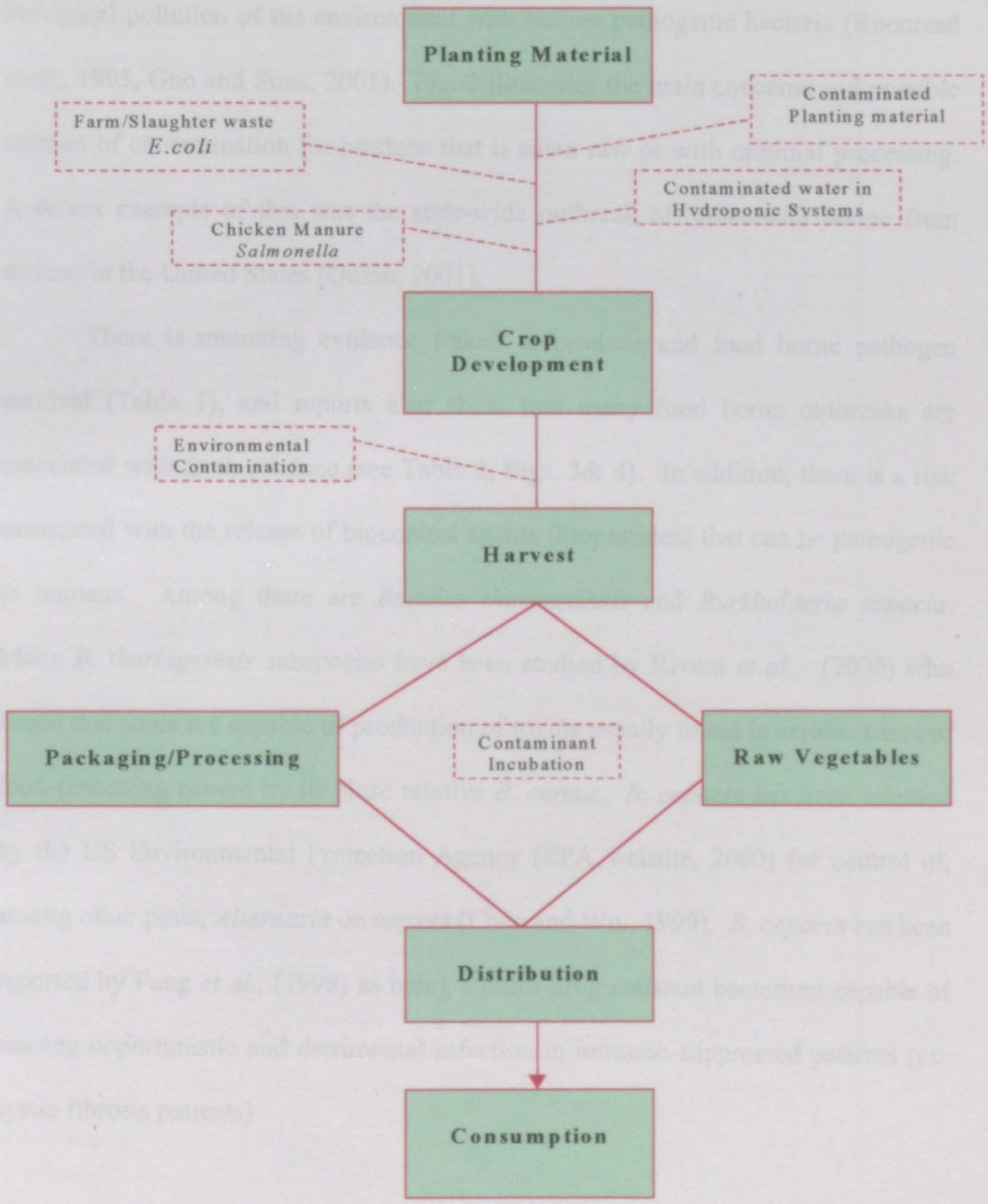


Fig. 2. Flow diagram of the possible sources of contamination for raw/minimally processed produce. Green boxes are the stages of production and dashed boxes are areas of risk.



of environmental concerns (Ahvenjarvi and Hakkila, 1997). The recycling of processing water; the discharge of contaminated processing water, land drilling of meat factory waste, and use of manure are factors underlying the increase in biological pollution of the environment with human pathogenic bacteria (Koenraad *et al.*, 1995, Guo and Sims, 2001). Fig. 2 illustrates the main concerns and possible sources of contamination for produce that is eaten raw or with minimal processing. A recent example of this was the state-wide outbreak of *Salmonella poona* from melons in the United States (Guinn, 2001).

There is mounting evidence linked to produce and food borne pathogen survival (Table 1), and reports also show that many food borne outbreaks are associated with fresh produce (see Table 2, Figs. 3& 4). In addition, there is a risk associated with the release of biocontrol agents (biopestides) that can be pathogenic to humans. Among these are *Bacillus thuringeinsis* and *Burkholderia cepacia.* Many *B. thuringeinsis* subspecies have been studied by Rivera *et al.,* (2000) who found that some are capable of production of toxins usually found in serious cases of food-poisoning caused by its close relative *B. cereus*. *B. cepacia* has been released by the US Environmental Protection Agency (EPA website, 2000) for control of, among other pests, *Alternaria* on carrots (Chen and Wu., 1999). *B. cepacia* has been reported by Fung *et al.,* (1998) as being a multi-drug resistant bacterium capable of causing opportunistic and detrimental infection in immuno-suppressed patients (i.e. cystic fibrosis patients).

Table 1. Bacterial pathogens isolated from raw vegetables in European countries.

Adapted from Beuchat (1996)

Vegetable	Country	Pathogen	Prevalence
Artichoke	Spain	*Salmonella*	3/25 (12.0%)
Bean Sprouts	Sweden	*Salmonella*	N/A
Beet leaves	Spain	*Salmonella*	4/52 (7.7%)
Cabbage	Spain	*Salmonella*	7/41 (17.1%
Cauliflower	Netherlands	*Salmonella*	1/13 (7.7%)
Cauliflower	Spain	*Salmonella*	1/23 (4.5%)
Celery	Spain	*Salmonella*	2/26 (7.7%}
Egg plant	Netherlands	*Salmonella*	2/13 (1.5%)
Endive	Netherlands	*Salmonella*	2/13 (1.5%)
Fennel	Italy	*Salmonella*	2/26 (7.7%)
Leeks	Spain	*L. monocytogenes*	4/89 (71.9%)
Lettuce	Italy	*Salmonella*	1/5 (20%)
Lettuce	Netherlands	*Salmonella*	82/120 (68%)
Lettuce	Spain	*Salmonella*	2/28 (7.1%)
Mustad Cress	UK	*Salmonella*	N/A
Parsley	Spain	*Salmonella*	N/A
Pepper	Sweden	*Salmonella*	1/23 (4.3%)
Potatoes	Spain	*L. monocytogenes*	2/12 (16.7%)
Prepacked Salads	N. Ireland	*L. monocytogenes*	3/21 (14.3%)
Prepacked Salads	UK	*L. monocytogenes*	4/60 (13.3%)
Prepacked Salads	UK	*L. monocytogenes*	N/A
Salad Greens	UK	*S. aureus*	13/256 (5.1%)
Salad Vegetables	Spain	*Aeromonas*	2/33 (6.1%)
Salad Vegetables	Spain	*L. monocytogenes*	21/70 (30%)
Salad Vegetables	Germany	*L. monocytogenes*	6/263 (2.3%)
Salad Vegetables	N. Ireland	*L. monocytogenes*	4/16 (25%)
Salad Vegetables	UK	*Y. enterolitica*	N/A
Spinach	Spain	*Salmonella*	2/60 (3.3%)
Vegetables	France	*Y. enterolitica*	4/58 (7%)
Vegetables	France	*Y. enterolitica*	15/30 (50%)
Vegetables	Italy	*L. monocytogenes*	7/102 (6.9%)
Vegetables	Italy	*Y. enterolitica*	8/103 (7.8%)
Vegetables	Spain	*L. monocytogenes*	8/103 (7.8%)
Vegetables	Spain	*Salmonella*	46/849 (5.4%)
Vegetables	UK	*L. monocytogenes*	4/64 (6.2%)

Fig. 3. Fruit and Vegetable outbreaks by Origin of Produce: 1990-1998. From Rangarajan *et al* 2000, reproduced with the kind permission of E. Bihn M.S., Good Agricultural Practices (GAPS) Project Co-Ordinator, Ithaca & Geneva Offices, Cornell University, USA Results compiled from the CDC in the United States which show that about 75% of the outbreaks were related to US domestically grown produce

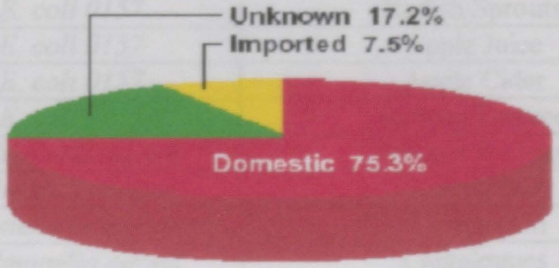


Fig. 4. Fruit and Vegetable Bacterial Outbreaks: 1988-1998. From Rangarajan *et al* 2000, reproduced with the kind permission of E. Bihn M.S., Good Agricultural Practices (GAPS) Project Co-Ordinator, Ithaca & Geneva Offices, Cornell University, USA. Most of the outbreaks were found to be caused by bacteria, *Salmonella* and *E. coli* species were found to be most common with *E. coli* O157:H7 being the most frequent of the *E. coli* species.

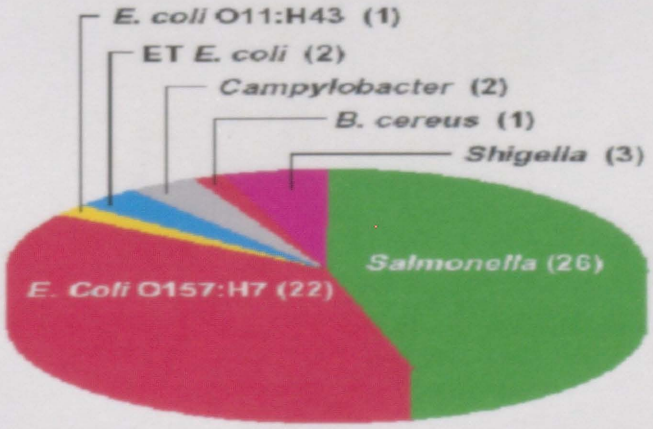
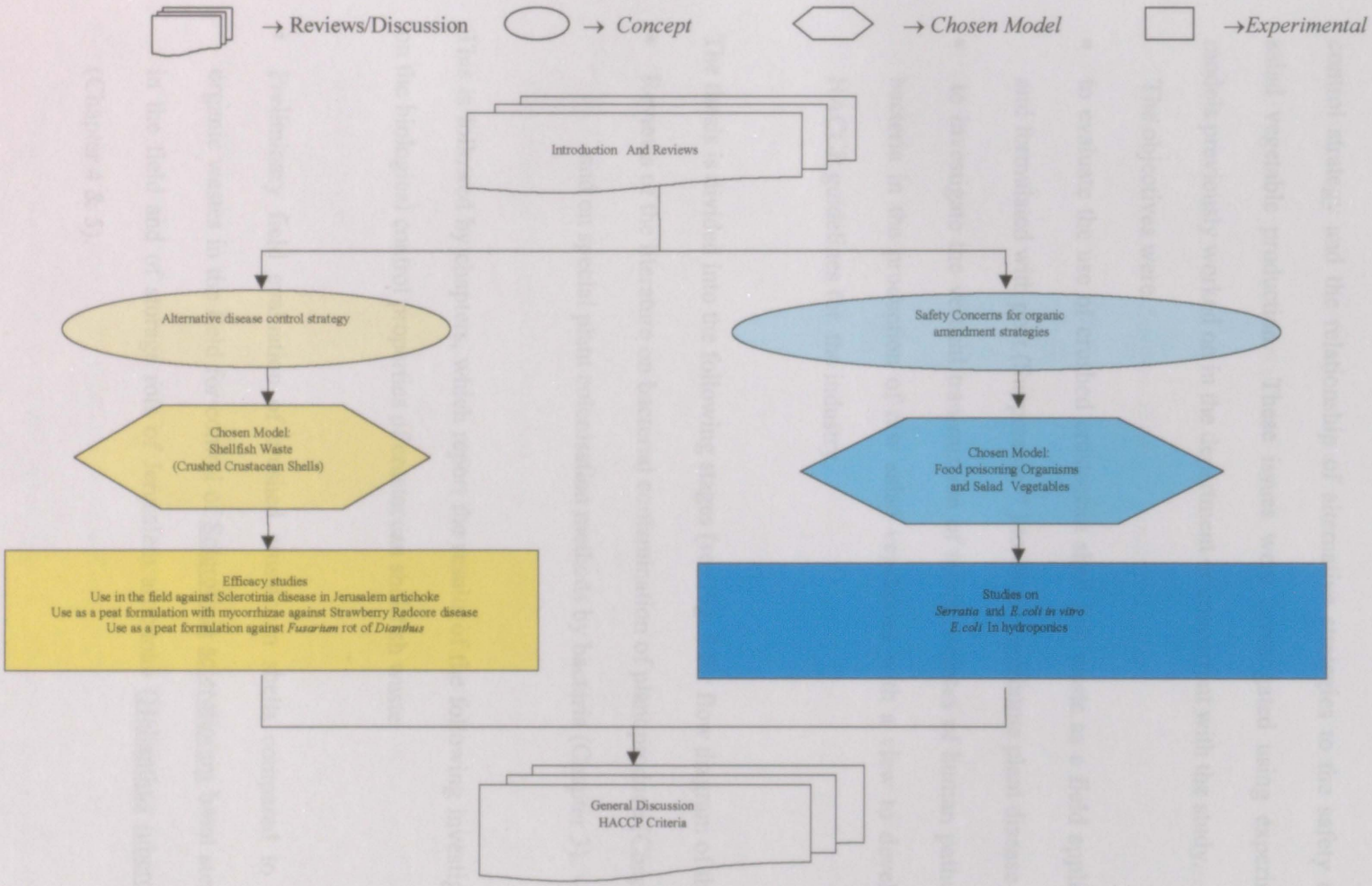


Table 2. Examples of pathogens assoicated with fruit and vegetables involved in outbreaks of foodborne disease. Adapted from WHO/FSF/FOS, 1998

Agent	Implicated Food
Bacillus cereus	Sprouts
Campylobacter	Cucumber
Campylobacter jejuni	Lettuce
Clostridium botulinum	Vegetable Salad
Cryptosporidium	Apple Cider
Cyclospora	Raspberries
E. coli 0157	Radish Sprouts
E. coli 0157	Apple Juice
E. coli 0157	Apple Cider
E. coli 0157	Iceberg Lettuce
Salmonella agona	Coleslaw & Onions
Salmonella miami	Watermelon
Salmonella oranienburg	Watermelon
Salmonella poona	Cantaloupes
Salmonella saint-paul	Bean Sprouts
Salmonella stanley	Alfalfa Sprouts
Salmonella thompson	Root Vegetables & Dried Seaweed
Shigella flexneri	Mixed Salad
Shigella sonnei	Iceberg Lettuce
Shigella sonnei	Tossed Salad
Vibrio chlolerae	Salad Crops & Vegetables

Fig. 5. Thesis flow diagram, showing progression from concept to chosen models to experimental work carried out.



Objectives of the project

The overall objective of this project was to evaluate the efficacy of a biological control strategy and the relationship of alternative strategies to the safety of raw salad vegetable production. These issues were investigated using experimental models previously worked on in the department or concurrent with the study.

The objectives were:

- to evaluate the use of crushed crustacean shell fish waste as a field application and formulated with peat (Suppressor™) to control soilborne plant disease
- to investigate the vertical transmission of model isolates of human pathogenic bacteria in the production of raw salad vegetables with a view to developing HACCP guidelines for the industry.

The thesis is divided into the following stages (see Fig. 5 for flow diagram of thesis)

- Reviews of the literature on bacterial contamination of plant produce (Chapter 2) and on special plant colonisation methods by bacteria (Chapter 3).

This is followed by chapters, which report the results of the following investigation on the biological control properties of crustacean shellfish waste:

- Preliminary field evaluation of crushed crustacean shells compared to other organic wastes in the field for control of Sclerotinia sclerotiorum basal stem rot in the field and of storage rots of Jerusalem artichoke (Helianthus tuberosus.) (Chapter 4 & 5).

- Stimulation of wild strawberry (*Fragaria vesca*) arbuscular mycorrhizas by addition of shellfish waste to the growth substrate: interaction between mycorrhization, substrate amendment and susceptibility to red core (*Phytophthora fragariae*) (Chapter 6).
- The identification and use of chitin-amended compost to suppress wilt disease in glasshouse-grown *Dianthus* 'mystère' plants (Chapter 7).

This next part of the work was aimed at investigating the risk to human health posed by bacterial contamination. Little is known about the ability of these the chosen model bacteria to persist in and on plants. To assess the risk to human health of the transmission of human pathogens in/on plants the following experiments were carried out:

- *Brassica* plants were inoculated in aseptic culture with model isolates of *E. coli* and *Serratia marcescens* (representative human food poisoning pathogens) and the bacterial persistence *in planta* and epiphytically, was studied. The plants were multiplied and inoculated with *E. coli in vitro*, established in hydroponic culture and host colonisation and bacterial plant interactions were studied (Chapters 8 & 9)

Chapter 10 is the final discussion of the results of the experimental work for this project. The last stage was to attempt to construct HACCP guidelines for the safe use of biological waste in raw salad production (Chapter 11).

While there have been increased reports of food poisoning due to consumption of raw salad vegetables, there is little information on the source of the

contamination, or the interaction between human pathogenic bacteria and plants, e.g. is colonisation endophytic thereby avoiding surface sterilization.

The disease problems selected for biocontrol investigation here have been the subject of on-going research in the Department of Plant Science or arose in parallel projects during the investigation (Cassells and Deadman, 1993, Cassells and Walsh, 1995, Dempsey, 1998). Endophytic bacterial contamination has been the subject of much research over the years in the department (Barrett and Cassells, 1994, Cassells and Tahmatsidou, 1996, Leifert and Cassells, 2000). Possible health risks associated with the discovery of *E. coli* in plants supplied with farmyard manure as an organic fertiliser (Cassells and Tahmatsidou, 1996), prompted the investigation into the possible risk of acquisition of human pathogen bacteria from organic material supplied to crops. The persistence of these bacteria endophytically in the plant would by-pass conventional surface washes of raw salad vegetables which are consumed uncooked or used as ingredients of prepared meals; they could possibly incubate and multiply in product distribution.

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Chapter Two

Human pathogens associated with plant produce

Preface to Chapter 2

This chapter is based on an invited lecture given at the International Commission for Radiation Research conference in Dublin, 1999. The lecture was subsequently published, after a peer review, in Radiation Research 2000, 2, 270-273.

HUMAN PATHOGENS ASSOCIATED WITH PLANT PRODUCE

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Introduction:

In recent years there has been an increase in foodborne incidences associated with fresh produce (1). Contributing factors include an increased rate in consumption of produce per capita, intensification of agricultural production, modern processing techniques and globalisation of the market (2).

Sources of contamination:

Numerous sources of microorganisms are present in food production. Contaminated soil, water, feed and manure are fundamental sources. Methods of introduction throughout the processing industry involve contaminated raw ingredients/ raw materials (e.g. packaging), unhygienic employees/surfaces, dirty process water, faulty air handling systems, and others (3).

Listeria monocytogenes, Clostridium botulinum, and *Bacillus cereus* can be naturally present in soils. *Campylobacter jejuni , Escherichia coli O157:H7, Salmonella* and *Vibrio cholerae* are more likely to contaminate produce through vehicles such as improperly composted manure or irrigation/wash water containing untreated sewage. Wild or domestic animals are another source of contamination.

Unhygienic surfaces and handlers can represent a potential basis for contamination from farm to fork (4). Investigators have long been concerned with

the threat posed from faeces fertilised produce. A 1912 Public Health Report called attention to the transmission of typhoid bacillus via fresh produce contaminated with human sewage (Cited by 5).

Recently several foodborne outbreaks have been linked to vegetables (See 6). Such reports have enhanced speculation that pathogens, present in agricultural manure, would pose a threat if applied to growing produce (5).

Water Transmission:

Use of contaminated irrigation water or inadequately treated water has been quoted as a vehicle of transmission for various food poisoning agents (20, 21). A major American producer of fresh-cut carrots now includes testing of irrigation and processing water for total coli-forms and *E. coli* (3). An interesting plant pathogenic case shows that bacterial spread through water is not uncommon. Potato brown rot disease is caused by *Pseudomonas solanacearum/Ralstonia solanacearum* biovar 2A. The bacterium has been found in most infected countries in surface water, ditch water (22), sterile surface water (23), and in the weed *Solanum dulcamara* growing along waterways (24). The pathogen can overwinter successfully in the roots (25), from which it can spread to potato crops when associated water is used for irrigation (26).

Bacterial Association with Plants:

Bacteria survive in association with plants in a variety of ways. They are commonly found as epiphytes but they also have more specialised methods of association.

Endophytic Survival: A method of avoiding the exterior stresses on a plant is to live within the tissue, which affords protection. Common endophytic isolates from plants include *Beijerinckia, Azotobacter, Erwinia, Klebsiella, Enterobacter, Bacillus (7), and Clavibacter* (8). Endophytes have been shown to survive in the following plant tissues: vascular tissue (9), roots (10, 11), stems and cotyledons/leaves (12, 13). Endophytic presence in aseptic tissue culture has also been noted (14). Systemic colonisation can afford protection for the bacterial endophyte from competition and environmental stresses such as washing and sterilisation procedures (15).

Biofilms: Various investigators have reported biofilms in the marine environs, implanted medical equipment and water distribution systems (16). Costerton (17), defines biofilms as: "Matrix enclosed bacterial populations adherent to each other and/or to surfaces or interfaces. The definition includes aggregates and flocculates and also adherent population within the pore spaces of porous media." It was noted that biofilm cells are at least 500 times more resistant to anti-bacterial agents than their planktonic counterparts. The control of biofilm bacteria has been the focus of vast amounts of applied and medical research. Why biofilm bacteria are less susceptible to usual lethal treatments is still unclear (17). Morris *et al* (18), observed biofilms directly on the leaf. The plant species chosen were all vegetables that are eaten raw (spinach, lettuce, Chinese cabbage, celery, leeks, basil, parsley and broad-leafed endive). Recovered biofilms using leaf washings and agar impressions revealed that they contained multiple species (19). Costerton (17) quotes studies on depth of biofilms, one homogenous biofilm studied was made up of *Vibrio parahaemolyticus*, a well-known food-poisoning agent. This would indicate that food poisoning agents could survive in this form.

Emerging Pathogens:

Various factors contribute to emerging pathogens including the globalisation of the food supply (3), as well as evolving microbial populations (27). Increasingly since the late eighties *Campylobacter* infection has risen to and surpassed that of *Salmonella* and campylobacteriosis is more common across the world (28). The Super family VI includes the genera *Campylobacter* and *Helicobacter*. These microorganisms are Gram negative, motile by means of flagella, spiral shaped and microaerophilic (29).

Campylobacter: During the past decade *Campylobacter* has emerged as a major cause of human enteritis (4,30,31,32,33). Patients, excreting the organism and healthy carriers such as poultry and pigs provide a constant flow of the bacterium into the environment. The application of natural or untreated water for irrigation of farmlands is a route of direct contamination. Waterborne outbreaks of Campylobacteriosis have been reported in Sweden, USA, Canada, England, Yugoslavia and Norway as cited by (21). Koenraad draws attention to the possible presence of Campylobacter species in water in a VBNC (viable but not cultivable) form (30). *Campylobacter* have been isolated from fresh market produce. 3.8% of the samples were positive for *Campylobacter* (21). Through analysis of diet histories Harris *et al* (34) cite Doyle *et al.,* (1986) as having isolated *Campylobacter jejuni* from a small percentage of commercial mushrooms (1.5%). Despite many investigations the sources of the majority of sporadic cases of human campylobacteriosis remains unproven. However, the major sources for *Campylobacter* in produce include, untreated waters and soil and manure. Poultry may have an important role in human infection but other sources cannot be ignored (31).

Helicobacter: H. pylori is the most common chronic infection in human kind and the major etiological agent for chronic active gastritis (29, 35). It is often present in ulcer disease and atrophic gastritis (36), it is being actively explored as a risk factor for gastric carcinoma. *H. pylori* is fastidious and requires three or more days for isolation, microaerophilic conditions must be constantly maintained (29). Little is known about environmental sources of *H. pylori* though the faecal oral route has long been suspected (35). That produce may be a vehicle in *H. pylori* transmission is based on serosurveys. A study in Chile showed a significantly higher prevalence in lower socio-economic groups. Since a key factor in enteric pathogen transmission in Chile is the use of sewage-contaminated irrigation water on produce, then it was thought that this might also be a route of transmission for *H. pylori* (Hopkins 1993 cited by 35). *Helicobacter* has been associated with waterborne transmission (37), probably in a viable but non-cultivable state (38). It is possible *Helicobacter* may not have been directly isolated from produce because of the difficulty in culturability and/or detection.

Conclusion:

Considering that bacteria are known to survive on salad vegetables as biofilms and as endophytes, this presents us with a risk that requires investigation. Whether human pathogens can survive on fresh produce requires further examination. Prevention of the transmission of human pathogens in the food industry involves taking action at all stages in the chain from farm-to fork. Properly composted manure and irrigation water from a clean source should be used on growing crops. All processing should include sanitary designed processing facilities, highly evolved HACCP plans, sanitation regimes, GMP, employee

training/monitoring in basic hygiene and perhaps to include irradiation as a final precautionary step (3), the latter should not be used on it's own or to process poorer quality raw materials. Research is necessary to understand more fully the survival mechanisms of pathogenic bacteria on fresh and minimally processed produce (3).

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Chapter Three

Colonisation of plants by bacteria as endophytes and biofilms

Preface to Chapter 3

This chapter covers areas briefly introduced in Chapter 2 in greater depth. The style follows that for reviews in the journal Plant Cell Tissue and Organ Culture.

Colonisation of Plants by Bacteria as Endophytes and Biofilms

Abstract

Bacteria are well-characterised inhabitants of the rhizosphere and phylloplane (Hallmann *et al.*, 1997a). It is now accepted that bacteria may occur also as endophytic colonisers of plants (Chanway, 1996). Bacteria may also survive both on roots and on the haulm in biofilms. It is recognised that bacteria in biofilms may be highly resistant to surface sterilizing agents while as endophytes they are immune from the effects of standard antiseptic treatments. Here, the literature on the colonisation of plants by bacteria is reviewed with emphasis on the implications of endophytic colonisation and biofilm formation, for the microbial safety of raw salad vegetables.

Endophytes

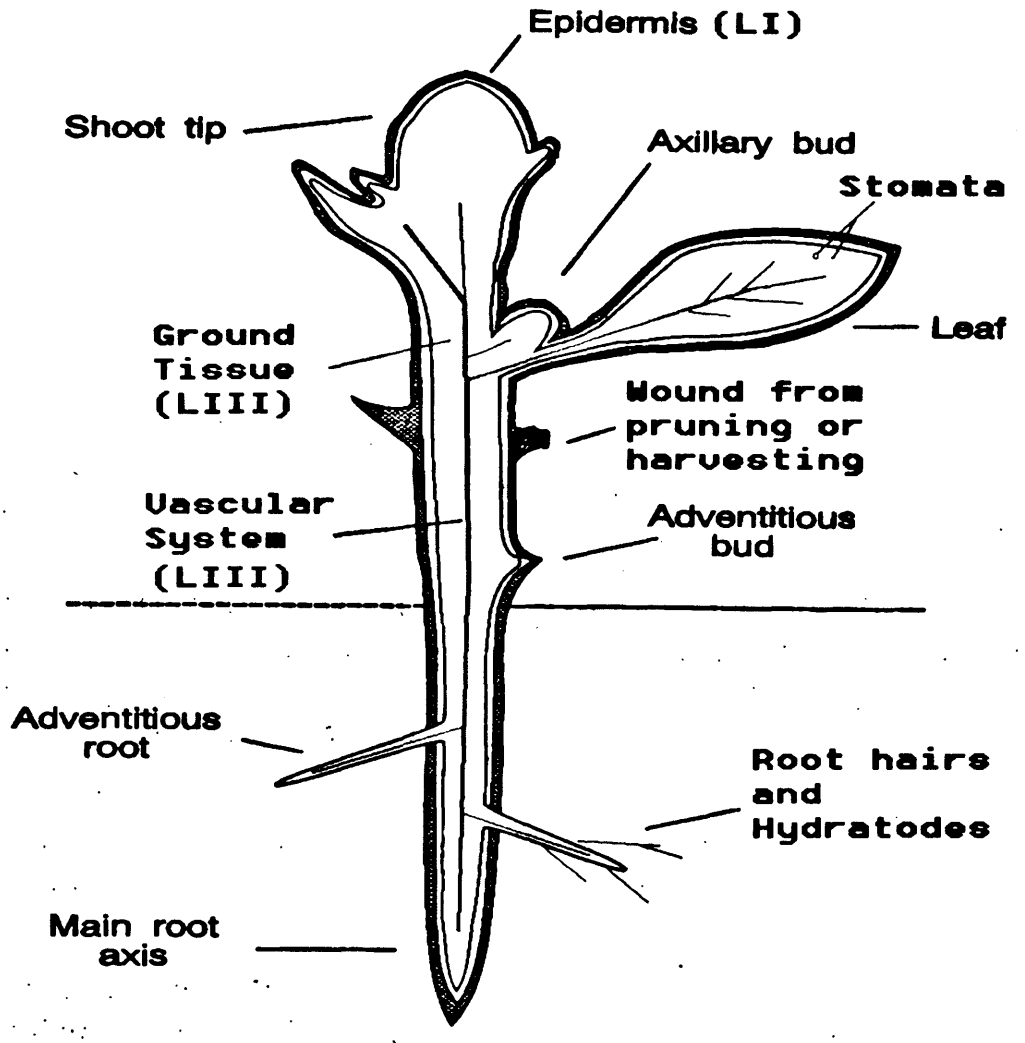
Host entry

Bacterial endophytes have been reported with cell counts up to 10^7 cfu/g of plant matter (Chanway 1998). Bacteria from very many genera have been found to reside within plant tissues without causing disease. Plants species from trees to grasses have been investigated and found to harbour endophytes. Table 1 lists many examples of bacteria and the plants tissues in which they have been isolated. As an endophyte, a bacterium is afforded protection from environmental factors such as UV, temperature, competition from other microbes, etc (Mahaffee *et al.*, 1994). The bacteria detected as endophytes have been shown to be present in the rhizosphere, phylloplane, planting material and seeds. The most likely primary route of entry is via the rhizosphere, with bacteria colonizing the germinating seed or vegetative propagule and spreading through the plant systemically. Studies to corroborate this

hypothesis have taken aseptic potato microplants and planted them in soil. The endophytes found subsequently in the tissues were similar to the saprophyte genera found in the soil (Kloepper and Beauchamp, 1992). While the endophyte communities originate from the rhizosphere bacteria, the root interface operates some selective barrier and/or the interior tissues represent a selective niche, as fewer genera have been reported as endophytes compared to the diversity of the bacterial flora in the rhizosphere (Hallmann *et al.*, 1997b). Endophytes may enter predominantly through the wound caused by lateral root emergence.

Stomata, lenticels, hydratodes and wounds are natural ports of entry for potential endophytes from the haulm/root epiflora. Most commonly, bacteria gain entry at secondary growth emergence zones, which form natural wounds for the bacteria to colonise, Fig. 1 illustrates some of the latter (Dane and Shaw 1996 , Hallmann *et al.*, 1997b, Reddy *et al.*, 1997, O Callaghan *et al.*, 1997). Natural husbandry of plants such as grafting, harvesting and pruning leaves create channels of entry as well as wounds caused by insects, fungi and nematodes (Hallmann *et al.*, 1997b). Tropisms that attract bacteria such as chemotaxis, electrotaxis and opportunistic colonisation as secondary colonists after pathogen colonisation, may also be significant factors. Weak forces adsorb the bacteria to the rhizoplane, followed by stronger forces leading to entry into internal tissues and epiphytic colonisation (Hallmann *et al.*, 1997b). Hallmann *et al.*, (loc. cit.) also report on another mode of active entry, which involves bacterial enzymatic systems. Cellulase and pectinase are produced by many endophytes and would support the hypothesis of plant wall degradation in order to gain access to the interior of the plant. Post-colonisation the bacteria can down-regulate the enzymes. This area needs closer examination order to elucidate the pathways used.

Fig. 1 Diagram showing examples of various points where entry of bacteria can occur: stomata, wounds, lateral root emergence, etc. Pathogenic bacteria can enter by digestion of the epidermis and cells walls. Endophytes can survive in ground tissue in intracellular spaces as well as travelling acropetally and basipetally in the vascular system. Biofilms can form on any part of the epidermis of the plant



Host colonisation

Nitrogen-fixing bacteria are commonly found in association with the roots of plants as endophytes (Neidhart *et al.*, 1990). Other than nitrogen fixers (eg *Bradyrhizobium japonicum*) common endophytic isolates from plants include: *Erwinia, Klebsiella, Enterobacter, Clavibacter, Bacillus* (Turner *et al.*, 1991 and Fuentes-Ramirez *et al.*, 1993). Bacterial endophytes have been shown to survive in every almost every tissue and organ of the plant from seeds to leaves. (Dane and Shaw 1996, Baldani *et al.*, 1997, Quandt- Hallmann and Kloepper 1996, Schloter and Hartmann, 1998). Generally endophytic bacteria are found in the intercellular spaces. There are some reports of intracellular endophytes but these are predominantly fastidious pathogens (Chanway, 1996). Reports of population numbers vary considerably, which might be explained by the as yet, crude methods of enumeration used, but a contributing factor to this is also the varying degrees of nutrient availability. Sugarcane intracellular spaces are reported as having sucrose in the fluid present, whereas the concentration of inorganic ions in the other wet intracellular spaces varies (Hallmann *et al.*, 1997b).

Reports of endophytes in the xylem do not always show whether the bacteria are growing or just surviving. Multiplication of endophytic pathogens in the vessels can cause blockages and constrictions but non-pathogenic endophytes generally remain below this threshold (Hallmann *et al.*, 1997b). Multiplication is difficult to demonstrate for endophytic colonists but long term survival of nitrogen-fixing endophytes suggests a dynamic association with the host tissues (Neidhart *et al.*, 1990, Fuentes-Raimerez *et al.*, 1993). In some studies where the hosts were deliberately inoculated, the bacteria reach a specific concentration, particular to the plant, regardless of the initial inoculum concentration (Chanway, 1998; Hallmann *et*

al., 1997b). These data would suggest that multiplication is regulated by host factors, or possibly nutrient availability maintains the bacterial population below the threshold for pathogenicity.

Bacterial movement within the plant

Endophytes may opportunistically colonise plants due to nutrient leakage (caused by extension during growth or other wounds) it could be assumed that the bacteria remain localised. However, studies by Quandt-Hallmann *et al.*, (1996) on *Enterobacter asburiae* JM22, using plating and immunochemical techniques to corroborate the results, showed that the inoculant *Enterobacter* was located in the internal tissues of roots, stems and cotyledons; the highest concentration was found in the roots following seed inoculation. When cotyledons and leaves were inoculated with JM22, bacteria were found internally in the cotyledons and the roots. Leaf inoculations also resulted in root colonisation (Quandt-Hallmann *et al.*, 1996). These data would support movement in both acropetal and basipetal directions. Similar conclusions were also drawn by McPherson and Preece (1978) previously, when they investigated the movement of the *Xanothmonas pelargoni* in *Pelargonium.*

Interactions with the host plant

In the last decade, interest in the interaction of non-pathogenic micro-organisms and plants has increased (Han *et al.*, 2000). Smith and Goodman (1999) recently reviewed plant-associated bacteria and listed nitrogen fixation, growth promotion, improvement of nutrient uptake and disease suppression among the benefits. As many variations in interaction occur in different plants and bacteria

combinations, it is suggested that plant genes are involved in the selection and/or support of such interactions. Research in the early nineties showed that *Pseudomonas* and *Serratia* species could induce systemic resistance in cucumber to various diseases when used as seed dressings. The selected strains remained localised in the plant distant from the induced systemic effects (Wei *et al.*, 1991, cited by Han *et al.*, 2000). Generally induction of resistance is termed Induced Systemic Resistance (ISR) when initiated by non-pathogens and usually involves ethylene or jasmonic signals. Systemic Acquired Resistance (SAR) more commonly involves induction by necrotising plant pathogens and leads to the build up of pathogenesis related (PR) proteins via the salicylic signalling pathway. (Han *et al.*, 2000). However, crossover between the two resistance pathways can occur and further studies on the plant proteins induced is required (Park and Kloepper, 2000). While much work has been done on bacterial inoculants and effects on plant disease suppression, little or no research has been carried out specifically aimed at elucidating endophyte-host interactions. Work usually is restricted to the disease under study and genes associated with the host response (Davila-Huerta *et al.*, 1995, Yoshimura *et al.*, 1998). There is a need for further study in this area in order to improve understanding and contribute to enhanced agriculture as well as safety of the plants for human consumption (Smith and Goodman, 1999; Han *et al.*, 2000).

Agrobacterium – a deliberate endophyte

Plant transformation can be achieved by various methods, but *Agrobacterium tumefaciens* mediated transformations are still the most popular (Fenning and Gartland 1995). However, it has been found that the antibiotics most useful for decontamination can quite often inhibit tissue regeneration (See Table 3)

The mechanism of transformation and choices of binary Ti vectors are well described elsewhere and is not the focus of this review (Bouzar *et al.*, 1995, Gartland and Davey 1995, Hellens *et al.*, 2000). Briefly, the presence of the *Ti* plasmid combined with marker genes that are easily selectable, enables systems that allow successful plant transformations. *Agrobacterium* mediated transformation requires co-culture, for a short time, of the bacteria and plants (or parts thereof). Successful transformants are detected by the reporter gene and must be treated with antibiotics for periods up to a few months to decontaminate progeny plants from the inoculant.

Leifert and Cassells (2000) have reviewed suppression of bacterial contaminants in tissue culture including *Agrobacterium* in transformed plants and found that very often despite media acidification and /or months of antibiotic therapy, that complete elimination is extremely difficult. In fact, in an SCRI study by Barrett *et al.* (1996), it is claimed that many laboratories do not check that *Agrobacterium* has been totally eradicated. The study indicates that 50% of plant material contained up to 10^7 cfu/g of plant tissue of the *Agrobacterium* binary vector system. This finding was made 6 months after the transformation (Barrett *et al.*, 1997). These studies show that once a plant is colonised with an endophyte, it can be very difficult to eliminate.

Biofilms

Biofilm structure

In many environments it is common to find assemblages of micro-organisms adhering to each other and/or to a surface and embedded in a matrix of exopolymers. These are referred to as biofilms (Morris, 1998). Costerton *et al.*, (1995) defines biofilms as:

A matrix enclosed bacterial population adherent to each other and/or to surfaces or interfaces. The definition includes aggregates and flocculates and also adherent population within the pore spaces of porous media.

As studied in the aquatic habitat, the microenvironment and physiology of biofilm bacteria are often markedly different from their planktonic counterparts because of some basic properties

- Biofilms are composed of an exopolymeric matrix and multiple layers of microbial cells leading to the creation of physical barriers and the establishment of chemical gradients
- They generally contain multiple species of micro-organisms fostering metabolic and genetic exchange
- Many biofilms are attached to a surface (biotic or abiotic including the surface of other micro-organisms or debris in the biofilm) which is requisite for the expression of certain genes (Costerton *et al.*, 1995).

Secondary levels of co-operation build up in biofilms which facilitate

- Physiological co-operativity between different bacterial species (Costerton *et al.*, 1995)
- Concentration gradients of molecules and ions may occur in viscous exopolysaccaharide matrix that supports the biofilm (Costerton and Lewandowski, 1997)

Costerton *et al.*, (1995) also state that direct observation has clearly shown that biofilm bacteria predominate numerically and metabolically in virtually all nutrient-sufficient ecosystems.

Biofilms and gene exchange

Sarand *et al.*, (1998) using transmission electron microscopy of the soil fungal interface showed ectomychorrhizae supporting morphologically diverse biofilms. Plasmid exchange was proven *in vitro* and was extrapolated to be more enhanced *in vivo* as the prevailing conditions would increase concentrations of bacteria present in such an energy rich environment. Gene exchange was also demonstrated to occur between marine bacteria in biofilms in reactor microcosms (Angles *et al.*, 1993).

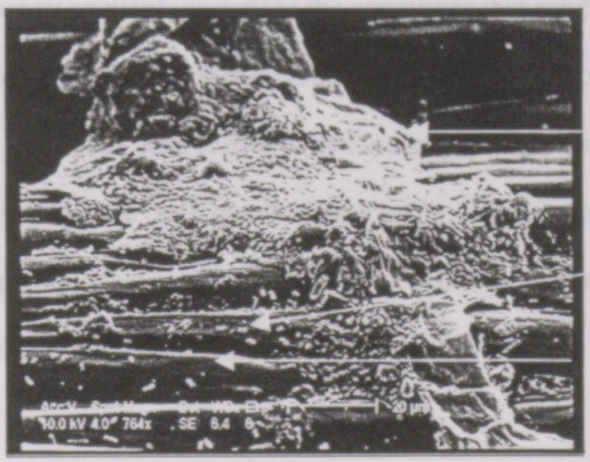
Biofilms and resistance to anti-microbial agents

Bacteria respond to their environment by varying their enzyme production, external structure and the composition of their cell walls. Biofilm bacteria differ greatly in their response compared to their 'normal' counterparts. Nickel *et al.*, (1985) carried out experiments on biofilms of *Pseudomonas* growing on catheter material. They found after isolation and culturing on agar that the *Pseudomonas* were killed after 8 hours treatment with 50μg ml^{-1} of tobramycin. However, the same bacteria growing as a biofilm were not killed after 12 hours contact with 1000μg ml^{-1}. It was their conclusion that biofilms are much more resistant to antibiotics and biocides than bacteria growing in non-biofilm conditions. Biofilms demonstrate an increased natural resistance to surfactants and antibiotic therapy. It is hypothesised that the strategy behind the formation of biofilms may be to enhance survival during nutrient shortages while retaining the capacity to return to the vegetative state when nutrients become available or conditions become more favourable. The distribution of biofilms is wide, Costerton *et al.*, (1995, 1997) report biofilms in medical, industrial and marine situations as well as in ecosystems.

Biofilms on salad vegetables

Scanning electron microscopy, confocal laser microscopy and epifluoresence microscopic techniques were used to observe biofilms on salad and herb vegetables. Biofilms were positively identified on basil, parsley, leek, celery, spinach, Chinese cabbage, lettuce and broad-leafed endive. All plants analysed were commercially produced and minimally processed (i.e. washed and chopped). The bacteria were not subjected to taxonomic identification in this study but isolation studies showed both Gram positive and negative bacteria to be present and display diverse cell and colony morphology (Morris *et al.*, 1998). See Fig. 2.

Fig. 2. SEM micrograph showing a bacterial biofilm on a lettuce leaf. (Morris, Personal Communication, 1999)



Examples of biofilms-forming species from other sources, including heterogeneous biofilms, that were subjected to taxonomic identification are shown in Table 2. Bacteria in this form can escape surface sterilisation due to their resistance to anti-microbials. When other epiphytes have been removed in the sterilisation process they are left in a non-competitive position to become epiphytic/endophytic

colonisers. They come from a mixed culture source capable of allowing gene exchange which implies that as a group they can carry and exchange resistance factors.

Conclusion

From the literature it is clear that internal tissues of plants can be colonised by diverse species of bacteria. As interest grows in of endophytic bacterial colonisers of plants, it is clear that further research on the elucidation of the mechanisms of entry and *in planta* regulation/suppression is required. This area is important to contributing to safety of plant health from the point of view of carrying potential human pathogens.

The study of biofilms on plants has yet to be fully pursued. Their formation and frequency on plants as well as the conditions that are conducive or inhibitory to their build up are aspects that need clarification. In addition, gene transfer in biofilms, which has been shown to occur cross-species, also requires study. This research would have consequences for transfer of resistance to anitmicrobials including antibiotics, as well as transfer of virulence factors between potential pathogens for plants and humans.

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Table 1 Non-pathogenic bacterial genera found in plants

Bacterial genera	Plant species	Tissue
Erwinia-like, *Pseudomonas*	Alfalfa (*Meidcago sativa* L)	Root
Acetobacter	Coffee (*Coffea arabica* L)	Root and stem
Acetobacter	Cameroon (*Pennisetum purpureum* Schumach)	
Bacillus, Burkholderia, Corynebacterium, Enterobacter, Klebsiella, Pseudomonas	Corn (*Zea mays* L)	Root and stem
Agrobacterium, Bacillus, Burkholderia, Clavibacter, Erwinia, Serratia, Xanthomonas	Cotton (*Gossypium hirsutum* L)	Root and stem
Agrobacterium, Arthrobacter, Bacillus, Burkholderia, Chryseobacterium, Enterobacter, Pseudomonas, Stenotrophomonas	Cucumber (*Cucumis sativis* L)	Root
Bacillus, Clavibacter, Comamonas, Curtobacterium, Enterobacter, Klebsiella, Moraxella, Pantoea, Pseudomonas, Rahnella,	Grapevine (*Vitis* spp)	

Rhodococcus, Staphlococus, Xanthomonas		
Bacillus Pseudomonas, Actnomyces, Staphlococcus	Hybrid spruce (*Picea glauca* x Engelmannii)	Root
Azoarcus	Kallar grass (*Leptochloa fusca* [L] Kunth)Root	Root
Bacillus	Lodgepole pine (*Pinus contorta* Dougl Ex Loud)	Root
Acidovorax, Acinetobacter, Actinomyces, Agrobacterium, Alcaligenes, Arthrobacter, Bacillus, Capnocytophaga, Cellulomonas, Clavibacter, Commamonas, Corynebacterium, Curtobacterium, Deleya, Enterobacter, Erwinia, Flavobacterium, Kingella, Klebsiella, Leuconostoc, Micrococcus, Pantoes, Pasteurella, Photobacterium, Pseudomonas, Psychrobacter, Serratia, Shewanella, Sphingomonas, Vibrio, Xanthomonas	Potato (Solanum tuberosum L)	Tuber

Acidovorax, Agrobacterium	Red clover (*Trifolium pratense* L)	Leaves, root and stem
Arthrobacter, Bacillus, Bordetella, Cellulomonas, Commamonas, Curtobacterium, Deleya, Enterobacter, Escherichia, Klebsiella, Methylobacterium, Micrococcus, Pantoea, Pateurella, Phyllobacterium, Pseudomonas, Pschrobacter, Rhizobium, Serratia, Sphingomonas, Variovorax, Xanthomonas	Rice (*Oryza sativa* L)	Root and stem
Achromobacter, Alcaligenes, Moraxella, Acinebacter, Actinomyces, Arthrobacter, Bacillus, Citrobacter, Corynebacter, Enterobacter, Flavobacterium, Klebsiella, Providencia, Pseudomonas, Serratia, Vibrio, Yersinia, Rickettsia-like	Rough Lemon (*Citrus jambhiri* Lush)	Root
Herbaspirillum	Sorghum bicolor L Moench	Shoot
Bacillus, Corynebacterium,	Sugar beet (*Beta vulgaris* L)	Root

Erwinia, Lactobacillus, Pseudomonas, Xanthomonas		
Acetobacter, Herbaspirillum	Sugar cane (*Saccharum officinarum* L)	Root and stem
Klebsiella	Teosinte (*Zea luxurians* Itins and Doebley)	Stem

(Adapted from Chanway, 1998)

Table 2 Examples of identified Bacteria from heterogeneous and homogenous biofilms

Identified Biofilm Bacteria	**Reference**
Actinomycetes species	Astier *et al.*, 1995
Bacillus	Quintern *et al.*, 1992
Campylobacter jejuni	Somers *et al.*, 1994
Citrobacter diversus	Stickler & Hewett, 1991
E. faecalis	Stickler & Hewett, 1991
E. coli	Evans *et al.*, 1991
E coli O157:H7	Somers *et al.*, 1994
Hyphomicrobium species	Banks and Bryers, 1991
Klebsiella pneumoniae	Siebel & Characklis, 1991
Listeria monocytogenes	Ronner & Wong, 1993, Somers *et al.*, 1994
Pseudomonas aeruginosa	Siebel & Characklis, 1991, Evans *et al.*, 1991, Nickel *et al.*, 1985
Pseudomonas fragi	Zanyk *et al.*, 1991
Pseudomonas putida	Gunning *et al.*, 1996, Shreve *et al.*, 1991
Staphylococcus aureus	Anwar *et al.*, 1992
Salmonella typhimurium	Ronner & Wong, 1993 Somers *et al.*, 1994

Table 3 Plants inhibited by antibiotics

Plants inhibited by Cefotaxime @ 500mg/L	Plants inhibited by Carbenicillin @ 250-500mg/L
Arabidopsis	*Antirrhinum*
Daucus carota	*Beta vulgaris*
Malus	*Nicotinia tabacum*
Solanum tuberosum	*Picea glauca*
Picea glauca	*Solanum tuberosum*
Pyrus communis	*Datura*
Triticum aestivum	*Arabidopsis*
	Delphinium
	Vitis

(Adapted from Nauerby 1997)

Chapter Four

Effects of calcium fertilisers on Sclerotinia disease in Jerusalem artichoke

Section B: Investigation of the biocontrol properties of chitin-containing crustacean shellfish waste

Preface to Chapter 4

This section is concerned with the evaluation of the biocontrol potential of chitin-containing crustacean shellfish waste. Calcium is reported to be a host resistance factor to Sclerotinia disease (see introduction to Chapter 4). As crustacean shellfish waste (CCS) contains both calcium and chitin, a preliminary experiment was carried out here to determine whether increase in calcium fertiliser affected Sclerotinia disease susceptibility in Jerusalem artichoke. The fieldwork was carried out in collaboration with R. Dempsey. Chapter 5 reports on the biological control potential of shellfish waste. The style is that of the journal Applied Soil Ecology

Effects of calcium fertilisers on Sclerotinia disease in Jerusalem artichoke

Abstract

In Ireland, basal stem and cottony tuber rot of Jerusalem artichoke (Helianthus tuberosus) caused by Sclerotinia sclerotiorum is most the serious disease threatening stable crop production. The longevity of sclerotia makes rotation impractical. Chemical soil treatment costs are prohibitive; foliar application is impractical due to the height of the canopy. Here, calcium fertilisers were evaluated for their effects on incidence of Sclerotinia disease in Jerusalem artichoke (Helianthus tuberosus L.). Calcium ammonium nitrate was used at the recommended rate and at twice this rate. A negative control with no calcium was also included (ammonium sulphate nitrate). *Sclerotinia* disease was reduced significantly when fertilised with the higher calcium application

Keywords; Sclerotinia, Helianthus tuberosus, calcium fertiliser, disease suppression.

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1 Introduction

Sclerotinia species cause disease in a very broad range of crop species world-wide. A list of hosts by Purdy (1979) included 64 families and 225 genera. S. sclerotiorum is ubiquitous and has the widest range of hosts. The disease can affect all stages of growth from damping-off at seedling stage to rot of harvested produce. In the case of Helianthus tuberosus the disease causes a stem rot in the field and

tuber rot in storage (Deadman and Cassells, 1993). For Sclerotinia diseases there is usually a direct relationship between inoculum density and disease incidence (Twengstrom et al., 1998). The sclerotia germinate in the top 2.5cm of the soil when temperatures reach 6-10 ° C in Spring, resulting in ascospores borne on apothecia, ejected into the atmosphere (carpogenic germination). Most spores invade by colonising dead or dying tissues (Purdy 1979). Forecasting methods are discussed by Twengström et al., (1998) which guide when to spray against carpogenic germination. This allows for only necessary use of expensive fungicide and reduction of yield losses. Sclerotia can cause infection of the below ground parts via production of mycelium which directly invades the tissues (myceliogenic germination). Oxalic acid is a pathogenicity factor which lowers the pH of the tissues to about 4, which is the optimum for the cell wall degrading enzymes produced by the pathogen (Godoy et al., 1990). Oxalic acid also chelates calcium ions into a calcium oxalate complex which also can aid the invasion of the tissue by Sclerotinia (Dempsey, 1998). Govrin and Levine (2000) report that necrotrophic pathogens such as Sclerotinia sclerotiorum can invade healthy tissue and evoke the hypersensitive response (HR). This triggers an oxidative burst that leads to plant cell death. The mechanism usually cuts off food supply to biotrophic pathogens, however necrogens can utilise dead tissue and hence exploit the plant's defences to further colonise the host tissues.

Control of soilborne plant pathogens is difficult and soil fumigants e.g. 1,2 dibromochloropropane (DBCP) or ethylene dibromide (EDB) have been suspended most countries. Methyl bromide, which is the most widely used fumigant, is now being restricted and will eventually be, discontinued (Gamliel et al., 2000). These chemical treatments were very expensive not always successful (Akhtar & Malik

2000, Gamliel et al., 2000). Steadman (1979) and Expert and Digat (1995) have discussed the lack of progress in breeding for sclerotinia resistance and in the development of effective chemical controls. Recent publications have elucidated *Sclerotinia* resistance mechanisms in sunflower (Urdangarín et al., 2000, Giudici et al., 2000). Kesarwani et al. (2000) reported that overexpression of a transgene for oxalate decarboxylase in tomato and tobacco conferred resistance to S. sclerotiorum. Oxalate decarboxylase catabolises oxalate and hence maintains the pH above the optimum for pathogen-produced host cell wall degrading enzymes. This strategy has potential for other susceptible hosts including H. tuberosus but consumer acceptance of GMO products has resulted in a moratorium on GMO trials in the EU.

Here, an alternative strategy for S. sclerotiorum disease control was evaluated based on attempts to manipulate host leaf calcium by calcium fertilizer application. Calcium is a host resistance factor inhibiting the activity of the pathogen's cell wall degrading enzymes (Cassells and Barlass, 1976, 1978). Previously, it was shown that high calcium-accumulating mutants showed enhanced field resistance to S. sclerotiorum (Walsh 1994), however, the cost of mutation breeding for individual varieties is a limitation on this strategy. The approach here was to attempt to manipulate leaf calcium by exploiting the counter ion effect where calcium uptake is inhibited by competition with ammonium (a cation), and promoted by nitrate (an anion). To achieve this, fertiliser was applied as formulations of ammonium sulphate nitrate and calcium ammonium nitrate.

2. Materials and methods

2.1. Field trial

The trials site was sprayed with glyphosate (Roundup; Monsanto (Irl.) Ltd, Dublin, Ireland) and 3 weeks later ploughed to a dept of 20 cm. The site was rotavated and fertilized with calcium ammonium nitrate or with the soil amendments.. Controls, were fertilised with calcium ammonium nitrate-CAN- (10.4 gN/m^2 and 1.6 gCa/m^2) 400 kg/ha, which is the standard fertiliser used in this temperate region (Cassells and Deadman, 1993). Double the usual concentration of calcium ammonium nitrate-Hi CAN- (20.8 gN/m^2 and 3.2gCa/m^2) was used, 800kg/ha. Negative control plots were treated with Ammonium sulphate nitrate – ASN-(10.4 gN/m^2, 0g Ca/m^2), 400kg/ha, which contained no calcium. Trials were planted at the end of March. Seed tubers of cv. Nahodka were produced from micropropagated stock (Cassells et al., 1988). Blocks were planted for all treatments. Each block consisted of approximately 600 plants with interplant spacing of 30 cm and inter-row spacing of 70 cm, equivalent to approx. 46,200 plants/ha. Blocks were not replicated in this trial.

2.2. Disease monitoring

Disease surveys were carried out at the end of the growing season. Sclerotinia-infected plants were recognised by necrotic basal stem lesions associated with characteristic cottony mycelium and the presence of sclerotia. A sample harvest was carried out of healthy and diseased plants, it was determined by random sampling of the plots. 10 healthy plants and 10 infected plants from each block were

lifted at random avoiding the margins. Tubers were washed, dried and the fresh weight recorded.

2.3 Mineral analyses

Five samples of bulk soil (approximately 100g) were taken from the top 3 cm of soil in each plot. The soil was collected randomly across each block and 5 samples from each block were pooled into a single plastic bag and tied. Samples were kept at 4°C until required. Leaves were collected at random from each of the blocks and sent for analyses. A commercial laboratory (Teagasc Johnstown, Co. Wexford), carried out soil and leaf analyses.

2.4 Data Analyses

A chi-squared analysis was carried out on the numbers of diseased plants observed in the plots and the percentage data was graphed for presentational purposes. All correlations were carried out using linear regression analyses in the GraphPad Prism ™ 2.0 software package. R^2 values given were checked for statistical significance by checking the R value at the 5% level on the Critical Values for Correlation Coefficient Table (Zar 1996). All correlation graphs presented are significant at this level.

3. Results

3.1 Effect of fertiliser on disease levels and yield

Yield comparisons for healthy plants are shown in Fig. 1. The Hi CAN and CAN treatments show the highest yields at 33,949kg/ha and 33,445kg/ha respectively. While the lowest was given by the crop fertilised with ASN at 26,481 kg/ha.

Included on this graph are yields corrected for the percentage disease loss observed. This shows a small difference between healthy and corrected yields for Hi CAN, differences in the other treatments are greater. Chi squared analyses showed that significantly fewer plants were diseased when Hi CAN was used ($p<0.01$) (Fig 2). The CAN and ASN treatments did not differ significantly.

3.2 Leaf and Soil analyses

The results received for leaf calcium and soil analyses results are shown in Tables 1 and 2 respectively. The leaf analyses shows that the highest of the treatments is Hi CAN leaves which had 2.86PPM, CAN had 2.76, while the lowest result was from the negative control plots at 2.52. The soil analyses showed that for the phosphorus analyses the Hi CAN had 19 mg/l and ASN had 18 mg/l. The lowest phosphorus result came from the control plots at 13.8 mg/l. Soil analyses showed that the pH for all treatments was pH 6.1/6.2

3.3 Interaction analyses between fertiliser levels used and disease.

Calcium leaf and soil levels (Fig 3) showed a positive correlation, significant at the 10% level ($p< 0.10$). Soil nitrogen and plant calcium showed a significant interrelationship shown in Fig 4. Calcium fertiliser rates were aligned with the leaf calcium concentrations and also the percentage loss observed at harvest. Both relationships were found to be significant at the 5% and 10% levels respectively (Figs 5 and 6). The last analysis shown (Fig 7) found that there was a correlation between the fertiliser nitrogen and the percentage losses seen in the field due to S. sclerotiorum ($p< 0.05$).

4. Discussion

Linear analysis showed a significant correlation between the calcium present in the fertiliser and the disease reduction observed in the artichoke crop (Fig 6). A significant relationship was seen between the calcium rates applied and the calcium present in the leaf (Fig 5). As calcium rates were increased so was the uptake, which would contribute to resistance. Both calcium (cation) and nitrate (anion) were doubled for the Hi CAN treatment and could have created a counter ion effect and increased calcium (cation) assimilation. Such conditions usually promote optimal growth and hence contribute to disease resistance (Marschner, 1988). In addition a significant negative correlation was found to exist between the amount of nitrogen in the fertilisers and the disease incidence. Other authors would agree with this finding as increased nitrogen addition to the soil usually increases plant vigour and yield and also resistance (Gamliel et al., 2000).

Both the CAN and ASN costs were in the region of Euro63/Ha, while the Hi CAN treatment was twice this at Euro127/Ha. Costs of artichokes are currently extremely high due to the lack of domestic suppliers. If production was started a comparable market might be the potato one. Ware potatoes trade in extremely high volume at a cost of Euro127/tonne (Personal Communication, Dr. Leslie Dowley, Potato Research, Teagasc, Ireland.). However trade is unlikely to reach such high volume for the Jerusalem artichoke crop and so comparisons on market price were made based on seed potato prices. The seed potato and artichoke markets may be similar in terms of a more specialised niche market. The average price per tonne of seed potato is Euro381 (Personal Communication, Dr. Leslie Dowley, Potato Research, Teagasc, Ireland.). Currently the artichoke market is much smaller and the prices per tonne are extremely inflated due to short supply (~Euro1270/tonne-

Superquinn Supermarkets, personal communication, July 2001). Prices of treatments and market price of yields achieved is shown are Fig. 8. It can be deduced from the graph that the price of the most expensive treatment, Hi CAN, could be absorbed easily if the crop was sold at Euro381/t?.

The results indicate that increased calcium fertiliser application has potential to control Sclerotinia disease in Helianthus tuberosus. This in agreement with the finding of an earlier mutation breeding progamme for increased calcium uptake in H. tuberosus. Lines with improved Sclerotinia disease resistance were shown to accumulate high levels of calcium (Cassells and Walsh, 1995). While relatively inexpensive compared to hybridization, mutation breeding is expensive for a minor crop like H. tuberosus and it has to be repeated for each commercial variety. The calcium fertiliser treatment appears to offer an economical treatment which should be applicable to a wide range of varieties and crops susceptible to Sclerotinia disease. This hypothesis needs further testing.

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Table 1. Calcium Leaf analyses.

Treatment	ASN	CAN	Hi CAN
Calcium concentration / PPM	2.52	2.76	2.86

Table 2. Post harvest Soil Analyses

	ASN	CAN	Hi CAN
Nitrogen (NO_3N) PPM	6	7.5	8
Calcium mg/L	1500	1510	1530
Phosphorus mg/L	18	13.8	19
Potassium mg/L	208	197	196
Magnesium mg/L	84.1	86.4	82.6
pH	6.1	6.2	6.2

Fig. 1: Yields of healthy plants and yields corrected for disease loss, from plots with different levels of calcium in the fertilisers. Treatment codes: CAN, Calcium ammonium nitrate; Hi CAN, Double Calcium ammonium nitrate; ASN, Ammonium sulphate nitrate

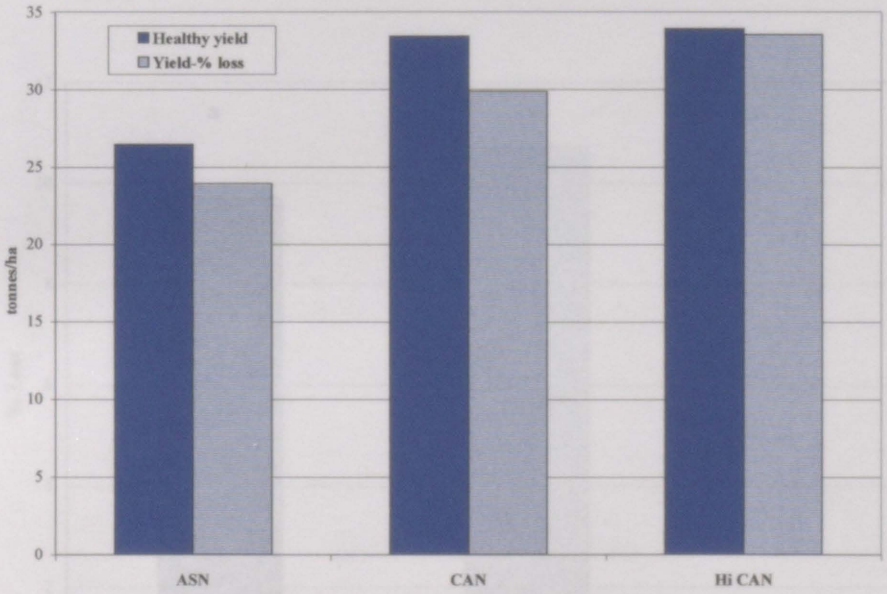


Fig. 2: Percentage Disease observed at Harvest in the plots with different levels of calcium fertilisers, codes as for Fig. 1. Chi squared analyses showed that Hi CAN was significantly different from the ASN and CAN treatments ($p > 0.01$) (Count data was analysed but for presentational purposes percentage data graphed)

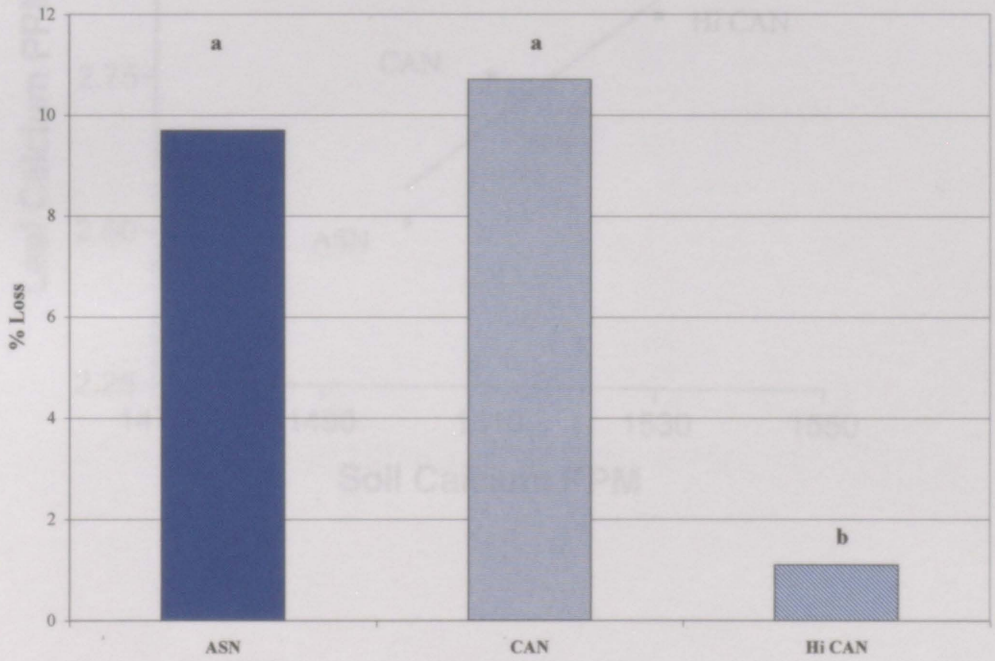


Fig. 3: Correlation of Leaf and Soil Calcium levels, codes as for Fig. 1. Linear analysis showed this to be significant at the 10% level.

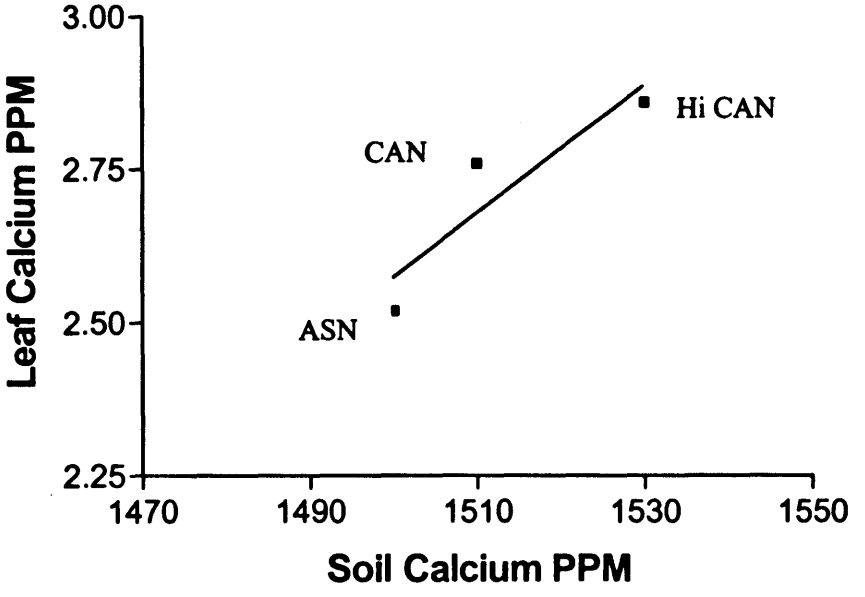


Fig. 4: Correlation of Soil Nitrogen and Plant Calcium levels, codes as for Fig. 1. Linear analysis showed this to be significant at the 5% level.

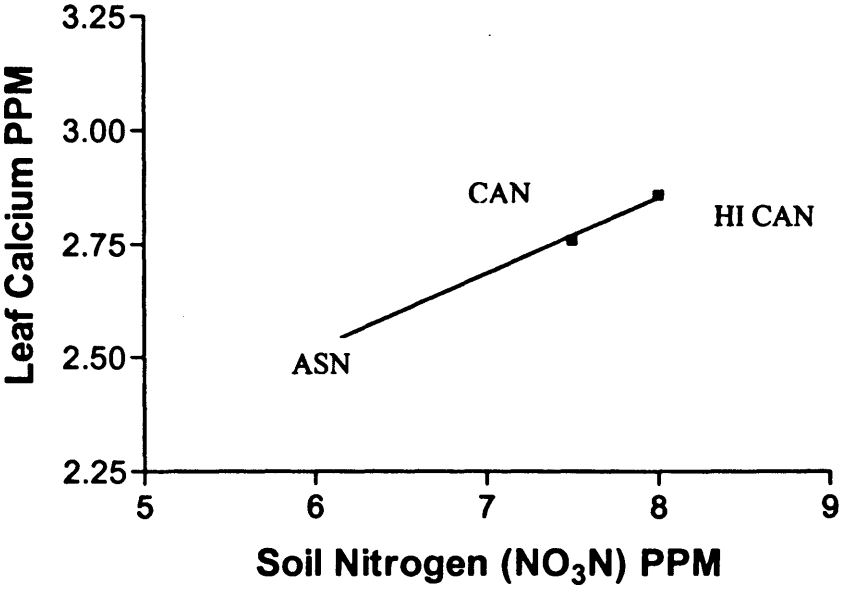


Fig. 5: Correlation of the Calcium rates used per treatment and leaf Calcium, codes as for Fig 1. Linear analysis showed this to be significant at the 5% level.

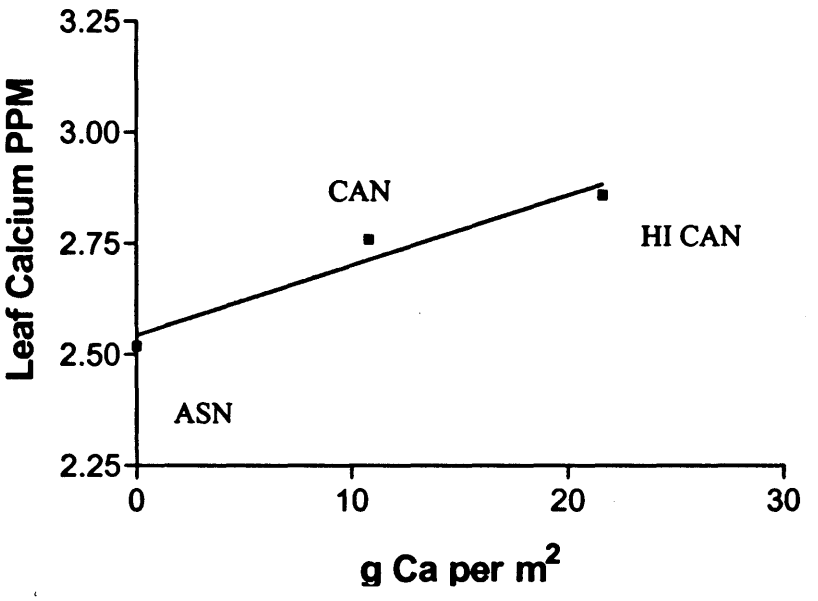


Fig. 6: Correlation of the Calcium rates used per treatment and Percentage loss observed at harvest, codes as for Fig 1. Linear analysis showed this to be significant at the 10% level.

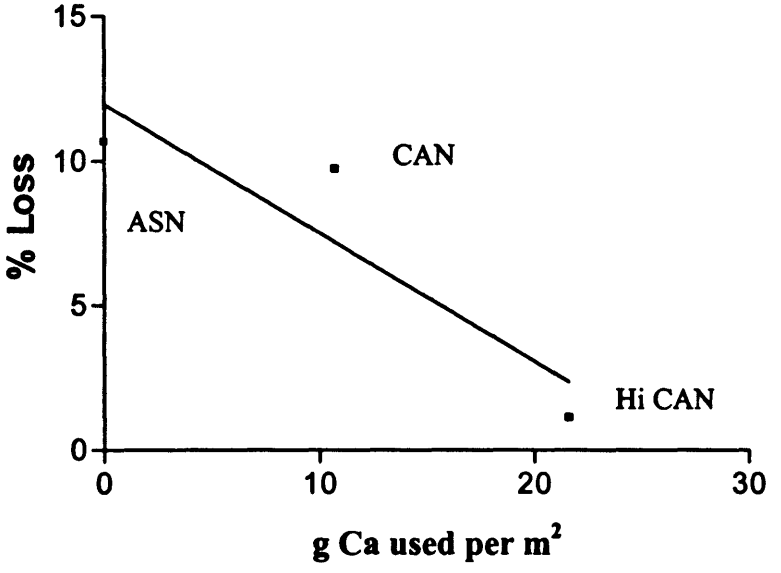


Fig. 7: Correlation of the Nitrogen rates used per treatment and Percentage loss observed at harvest., codes as for Fig 1. Linear analysis showed this to be significant at the 5% level.

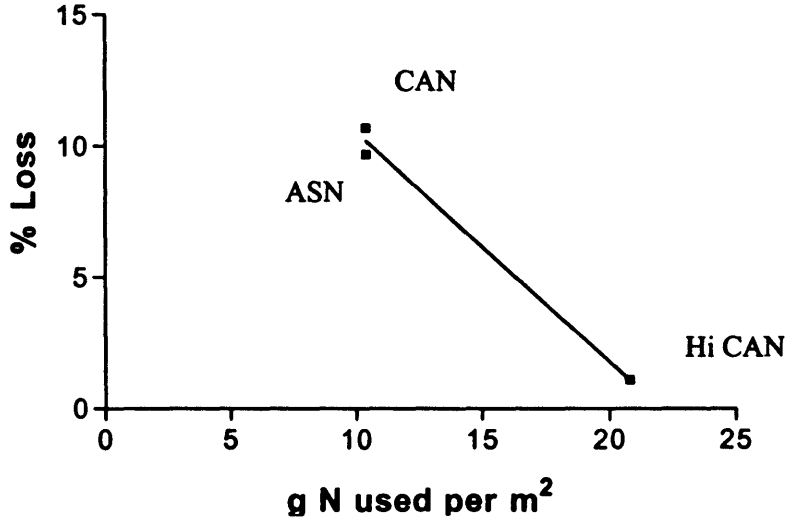
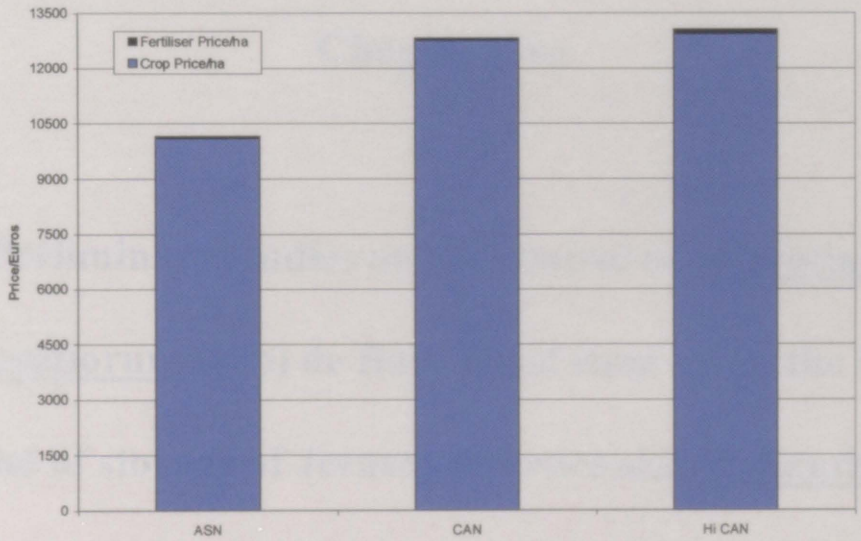


Fig. 8 Cost per hectare of the fertilisers used against the market price of the crop per hectare, codes as for Fig. 1



Chapter Five

Preliminary studies on the control of Sclerotinia sclerotiorum (Lib) de Bary basal stem rot in the field and of storage of Jerusalem artichoke (Helianthus tuberosus L.) using chitin-continuing shellfish waste

Section B: Investigation of the biocontrol properties of chitin-containing crustacean shellfish waste

Preface to Chapter 5

This chapter investigates the biological control potential of crushed crustacean shells (CCS) as a soil amendment in the field. The preliminary trial reported was carried out in parallel with the calcium trials in the previous chapter (4). The style is that of the journal Applied Soil Ecology.

Preliminary studies on the control of Sclerotinia sclerotiorum (Lib) de Bary basal stem rot in the field and of storage rots of Jerusalem artichoke (Helianthus tuberosus L.) using chitin-containing shellfish waste

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Abstract

Chitin-containing shellfish waste, calcified seaweed and organic fermentation waste, the latter used in combination, were tested as soil amendments for their effects against Sclerotinia disease of Jerusalem artichoke in the field. Furthermore, peats amended with shellfish waste, cellulose and nitrogenous fermentation waste, were evaluated for their effects against tuber storage rots. Soil amended with shellfish waste and peat formulated with shellfish waste, suppressed Sclerotinia disease in the field and storage rots, reducing the number of Sclerotinia-infected plants in the field by 54% and the number of rotted tubers by 58% compared to the controls. The biological control promoted by shellfish waste was correlated with stimulation of antagonists in the respective substrates. The potential of shellfish waste in the organic production of Jerusalem artichoke ware, processing and seed tubers is discussed.

Keywords: Cellulase, chitin, chitinase, Helianthus tuberosus, pathogenesis-related proteins, soil antagonists, suppressive soil

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1. Introduction

In the 1980s the potential of Jerusalem artichoke was evaluated in Europe as a biomass crop for industrial uses such as ethanol production, based on its high yield, hardiness and low production cost (Denoroy, 1996). Jerusalem artichoke stores carbohydrate in its tubers in the form of inulin, a fructose polymer. More recently, Jerusalem artichoke has attracted attention as a source of functional food ingredients. In human nutrition, inulin functions as a dietary fibre and fructose polymers of low chain length are selective substrates (neutraceuticals or 'pre-biotics') for beneficial bifidobacteria in the human colon (Modler et al., 1993).

Jerusalem artichoke, a native of North America and close relative of sunflower (H. annus L.) (Kohler and Friedt, 1999), grows well in temperate regions. Yields of 50-70 t/ha and higher, have been reported in southern Europe but to achieve this, irrigation is required (Denoroy, 1996). The crop yields 55-65 t/ha without irrigation in the more maritime regions of Europe (Cassells and Deadman, 1993). The growing Jerusalem artichoke crop is prone to the same spectrum of field diseases as its relative, the widely cultivated sunflower (McCarter, 1984). The most important of these in cool maritime regions is Sclerotinia stem rot (Cassells et al., 1988). Sclerotia in the soil can cause infection of the stem base via production of mycelia which directly invade the tissues (myceliogenic germination). Myceliogenic infection was present at the trial site where disease was seen to occur in discreet areas of the field as opposed to a more random distribution that would indicate infection by ascospores (Quinlan, 1992).

Stored tubers of Jerusalem artichoke, because of their poorly developed periderm are particularly vulnerable to cottony rot caused by Sclerotina sclerotiorum and other

storage/wound rots caused by fungi and bacteria (Cassells et al., 1988). Aside from storage rot caused by S. sclerotiorum, contaminating sclerotia and mycelium may be transmitted with seed tubers to infect the new crop (Masirevic and Gulya, 1992). Consequently, control of Sclerotinia is critical for ware, processing and seed tuber production.

Sclerotinia causes disease in 64 families and 225 genera (Purdy, 1979) and is widespread in agricultural soils and in temperate crop rotations e.g. involving oil seed rape (Bailey et al., 2000) and sunflower (Masirevic and Gulya, 1992) and so there is a high probability that the soil will contain inoculum. Soil chemical sterilisation to reduce or eliminate inoculum is not an economic option and is now problematic due to the imminent withdrawal of methyl bromide from the market (Akhtar and Malik, 2000; Gamliel et al., 2000). Washing the tubers prior to storage to remove sclerotia can result in high losses due to damage caused by facultative/wound pathogens, a consequence of the thin periderm. This crop has the potential to be grown organically/ ecologically (Anon., 1995), as its rapid growth and dense, tall canopy effectively smothers weeds, eliminating the requirement for herbicides. Other than Sclerotinia, there are no significant haulm or foliar diseases or pest infestation requiring pesticides, at least in the cool maritime regions (Denoroy, 1996; Cassells and Deadman, 1993). While successful chemical treatments have been developed for the control of Sclerotinia in canola (Bailey et al., 2000), the canopy height of 2-3 m, depending on cultivar, makes chemical application impractical for Jerusalem artichoke. Organic or ecological production, may be impractical, where there are limitations on the availability of certified land (Anon., 1995) free of Sclerotinia, because rotations of up to a 10 years are recommended for the elimination of S. sclerotiorum (Masirevic and Gulya, 1992).

Continuous cropping has been reported to result in Sclerotinia 'decline' in sunflower after a peak in disease incidence after 5-6 years (Huang and Kozub, 1991). This approach merits investigation for the control of S. sclerotiorum in the cool maritime regions.

Here, the objective was to evaluate the potential of disease control based on the use of a soil amendment, namely shellfish waste in the form of crushed shells of shrimp and crab. It was chosen as it is in plentiful supply and is a source of chitin which has been shown to exert biological control through its promotion of antagonistic soil micro-organisms (Mitchell and Alexander, 1962). Chitin (Evans, 1993; Ren and West, 1992) and its derivatives (Akiyama et al., 1995; Gagnon and Ibrahim, 1997; Pearce et al., 1998) are also reported to induce plant disease resistance. In the first year's trial a decrease in percentage disease was observed when the crushed crustacean shells (CCS) were incorporated in the soil. The trial was repeated in a second year to confirm the result and to assess whether the affects were attributable solely to nutritional factors associated with the shellfish waste or whether biological control was promoted. Calcified seaweed (approved for organic production by Anon. (1995) and NitroGro III (organic waste product from citric acid fermentation, formulated as an organic nitrogen fertiliser) were used as sources of calcium and organic nitrogen, respectively. Calcium has been implicated as a host resistance factor to Sclerotinia disease (Cassells and Walsh, 1995) and calcified seaweed has been used as a soil conditioner (Tye, 1996). Application of organic nitrogen has also been reported to suppress soil borne diseases (Gamliel et al., 2000). Their use in combination was designed to provide equivalent nitrogen and calcium to the shellfish waste amendments and to check for possible interactive effects of organic nitrogen and calcium. It is important to emphasise that the shellfish waste

and calcified seaweed treatments used here were not supplemented with inorganic nitrogenous fertiliser as this would have breached the rules for organic production (Anon., 1995)

Based on experience from 14 years of field trials at different locations, a site with a predicted inoculum potential to reduce crop yield by c. 20% was chosen for the trials. This inoculum potential was arbitrarily chosen to represent the level predicted for the third year of continuous culture. In the second trial in order to elucidate the mechanism(s) of any suppressive effects on disease development in the field, effects on soil chitinolytic and proteolytic microorganisms (Bonmati et al., 1998) and soil chitinase and cellulase activities were determined (El-Tarabily et al., 2000; Nielsen and Sorensen, 1997). Tuber chitinase and cellulase levels were assayed as markers of induced resistance in the host plant (Jung et al., 1995).

In the storage trial sphagnum peat with shellfish waste, cellulose and NitroGro III amendments were also investigated as a dressing applied to the tubers going into store to suppress storage diseases. Shellfish waste was used to stimulate chitinolytic peat micro-organisms (Mitchell and Alexander 1962), cellulose (as an organic carbon source) and NitroGro III (as a source of organic nitrogen) amendments were added as substrates to evaluate their potential to stimulate native peat-based antagonists.

2. Materials and methods

2.1. Field trials

The trials site was sprayed with glyphosate (Roundup; Monsanto (Irl.) Ltd, Dublin, Ireland) and 3 weeks later ploughed to a depth of 20 cm. The site was

rotavated and fertilised with calcium ammonium nitrate or with the soil amendments. All amendments used were applied 4-6 weeks before planting to allow these to mature in the soil as recommended by manufacturers of commercial chitin biocontrol formulations e.g. Clandosan (Igene Biotechnology Inc., Columbia, MD, USA). Control plots, for both the first and second trials, were fertilised with calcium ammonium nitrate (27.5% nitrogen; 4 % calcium) 400 kg/ha, which is the standard fertiliser used in this temperate region (Cassells and Deadman, 1993). Experimental plots were treated with shellfish waste (3.5% nitrogen; 21% calcium; Landtech Soils Ltd., Nenagh, Co. Tipperary, Ireland) at 600 kg/ha in the first and second year or in the second year only, with calcified seaweed (0% nitrogen; 21% calcium; Celtic Sea Minerals, Strand Farm, Currabinny, Co. Cork, Ireland) at 600 kg/ha or NitroGro III (18% nitrogen, 0% calcium: ADM, Ringaskiddy, Co. Cork) at 117 kg/ha; with a combination of calcified seaweed and NitroGro III at the rates given above. The calcified seaweed amounts were chosen to mirror the calcium levels found in crushed crustacean shells and the rate of NitroGro III was chosen to reflect the nitrogen levels in the shell treatment. Trials were planted at the end of March. Seed tubers of cv. Nahodka were produced from micropropagated stock (Cassells et al., 1988) and were from the second field multiplication cycle. In the first year large blocks of both the control and the CCS treated artichokes were planted. Each block consisted of approximately 600 plants with interplant spacing of 30 cm and inter-row spacing of 70 cm, equivalent to approx. 46,200 plants/ha. Blocks were not replicated in this preliminary trial. However, in the second year the trial was set up in a randomised block design, consisting of 5 replicate blocks of 25m^2 each per treatment. Each block consisted of 100 plants with the same spacing as the first trial. The site was sprayed with paraquat (Gramoxone 100; Zeneca (Irl.) Ltd, Dublin,

Ireland) and terbutylazine (Opogard; Ciba Geigy, Dublin, Ireland) at 20% emergence at the manufacturers' recommended rates.

2.2. Disease monitoring

Disease assessments were made during the growing season (May and September). Sclerotinia-infected plants were recognised by necrotic basal stem lesions associated with characteristic cottony mycelium and the presence of sclerotia. In November, a final assessment was made and the fresh weight of tubers from healthy and diseased plants was determined at harvest by random sampling of the replicate plots. Three healthy plants and three infected plants from each replicate block were lifted at random avoiding the margins, that is, a total of 15 healthy and 15 diseased plants per treatment were sampled. Tubers were washed, dried and then weighed.

2.3. Soil microbiology

During the second trial, in May, when the plants were one month-old, and again in September (mid growing season) soil samples were taken and the chitinase- and protease-producing microbial population in the soil were determined.

Five samples of bulk soil (approximately 100g) were taken from the top 3 cm of soil in each plot. The soil was collected at 5 points from a W pattern across each plot and the 5 samples from each plot were pooled into a single plastic bag and tied. Samples were kept at 4°C until required. These samples were also used for enzyme analyses.

Selective media for chitinolytic bacteria was prepared according to the method of Friesman and Chet (personal communication): 3 g/l K_2HPO_4, 1.0g/l $MgSO_4.7H_2O$, 0.5 g/l $(NH_4)_2SO_4$, 0.8 g/l colloidal chitin, 2.0 g/l yeast extract, 20 g/l purified agar (Oxoid, Basingstoke, UK). Colloidal chitin was made up as follows: 0.18 l of conc. HCl was added with stirring to 20 g of chitin (Sigma Chemical Co. St Louis, MO 63178, USA, Cat no. C-7170). This was allowed to stand for 2 h with intermittent stirring. The solution was poured into a 5l container half filled with tap water, a suspension in water forms and the volume was brought up to 5l with tap water. The suspension was allowed to stand overnight and then washed in tap water. This was repeated 4 times followed by 3 washes in distilled water. The suspension was then passed through a sieve (0.1 mm mesh) to remove large particles. The resulting suspension had a pH of 5.5-6 and was stored in the dark at 4°C. Prior to autoclaving, the chitin concentration was determined gravimetrically after drying in an infra-red dryer. Soil samples were serially diluted in quarter strength Ringer's solution (Oxoid, Basingstoke, UK) and plated onto 3 replicate petri-dishes of medium. Petri-dishes were incubated at 25°C for 5-7 days and chitinase producers were characterised by clear zones around the colonies.

The following medium was used to enumerate protease producers: 30g of skimmed milk powder was autoclaved in 300 ml of distilled water for 10 min at 68.9 kPa. 12 g of agar in 700 ml of distilled water was autoclaved for 15 min at 103.4 kPa. These were mixed at 40-50°C after autoclaving. Soil samples were serially diluted in quarter strength Ringer's solution and plated onto 3 replicate petri-dishes of medium. Petri-dishes were incubated at 25°C for 5-7 days and protease producers were characterised by clear zones around the colonies.

2.4. Soil enzymology

The soil samples as described above were also used for enzyme analyses. The procedure for extraction of enzymes from soil was based on Wirth and Wolf (1992). 5 ml 0.5 M sodium acetate-acetic acid buffer, pH 5, per 1 g dry weight of soil, were mixed using a magnetic stirrer for 1 h. The suspension was then centrifuged at 28,950 g for 10 min at 4°C and the supernatant filtered through glass fibre filter paper. The supernatant was then stored at -20°C prior to analysis.

A colorimetric assay was used to determine endo-chitinase activity in the soil samples (Wirth and Wolf, 1992). The substrate was carboxymethyl-substituted soluble chitin (CM-chitin) covalently linked with Remazol Brilliant Violet 5R (RBV). The colorimetric assay is based on the precipitation of unreacted CM-chitin-RBV from buffered extract solutions with HCl (Wirth and Wolf, 1992). Based on the same principle, the substrate carboxymethyl-cellulose-Remazol Brilliant Blue 5R (CM-cellulose-RBB) was used for endo-cellulase assay (Wirth and Wolf, 1992; 1990). The substrates were obtained from Blue Substrates (Grisebachstrasse 6, D-3400, Göttingen, Germany). Assays were performed in 96-well microtitre plates (Costar Europe, High Wycombe, UK; cat no. 3590). Each well contained the following: 50 µl of substrate, 100 µl of extract, 50 µl of buffer (0.2 M sodium acetate - acetic acid buffer, pH5). Extract was added to the control wells after the acid addition; 4 control and 8 test replicates were assayed. Incubation was carried out at 40°C for 3 hours. The reaction was stopped by the addition of 50 µl of HCl (1N for CM-Chitin-RBV and 2N for CM-cellulose-RBB). Plates were cooled on ice and centrifuged (1,450 g for 10 min). 175 µl of supernatants were transferred to a 96-well half-size EIA plate (Costar, cat no 3690). Activity was read at 550 nm for Chitin-RBV and at 600 nm for Cellulose-RBB. Extracts with a reading > 0.1 were

diluted and assayed again as they were substrate limited. Calculation of relative enzyme activity was carried out using the following formula: Absorbance x 1000 x min^{-1}

2.5. Tuber storage trial

The trial was carried out in a ventilated shed at a mean temperature of +8°C (Max: +11.5 ^{0}C, Min: +4.6 ^{0}C). Tubers of cv Nadhodka were collected from control plots, excess soil was removed by hand and the tubers were arranged next to each other horizontally and vertically in layers, in Curver ™ nestable plastic containers each with a capacity of 0.037 m^3. Blank and control treatments were set up using tubers stored with no treatment and stored in unamended peat, respectively. Tubers were placed in a single layer and then the appropriate peat formulations were shaken over them, the next layer of tubers were placed on top and covered with peat again. The tubers were stored in a commercial peat amended with shellfish waste (Suppressor™; Landtech Soils Ltd., Nenagh, Co. Tipperary Ireland); in peat amended with 15 g/l cellulose or 3 g/l NitroGro III. These were allowed to mature for 4-6 weeks before use. There were five replicates of each treatment, each replicate contained 30-50 tubers (numbers differed reflecting variation in tuber sizes). The containers were covered with black polythene bags and loosely tied with twine. Containers were stacked in a non-insulated metal shed and stored at ambient temperature (mean +8^{0}C) and examined every 2 weeks for signs of infections. Final results were taken after 10 weeks.

2.6. Tuber enzyme extraction and analyses

Tuber enzyme was extracted after washing and peeling tubers, macerating them in a juice extractor and sieving through cheesecloth. Bisulphite solution (10 µl/ml) was added to the sap as an antioxidant (Appel *et al.*, 1995). Centrifugation was carried out at 13,000 g for 10 min and supernatants stored at -20°C until analysed. Enzyme analyses was carried out as described for the soil extracts.

2.7. Microbiology of crushed crustacean shells

The crushed shells used in this trial were sent for independent testing for pathogens associated with shellfish and food poisoning, to the Department of Clinical Microbiology, Central Research Laboratory, St James Hospital, University of Dublin, Dublin 8.

2.8. Statistical analyses

The initial field trial was not replicated and so count data were subjected to chi-squared analyses. For the purposes of graphical representation the percentage disease is presented and values sharing the same letter are not significantly different ($P<0.05$). For the second trial, all data were found to be non parametric and most could not be normalised (except storage data) and so was subjected to the Kruskal-Wallis analysis (non-parametric ANOVA). Median quantities are presented in the case of non-parametric data. Statistical letters are indicated on the graphs and tables and those medians sharing the same letter are not significantly different ($P<0.05$). In the case of the storage trial, the data were normalised using the square root function and were subjected to a one way Anova analysis using Data Desk™ software. Data

presented show the mean values and those sharing the same letter are not significantly different ($P<0.05$).

3. Results

3.1. The effects of treatment on Sclerotinia disease development

The preliminary trial set up in 1996 was to investigate the effects of crustacean shellfish waste on suppression of the Sclerotinia disease. The results showed that there was a suppression of disease (Fig 1). It decided to repeat the trial in the second year in a randomised replicate block design and also to use other forms of organic amendments to determine whether the effects were nutritional or involved biological control. The development of S. sclerotiorum disease in the field for the second trial is shown in Fig. 2. It can be seen that 20% of the plants in the control plots were affected by S. sclerotiorum based on visual examination for the presence of sclerotia. Disease was suppressed by all the treatments, with the highest suppression, approx. 11% of the plants diseased, in the shellfish waste soil amendment. This a 54% reduction in diseased plants compared to the control. The effects of the treatments on tuber yield are shown in Table 1. The data show no significant difference in yield between healthy plants in the treatments and those in the control and there were no significant differences between tuber yields from infected plants and those in the control. Yields from infected plants were reduced on average by approximately 85%.

3.2 Effects of treatments on soil chitinase and protease-producing micro-organisms

Soil chitinase- and protease-producing micro-organisms were assayed in May and September. Chitinase producers in the control were at the threshold of detection in the May samples (Table 2). A higher level of activity was detected in September. In the shellfish waste amended treatment, chitinase producers were raised to c. 23 x 10^6 cfu/ml in the May assay and declined to 0.45 x 10^6 in the September soil extracts. Calcified seaweed amendment significantly increased soil chitinase activity in May but the levels in September were less than the control. NitroGro III did not affect chitinase levels, however, the combination of calcified seaweed and NitroGro III significantly increased chitinase activity in May but much less than that induced by shellfish waste; in September the value had declined to that of the control.

The numbers of protease-producing micro-organisms were lower in the control soil extracts in May compared with all treatments except for the NitroGro III treatment; they were higher in September than the NitroGro III and combined NitroGro III and calcified combined treatments. Addition of CCS increased protease producers in May and the number was still higher than others in September though lower than those in May. (Table 3).

3.3. The effects of treatments on soil enzymes

The effects of the soil amendments on soil chitinase and cellulase are shown in Tables 4 and 5. The data show that all treatments except calcified seaweed increased soil chitinase in May and the shellfish waste and NitroGro III treatments also in September, compared to the control with the highest increases in the shellfish waste treatment (Table 4; see Table 2 for comparative data for soil microorganisms). Cellulase levels were not significantly altered in the treatments (Table 5).

3.4. Effects of amendments on host plant chitinase and cellulase activity

Tuber chitinase and cellulase activities did not differ between healthy plants from the controls and treatments (data not shown). However, chitinase and cellulase were in significantly greater amounts in diseased tubers from the shellfish waste treatment, as was chitinase in the calcified seaweed treatment, compared with the control (Figs 3 and 4, respectively). There were significant decreases in chitinase in the diseased tubers from the NitroGro III treatment and the combined NitroGro III and calcified seaweed. There was a significant increase in cellulase in the latter treatment in the diseased tubers compared to the diseased control tubers.

3.5. Effects of treatments on disease development in store

Tubers were stored alone, in peat and in peat amended with shellfish waste (Suppressor™), cellulose, and NitroGro III, respectively. The mean storage temperature was +8°C. Infected tubers were recorded as having cottony, brown and soft rot and were examined for bacterial or fungal rots. The results show that 95% of the tubers stored without treatment developed disease (Fig. 5). Disease incidence was significantly reduced to 37% in the shellfish waste amended peat compared to the blank untreated and peat dressed controls but not significantly reduced in the other amended peats. When sampling the stored tubers it was noted that infection in the shellfish waste amended peat containers remained localised. Infection was seen to spread in the other treatments. Sprouting had occurred in all the treatments by the end of the trial. It was observed that the sprouts were killed, corresponding to the level of infection, in all the treatments except in the shellfish waste amended peat. A comparison of tubers from the shellfish waste and the blank (control, no peat)

treatment, showing the healthy sprouts in the former and the necrotic sprouts in the latter is presented in Fig. 6.

3.6. Microbiology of the crustacean shellfish waste

Tests were carried out for total viable bacterial counts and for shellfish-associated human pathogens: Staphyloccus aureus, Salmonella spp., Listeria spp., Campylobacter spp., Helicobacter pylori, Eschrichia coli and Vibrio spp. The total viable count was 20,000 cfu/g. None of the specified pathogenic genera/species were detected.

4. Discussion

In fourteen years of field trials in Ireland, Sclerotinia sclerotiorum has been identified as the only major biotic threat to stable crop production. Due to the ubiquity of S. sclerotiorum in agricultural soils and the increased acreages of susceptible crops, e.g. oil seed rape in rotations, disease outbreaks are highly likely. Given the longevity of sclerotia of up to 10 years in soil, crop rotation is not a practical strategy for disease elimination. Furthermore, the poor storage characteristics of the tubers, exacerbated by tuber washing (Rafferty, unpublished) increases the likelihood of significant losses in store and inoculum transmission with the seed tubers. Here, an organic soil amendment has been evaluated to reduce Sclerotinia disease losses in the field and Sclerotinia and other disease development in store. It has been shown that amendment of the soil with abundant, organic-production compatible, shellfish waste may have the potential to reduce both field

incidence of Sclerotinia field infection (Fig. 2) and to effect a more general reduction of storage losses (Figs 5 & 6).

Shellfish waste appears to exert biological control over inoculum in the field by promoting the growth of antagonistic chitinase- and protease-producing soil micro-organisms (Tables 2 & 3) and by increasing the levels of anti-fungal chitinolytic activity in the soil (Figs 3 & 4). There are strong correlations between the levels of these microorganisms and biological control of soil fungi (Cook and Baker, 1983).

Another mechanism whereby disease development may be restricted is by the action of chitin and chitin-breakdown product as elicitors of host anti-pathogen defence mechanisms. Enzyme assays of the infected tubers from plants grown in shellfish waste amended soil, but not in the control or other treatments, show statistically significant increases in the activity of chitinases and cellulases in the tubers (Figs. 3 & 4). Elevated chitinase but not cellulase was also detected in infected tubers from the calcified seaweed amended soils but not in the control or other treatments (Figs. 3 & 4). These results indicate that plants in the shellfish amended soil may have been sensitised to pathogen attack (Lusso and Kuc, 1995) and rapidly up-regulate the synthesis of pathogenesis-related proteins on infection, where chitinase and cellulase activities are markers of the latter. However, there was no correlation between the elevated chitinase/cellulase activity and tuber yield loss in infected tubers (Table 1). The mechanism of increase in chitinase without concomitant increase in cellulase activity in the calcified seaweed treatment requires further elucidation.

Storage of harvested tubers in shellfish waste-amended peat reduced storage rots, including Sclerotinia cottony rot, from almost total tuber loss in the controls and other treatments to 37% in the shellfish treatment (Figs. 5 & 6). These very high

losses emphasise a considerable problem particularly in the production of high quality propagation material where the tubers are lifted and stored over the intercrop period in mild temperate climates. The mean temperature during storage was +8°C (Max: +11.5 °C, Min: +4.6 °C) which is just over 2°C higher than the average November-February mean, +5.9°C (Max: +8.4°C, Min: +3.3°C) for the region (south west Ireland) which may have influenced disease severity. While the white cottony mycelium of Sclerotinia was ubiquitous on the diseased tubers in control and all the treatments, other fungi and bacteria also contributed to tuber decay. Due to the rapid onset of the tuber rots it was not possible to distinguish the primary and secondary causal agents and consequently, no attempt was made to isolate the putative pathogens previously reported (Cassells et al., 1988).

An important consideration when applying biological waste to crops is the possibility of contamination with potential human pathogenic bacteria (Beuchat, 1996). This risk is now becoming more widely recognised where the plant material is consumed raw (Rafferty and Cassells, 2000). The risk in the case of Jerusalem artichoke is relatively low as the tubers are usually cooked before consumption but the vegetable may be eaten raw, for example, shredded on salad. Health risks associated with shellfish are widely recognised (Huss et al., 2000) and so here, the commercial material was examined for human pathogenic bacteria to an accredited hospital laboratory. Total viable counts were low and the tests for food poisoning pathogens were negative.

Partial biological control of Sclerotinia has been reported in sunflower by treating the seed with bacterial inoculants (Hebbar et al., 1991) or by application of Talaromyces flavus and Coniothyrium minitans to the soil at seeding time (McLaren et al., 1994). The latter strategies are based on the introduction of antagonists

directly into the soil or on the planting material, and their success may be dependent on the resident soil microorganisms at the site of application. The strategy advocated here depends on semi-selective stimulation of native soil residents, which arguably, is less inoculant-soil dependent (Nelson and Craft, 2000).

While soil amendment with shellfish waste has some potential to reduce Sclerotinia disease development in the field by reduction of pathogen inoculum, this may not be cost effective. It will depend on the volume-dependent cost of the shellfish waste and the market price for the crop when it is traded in high volume. It is also recognised that biological control strategies are difficult to reproduce due to variability in the soil microbiological environment (Cook and Baker, 1983), that is, in the resident soil microflora in different soil types and the efficacy of the treatment would have to be confirmed in multi-site trials. The shellfish treatment could possibly be improved e.g. by supplementing with nitrogen to evaluate effects on yield. Here, the control nitrogen fertiliser application was optimised for the crop based on previous trials (Cassells and Deadman, 1993). The nitrogen content of the shellfish waste was approximately 20% of the control but was not supplemented as this would have compromised the organic production strategy. However, yield data for the different treatments did not indicate any mineral deficiencies.

The results show that long term-storage of Jerusalem artichoke tubers for processing or for seed presents major problems in mild regions. Jerusalem ware tubers are usually lifted and sold fresh before the land becomes unworkable. In previous years trials here, tubers were stored over winter in the soil for seed and processing but lifting in spring may be problematic due to unworkability of the land and seed losses may be high where sprouting has begun. Chemical treatment at lifting (Denoroy, 1996) not investigated here and storage in ventilated, temperature-

controlled potato stores may help prevent disease development but the former is not an option for organic growers and seed producers. Storage in peat amended with shellfish waste or other biocontrol treatments may be economic where organic certification is required.

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Fig. 1. Preliminary trial results showing the percentage disease in the Control and CCS (Crushed Crustacean Shells) plots. Chi-squared analyses was used on the count data and no significant difference was found (P> 0.5)

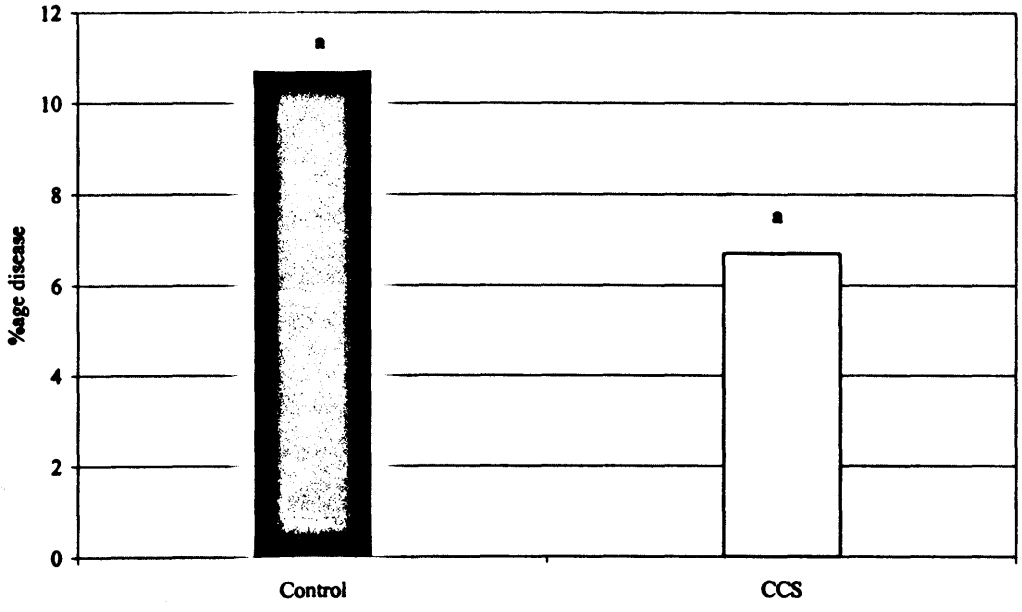

Fig. 2. The percentage diseased plants in the control and treatment plots. CCS- Crushed Crustacean Shells; CaS – calcified seaweed; NIII – NitroGro III, CaS and NIII combined calcified seaweed and NitroGro III amendments, respectively.

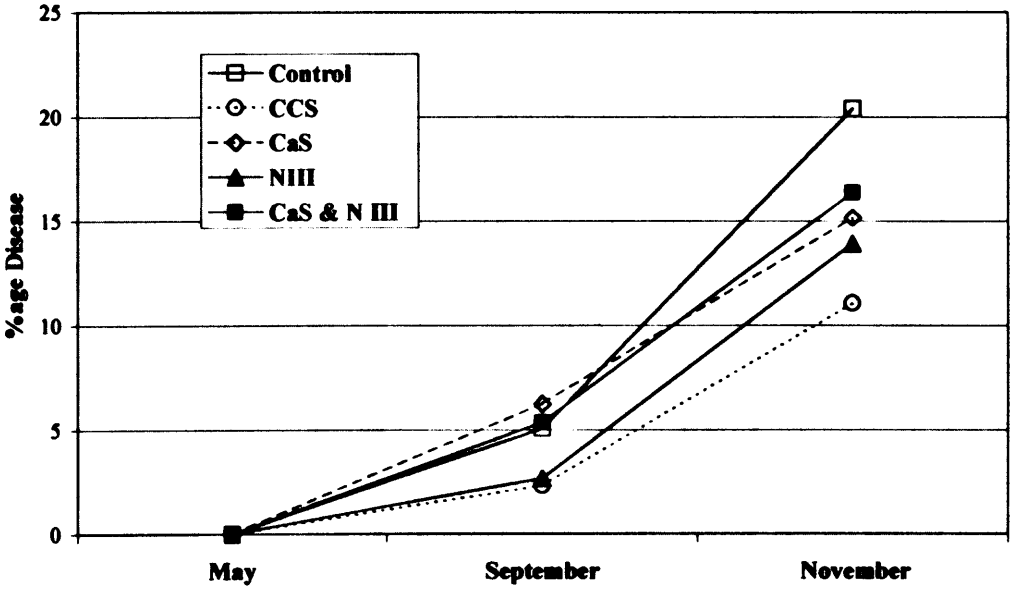

Fig. 3. Chitinase activity in tubers from infected plants. Treatment codes as Fig. 2. Data was subjected to the Kruskal-Wallis analysis. Columns sharing the same letter are not significantly different ($P < 0.05$).

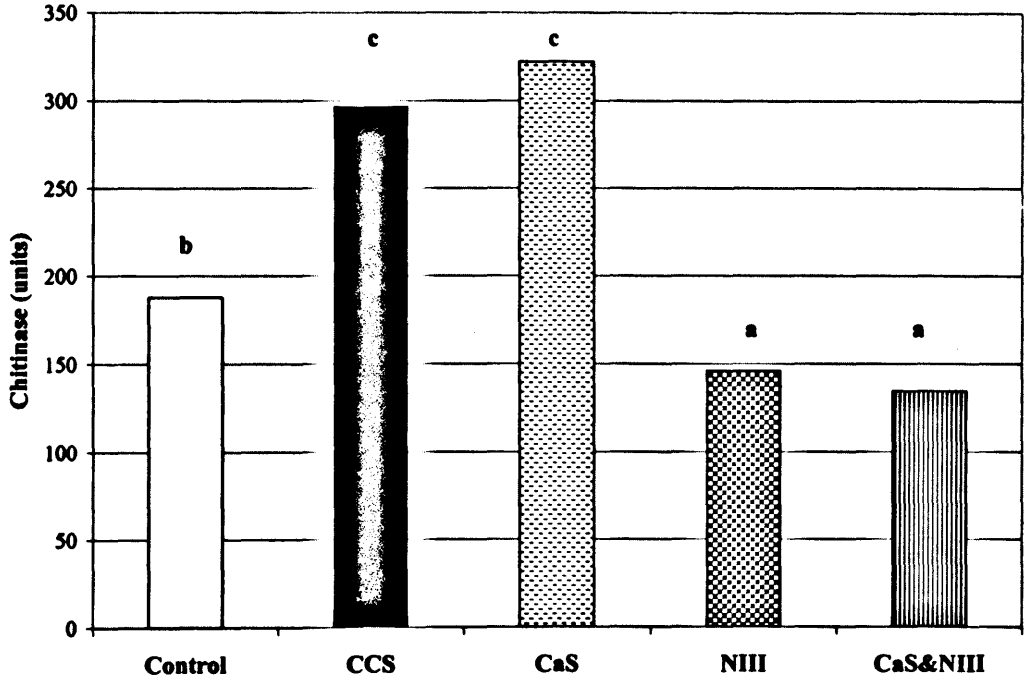

Fig. 4. Cellulase activity in tubers from infected plants. Treatment codes as Fig. 2. Data was subjected to the Kruskal-Wallis analysis. Columns sharing the same letter are not significantly different ($P < 0.05$).

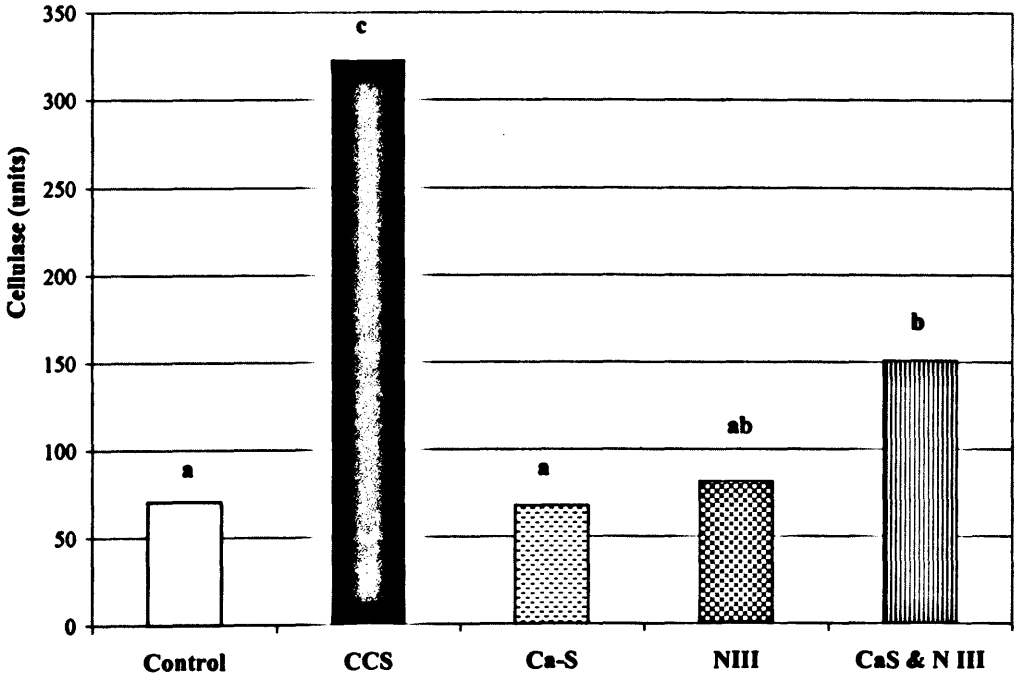

Fig. 5. Percentage of infected tuber in the control and treatments in the storage trial. Data was normalised using the square root function and subjected to a one way Anova analysis using Data Desk™ software. Columns sharing the same letter are not significantly different ($P < 0.05$).

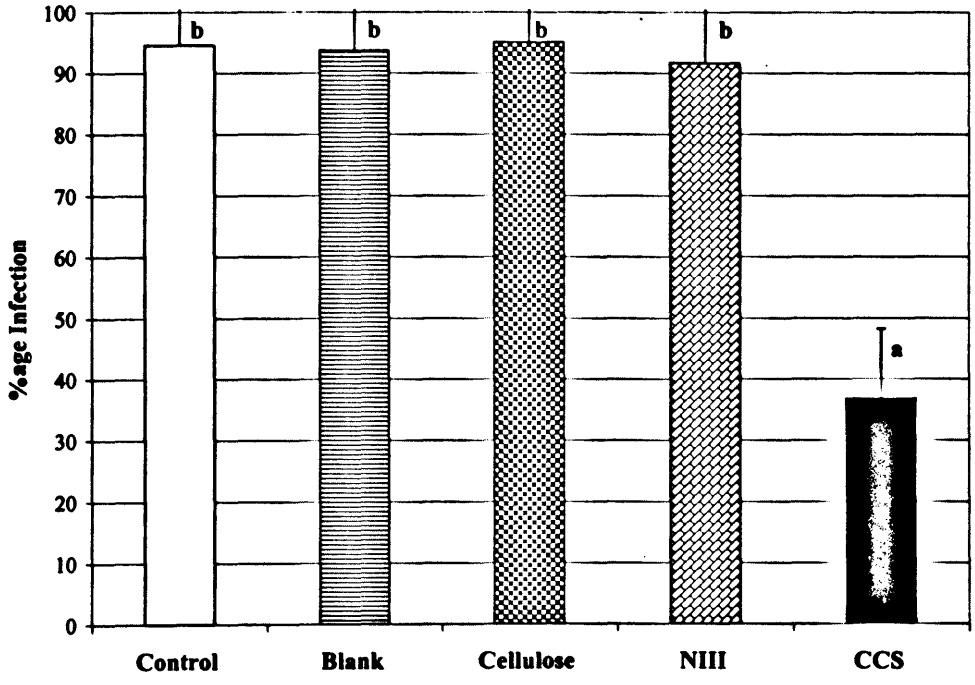

Fig. 6. Photograph of tubers from the control and Suppresser™ (peat amended with chitin) trials. Note the healthy sprouts in the Suppresser™ treatment (*top*) compared to the rotted sprouts in the blank control (*bottom*).

Table 1 Comparison of Yields for Second Trial

	Control	CCS	CaS	NIII	CaS & NIII
HT	62.88a	50.96a	52.02a	52.76a	51.2a
IT	10.03a	10.49a	5.41a	12.34a	4.48a

HT: Tubers from healthy plants IT: Tubers from infected plants

Data was non-parametric so values presented are median tonnes/ha

Data from healthy and infected plants were statistically analysed separately.

Kruskal-Wallis analysis was carried out and values sharing the same letter are not significantly different ($P < 0.05$)

Table 2 Quantification of chitinase producers in soil

	Control	CCS	CaS	NIII	CaS & NIII
May Chitinase Producers	0.000 a	22.522 c	0.333 bc	0.000 a	1.454 bc
Sept Chitinase Producers	0.047 bc	0.467 bc	0.000 a	0.007 a	0.107 b

Data was non-parametric so values presented are median cfu x 10^6

Kruskal-Wallis analysis was carried out and values sharing the same letter are not significantly different ($P < 0.05$). The high number of zero readings on selective media created a tied value for most data during ranking, hence there is no difference statistically between the SFW (May) reading of 22.522 million cfu/g and other readings greater than 0 (e.g. the Control (Sept) reading of 0.0467 million cfu/g).

Table 3 Quantification of protease producers in soil

	Control	CCS	CaS	NIII	CaS & NIII
May Protease Producers	29.260 ab	45.867 d	14.280 c	22.720 bc	20.3420 cd
Sept Protease Producers	4.399 bc	8.13 cd	3.333 bc	0.853 a	0.740 a

Data was non-parametric so values presented are median cfu x 10^6

Kruskal-Wallis analysis was carried out and values sharing the same letter are not significantly different ($P < 0.05$)

Table 4. Soil Chitinase Activity

	Control	CCS	CaS	NIII	CaS & NIII
May Chitinase (units)	0.380a	1.110cd	0.579abc	0.754bcd	0.733bcd
Sept Chitinase (units)	0.451ab	1.518d	1.324cd	0.665bcd	0.423a

Data was non-parametric so values presented are medians

Kruskal-Wallis analysis was carried out and values sharing the same letter are not significantly different ($P < 0.05$)

Table 5 Soil Cellulase Activity

	Control	CCS	CaS	NIII	CaS & NIII
May Cellulase (units)	0.378ab	0.335ab	0.336ab	0.395ab	0.496b
Sept Cellulase (units)	0.388ab	0.283a	0.307a	0.395ab	0.516b

Data was non-parametric so values presented are medians

Kruskal-Wallis analysis was carried out and values sharing the same letter are not significantly different ($P < 0.05$)

Chapter Six

Stimulation of wild strawberry (Fragaria vesca) arbuscular mycorrhizas by addition of shellfish waste to the growth substrate: interaction between mycorrhization, substrate amendment, and susceptibility to redcore (Phytophthora fragariae)

Preface to Chapter 6

This work for this chapter was carried out in collaboration with John Murphy.

The chapter is based on a lecture given by S. Rafferty at an Inter-COST (COST actions 8.21, 8.22, 8.30 and 8.31 with the ISHS Group on Quality Management in Micropropagation) conference in Edinburgh, September1998. The lecture was then published after peer review in Applied Soil Ecology 2000, 15; 153-158.

Stimulation of wild strawberry (*Fragaria vesca*) arbuscular mycorrhizas by addition of shellfish waste to the growth substrate: interaction between mycorrhization, substrate amendment and susceptibility to red core (*Phytophthora fragariae*)

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Abstract

Wild strawberry (Fragaria vesca) microplants were inoculated at establishment in the glasshouse with the commercial inoculants Endorize IV, Vaminoc and Glomus mosseae. After two weeks, plants were transferred to control peat-based growth substrate and Suppressor®, a commercial peat substrate amended with chitin-containing shellfish waste. Percentage root length colonisation (%RLC) by Vaminoc and G. mosseae, but not Endorize IV, was stimulated significantly after 4 weeks growth in the amended substrate but there were no significant differences for any of the inoculants at 8 weeks. Runner production in Vaminoc-inoculated plants was unaffected by either growth substrate. Runner production was significantly reduced in Endorize IV and G. mosseae treatments in the control growth substrate, other growth parameters were not significantly affected. Disease resistance to red core was increased by growth of the Vaminoc-inoculated plants for 4 weeks in Suppressor® before challenge in control compost. Neither Vaminoc inoculation nor growth in Suppressor® resulted in increased disease resistance.

Key words; Chitin, commercial mycorrhizal inoculants, Suppressor®, red stele.

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1. Introduction

Inoculation of micropropagated plantlets with arbuscular mycorrhizal fungi (AMF) has been shown to increase establishment and to stimulate plant growth (Wang et al., 1993; Puthur et al., 1998). In general, when inoculating plants, consideration should be given to the interaction between host genotype, AMF isolate and growth substrate composition in order to optimise plant performance (Gianinazzi et al., 1990). Perrin et al. (1988) discussed the importance of characterising efficient AMF strains and the substrate receptiveness to mycorrhizal inoculum; this is described as the ability of a substrate to allow mycorrhizal association development on host plants from introduced inoculum. Azcón-Aguilar and Barea (1997) discussed the selection of growth substrates which favour the formation and functioning of mycorrhizae and the interaction between AMF and other components of the microbiota of the growth substrate, in relation to the biological control of root diseases. The complexity and variability of responses following the addition of organic amendments to the growth substrate is another factor which must be taken into consideration when examining plant-substrate-AMF interactions (Gryndler and Vosatka, 1996).

Here, the interactions are investigated between wild strawberry (Fragaria vesca L.), three commercial AMF inoculants and two peat-based substrates, one of which had been amended with shellfish waste, namely Suppressor®. The use of shellfish waste, an inexpensive source of chitin (Sugimoto et al., 1998), is based on well established observations of biological control properties against soil fungi (Fusarium solani f. phaseoli) described by Mitchell and Alexander (1962) and due to the stimulatory effect reported towards AMF colonisation (Gryndler and Vosatka, 1996).

2. Materials and methods

2.1 Plant material and growth conditions

Aseptic seedlings of the outbreeding wild strawberry (Fragaria vesca L.) were produced by aseptically germinating seeds (Chiltern Seeds, Ulverston, Cumbria, UK.) for 12 days on water agar before transferring them for four weeks to half-strength Murashige and Skoog (1962) medium in vitro as described in Mark and Cassells (1996). The aseptic seedlings were acclimatised for 2 weeks (in plastic covered vented weaning trays) in a glasshouse into a Peat Vermiculite Sand (PVS); [8:1:1 (v/v/v)] substrate which had been steam sterilised for 1 hour at 121°C over three consecutive days and allowed to rest for a further week before use. The PVS was fertilised (NPK, 16:8:12) with 9 month Osmocote Plus® 1g/l (Grace Sierra B. V. Herleen, The Netherlands) and limed (CaO, 5g/l) to a pH of 6.2. For sterilised PVS the lime and osmocote were added after final autoclaving and cooling (Mark and Cassells loc. cit.) On acclimatisation, plants were inoculated with three commercial mycorrhizal inoculants; Vaminoc, Glomus mosseae (both from MicroBio Division, Herts. UK.) and Endorize IV (Biorize, Dijon, France). The mycorrhizal inoculum was placed in the planting hole in direct contact with the plant root system, the amount of inoculum used was as recommended by the suppliers, i.e. 1g of Vaminoc and G. mosseae inoculum per plant and 5% by volume (equivalent to 2.5 ml per 50 ml plug tray) for Endorize IV. The PVS substrate used for the acclimatisation stage was not amended with a chitin source as previous experimental work (unpublished) showed incompatibility with the chitin amended compost and microplants of F. vesca at acclimatisation.

Following acclimatisation mycorrhizal and control microplants were potted up in PVS substrate as described above (87 mm pots, Omnipot 9F, Congleton Plastic Co. Ltd., Cheshire, UK.) and in a PVS substrate which had been amended with a source of chitin (Suppressor®, Landtech Soils Ltd., Tipperary, Ireland) with a minimum of 16 plants per treatment. The treatments were randomly arranged in

separated blocks on potting benches (which had been covered with plastic to prevent cross-contamination of the treatments) in a glasshouse at an ambient temperature of 15-25°C. Plants were grown with a 16 hour photoperiod under high-pressure sodium lamps 400W, 290/240 volts, Thermoforce Ltd., Essex, UK.).

2.2 Plant Monitoring

Plants were assessed 4 weeks after potting up for early vegetative growth responses to AMF inoculation by counting the numbers of leaves per plant. Chlorophyll meter readings were taken weekly in order to assess the nutritional and health status of the plants using a Minolta Chlorophyll SPAD-502 meter (Minolta Camera Ltd. Osaka, Japan). The percentage root length colonisation was assessed at 4 weeks and at 8 weeks after potting up following clearing in 10% (w/v) KOH and staining with 0.05% w/v aq. trypan blue, (Phillips and Hayman, 1970) and quantifying AMF presence using the magnified hairline intersect method of McGonigle et al. (1990) using a compound microscope at x100 magnification.

Vegetative growth responses were assessed by taking runner counts four weeks after potting up, these were mechanically removed and runner re-growth was quantified after a further 4 weeks. The number of crowns per plant and the % shoot dry matter content were recorded at week 26. Flowering onset was monitored weekly in order to assess the effects of mycorrhizal application and of the substrate amendment.

2.3 Infection with Phytophthora fragariae

A challenge with oospore inoculum of Phytophthora fragariae Hickman [from the Culture Collection, Department of Plant Pathology, National University of Ireland Dublin, Ireland] was carried out on control plants and on plants which had been inoculated with Vaminoc on control and Suppressor® substrates. Plants which

had been inoculated with Vaminoc and grown in Suppressor® for 4 weeks were divided into two batches, one of which was grown on in Suppressor®; the other batch was re-potted in non-amended substrate after 4 weeks. The plants were challenge inoculated with oospores at the end of this 8 week period.

The oospore inoculum was produced by inoculating acclimatised aseptically germinated seedlings of F. vesca with P. fragariae (from a culture which had been maintained on lima bean agar) in steam sterilised vermiculite and allowing the infection to develop as described in Mark and Cassells (1996). The oospore inoculum used was standardised by comminuting infected root material in an electric blender (Kenwood Ltd., Hants, UK.), and had an estimated oospore concentration of $2.5x10^3$ oospores per ml of inoculum, 5 ml of P. fragariae inoculum were used to inoculate each test plant in the disease challenge. After adding the P. fragariae inoculum to an inoculation hole made near the stem base of each plant being inoculated the plants were transferred to a controlled environment growth chamber and incubated for 2 weeks at 13-15° C, 12h photoperiod with PAR 9 μmol m^{-2} s^{-1}, after this period the temperature was reduced to 6^0 C and the vermiculite was allowed to dry out in order to induce oospore production (Mark and Cassells, 1996). Test samples were cleared and stained as for AMF detection (see above) and the response to the pathogen was assessed using Disease Severity indexes (DSI) as described by Milholland et al. (1989). This index is calculated by multiplying the number of oospores present per 1.0 cm root segment sampled by the % root length infected and dividing by 100, any sample found to have a DSI of less than 1.0 is said to be resistant to P. fragariae where as any value greater than 1.0 is considered susceptible. This method is an alternative to visual assessment which is viewed as being too subjective, Milholland and Daykin (1993).

2.4 Statistical Analysis.

The Mann-Whitney (Comparison of 2 treatments) and the multiple comparison Kruskal Wallis tests were used for non-parametric data which were

analysed with the aid of Data Desk® 5.0 (Data Description, Inc., N.Y., USA). Median values were used to represent the central tendency in non-normal data.

3. Results

3.1 The effects of shell-fish waste amendment on mycorrhizal colonisation

Growth of microplants in Suppressor®-amended-PVS resulted in increased percentage root length colonisation of F. vesca by all three AMF isolates, this increase was significant for Vaminoc and G. mosseae (Table 1) four weeks after potting up. There were no differences detected in Suppressor® at week 8, this indicates that the acceleration of colonisation induced by substrate amendment occurred within four weeks of transfer to this medium. Vaminoc-associated colonisation reached a plateau by week four without further increase at week 8. The same result was obtained for F. ananassa cv. Tenira (data not shown).

3.2 The interaction between substrate amendment and mycorrhization on plant growth

Table 2 shows that significant plant growth effects occurred in Suppressor®-amended-PVS. The number of runner plants was significantly lower in uninoculated plants, plants inoculated with Endorize IV and with G. mosseae. The depressive effect of the substrate amendment on runner production was not observed with Vaminoc inoculated plants. The runner counts recorded at week 8 show a similar pattern. This indicates that a depression rather than a delay in runner production occurs as a result of the substrate amendment. Other growth parameters monitored, namely, leaf number, chlorophyll content, % shoot dry matter and crown count

showed no significant differences, except for Endorize IV inoculated plants which produced significantly more runners independently of growth substrate composition.

A slight reduction occurs in the % flowering in the non-mycorrhizal plant population, but not significantly so, the differences are also not significant between any of the AMF treatments (Fig. 1). G. mosseae plants grown in Suppressor® had a higher % flowering, this is not significantly higher.

3.3 The effect of substrate amendment and mycorrhization on the severity of redcore

The Vaminoc inoculant was used here as it had shown the highest positive response in the mycorrhizal inoculum – substrate amendment trial above. The disease severity indexes for all six treatments studied, namely, Vaminoc, plus and minus substrate amendment, at 4 and 8 weeks, are shown in Table 3. The treatments are ranked in increasing disease severity, mean values are included for clarity. The lowest DSI is observed for Vaminoc inoculated plants which were grown in Suppressor®for four weeks before transfer to non-amended substrate (Plants were transferred as the stimulatory effect of amended substrate on %RLC reached a plateau at 4 weeks; see 3.1). This is the only treatment which results in a DSI of less than 1.0 which is under the resistance threshold, this value differs significantly from the median DSI value for similar plants which were grown on in Suppressor®. Vaminoc and Suppressor® separately are seen to reduce disease severity but not significantly from their respective control treatments. Interaction analysis of variance (ANOVA) confirms that a significant interaction occurs between growth substrate type and AMF inoculation.

4. Discussion

Vestberg (1992) found that of six AMF strains used to inoculate commercial strawberry, three were found to be highly efficient and the three others were less efficient. Here, the vegetative response of F. vesca, to AMF inoculants containing different isolates was shown to vary confirming previous results with this species (Mark and Cassells, 1996). Suppressor® , the shell-fish waste amended growth substrate used here was found to increase the % root length colonisation confirming the findings of Gryndler and Vosatka (1996). Stimulation of mycorrhizal colonisation, however, was not associated with significant growth increases or earlier flowering (Fig. 1), as reported by Wang et al., (1993). A depression of runner plant production was seen to be associated with the inoculant - Suppressor® interaction, except for Vaminoc. This may be due to a genotype-dependent interaction of the AMF inoculant with the substrate. The lack of variation in the other growth parameters monitored such as early leaf count and crown numbers (Table 2) and in % dry matter content, indicate that the quality of the mycorrhized plant material in control and shell-fish waste amended growth substrate is not generally adversely affected. The shell-fish waste amendment did not alter the nitrogen content of the host plant to a level detectable with the chlorophyll meter. This also agrees with the findings of Gryndler and Vosatka (1996). This parameter is important as nitrogen affects root colonisation by AMF and nitrogen stress, like phosphorus stress, promotes root colonisation by AMF (Sylvia and Neal, 1990).

Caron (1989) recommended environmental manipulation in order to trigger and enhance the activities of biocontrol agents. The interaction of the host genotype-AMF-growth substrate composition with the root disease P. fragariae (Table 3) indicates that manipulation of the growth substrate composition may result in a significant reduction in disease severity. Azcón-Aguilar and Barea (1997) reported that enhancement of root resistance or tolerance to pathogen attack is not expressed in all substrates. The variation in disease severity indexes (Table 3) seen here

confirms the latter. An important factor is seen to be the timing of inoculum interaction with the amended growth substrate, which interact significantly.

The shell-fish waste amendment is also seen to accelerate as well as stimulate AMF colonisation by Vaminoc, exploitation of the shell-fish waste amendment is only possible 2 weeks after acclimatisation (due to toxicity to the young microplant) by which time early AMF infection has taken place (<10% data not presented). The most effective protection against P. fragariae occurs when Vaminoc inoculated plants were grown in Suppressor® for 4 weeks and then transferred to a non-amended substrate. In conclusion, positive interactions between the host plant, mycorrhizal inoculant and shell-fish waste amended growth substrate and resistance to P. fragariae have been demonstrated. However, the complexity of this interaction is such that commercial exploitation of this tripartite relationship would appear difficult, especially when confronted with the biological diversity of soils.

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Table 1. Effect of shell-fish amendment of the growth substrate on median percent root length colonisation (ϕ % RLC) at 4 and 8 weeks for. F. vesca. Ch^-, control PVS growth substrate; Ch^+ Suppressor®-amended substrate.

Four Weeks						
Treatment	ϕ % RLC	95 % C. I.	Treatment	ϕ % RLC	95 % C. I.	Effect
Endorize iv Ch^-	8.5	[5-17]	Endorize iv Ch^+	12.5	[5-17]	N.S.
Vaminoc Ch^-	17.5	[11.7-25.6]	Vaminoc Ch^+	37.0	[11-50]	S. ($p<0.05$)
G. mosseae Ch^-	5.0	[3-15]	G. mosseae Ch^+	18.7	[3-86]	S. ($p<0.05$)
Eight Weeks						
Treatment	ϕ % RLC	95 % C. I.	Treatment	ϕ % RLC	95 % C. I.	Effect
Endorize iv Ch^-	25.0	[14-43]	Endorize iv Ch^+	30.0	[16-47]	N.S.
Vaminoc Ch^-	24.5	[13-48]	Vaminoc Ch^+	37.0	[11-50]	N.S.
G. mosseae Ch^-	24.5	[9-46]	G. mosseae Ch^+	30.5	[12-50]	N.S.

N.S = not significant, S. = significant ($P < 0.05$, Mann-Whitney U test).

Ch^- = Without chitin amendment, Ch^+ = with chitin amendment (8 plants per treatment).

Table 2. Effect of shellfish amended growth substrate on the vegetative growth response in Fragaria vesca, median (♦) runner count data 4 and 8 weeks after potting up. (codes as Table 1).

Week 4			Week 8		
Treatment	Median	95 % C.I.	Treatment	Median	95 % C.I.
Control Ch^-	7.5 b	[3-10]	Control Ch^-	6.0 b	[3-9]
Control Ch^+	2.0 a	[1-4]	Control Ch^+	1.5 a	[0-4]
Endo. iv Ch^-	1.0 a	[0-2]	Endo. iv Ch^-	1.0 a	[0-2]
Endo. iv Ch^+	3.0 a	[0-5]	Endo. iv Ch^+	2.0 a	[0-8]
Vaminoc Ch^-	7.0 b	[5-10]	Vaminoc Ch^-	7.0 b	[5-9]
Vaminoc Ch^+	6.5 b	[4-10]	Vaminoc Ch^+	6.5 b	[4-9]
G. moss. Ch^-	2.0 a	[0-4]	G. moss. Ch^-	1.5 a	[1-3]
G. moss. Ch^+	0.0 a	[0-2]	G. moss. Ch^+	0.0 a	[0-3]

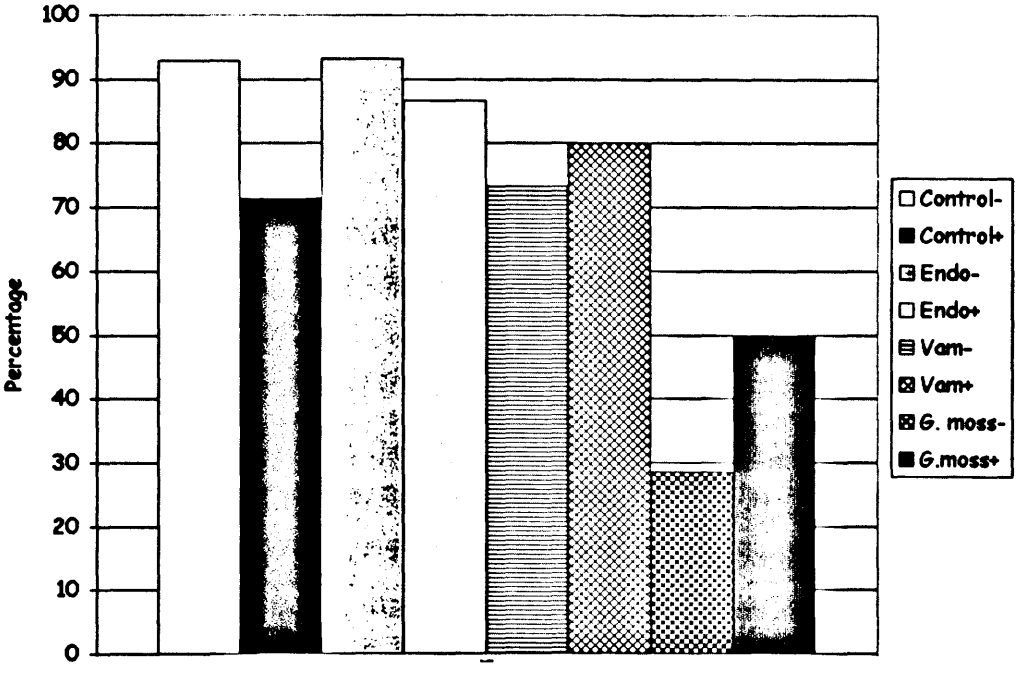
Median runner count values followed by the same letter (horizontally) are not significantly different ($P < 0.05$, 15 plants per treatment). C.I. = Confidence intervals

Table 3. Disease Severity indexes (DSI) following challenge with Phytophthora fragariae inoculum at week 8 of Vaminoc-inoculated plants grown in control substrate, and shell-fish amended substrate and in amended substrate for 4 weeks followed by return to control compost for 4 weeks before challenge.

Rank	Treatment	(Mean DSI)	Median DSI	95 % Confidence limits
1.	$Vam^+ Ch^+$(4 week)	(0.91)	0.47 a	[0.08-3.85]
2.	$Vam^+ Ch^-$	(3.62)	3.48 ab	[0.21-9.49]
3.	$Vam^- Ch^+$(4 week)	(3.68)	3.49 ab	[0.48-9.2]
4.	$Vam^- Ch^+$(8 week)	(4.45)	3.46 ab	[0.21-12.6]
5.	$Vam^- Ch^-$	(4.79)	3.47 b	[0.66-13.42]
6.	$Vam^+ Ch^+$(8 week)	(12.48)	9.33 b	[1.12-29.55]

Median values followed by a different letter (horizontally) were found to differ significantly following the Kruskal-Wallis test. ANOVA Significant interaction between Chitin and Vaminoc ($H = 7.43 > \chi^2 = 5.99$; $P < 0.05$).

Fig. 1. Percentage of plants in each treatment, which had flowered by week 26. Ch-, control PVS growth substrate; Ch+, PVS substrate plus Suppressor®; Endo- in PVS; Endo +, in PVS containing Suppressor®; similarly for Vam – and +, G. mossae – and +.



Chapter Seven

The identification and use of chitin-amended compost to suppress wilt disease in glasshouse-grown Dianthus 'Mystère' plants

Section B: Investigation of the biocontrol properties of chitin-containing crustacean shellfish waste

Preface to Chapter 7

This chapter is based on work done in collaboration with Ultan Cronin. The style is that of the journal Applied Soil Ecology.

THE IDENTIFICATION AND USE OF CHITIN-AMENDED COMPOST TO SUPPRESS WILT DISEASE IN GLASSHOUSE-GROWN DIANTHUS 'MYSTÈRE' PLANTS

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Abstract

The causal agent of a virulent wilt disease of mutant microplants and selected mutant lines of Dianthus 'Mystère' in the glasshouse was identified, and independently confirmed, as Fusarium oxysporum f. sp. dianthi. The disease was not controlled by fungicide application. Cultivation in compost amended by the addition of crustacean shellfish waste was effective in reducing disease incidence in heavily contaminated glasshouse conditions. In addition to positively influencing antagonists in the peat compost, chitin-amendment was shown to stimulate in planta chitinase levels and alter protein banding patterns.

Keywords; Biological control, cellulase, chitinase, Fusarium oxysporum, pathogenesis-related proteins, tissue culture

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1. Introduction

A wilt disease developed in a mutation breeding programme on Dianthus 'Mystère', a hybrid between D. caryophyllus and D. barbatus (Cassells et al., 1993). The disease caused losses in the glasshouse-grown both affecting established mutant microplants before selection and the maintenance of selected lines. Benolate and carbendazim fungicides are recommended for use against Fusarium oxysporum (Hanks, 1996). However the disease was not responsive to either of these systemic fungicides. Other authors have also found poor response to these chemicals and utilised different control strategies (Minuto et al., 1995, Lenteren, 2000) and so here an alternative method of stock block maintenance was investigated.

The initial symptom of the disease was a yellowing of the basal leaves of the plant's rosette of leaves. The rosette then became chlorotic, with patches of red-purple anthocyanin pigmentation evident on many of the leaves followed by wilting of the foliage (Fig. 1). Two to three weeks after the initial expression of symptoms the infected plants died.

A number of organisms, both bacterial and fungal, are reported to cause wilt diseases of Dianthus species (Fletcher, 1984; Smith et al., 1988; Whealy, 1992). The bacterial species, Pseudomonas caryophylli and Erwinia chrysanthemi pv. dianthicoli and the fungal species, Fusarium oxysporum f. sp. dianthi, Rhizoctonia solani, Phialophora cinerescens and Calonectria kyotensis are the causal agents of most of the common forms of Dianthus wilt disease. Fusarium wilt is the most important disease of species within Dianthus, and worldwide, causes severe economic losses for commercial growers. It is prevalent in the south of England which has similar climatic conditions to Cork (Chiocchetti et al., 1999; Carver et al., 1996; Whealy, 1992). Outbreaks of Fusarium wilt in a glasshouse or field bed are attributed to

germination of a dormant or recently introduced chlamydospore within the growth substrate (Nelson, 1981; Smith et al., 1988).

Several approaches to the control of F. oxysporumf. sp. dianthi have been reported elsewhere (Table 1), and these include biological control of F. oxysporum f. sp. dianthi. For example Vanpeer et al. (1991, 1992) had some success using a strain of Pseudomonas that induces systemic resistance in both carnation and radish. Carver et al. (1996) also reported suppression by Trichoderma, however this is limited as plant-pathogen biocontrol-agent specificity, as well as temperature specificity, were observed. Other fungal inoculants also showed some degree of success in carnation and chickpea, for example, non-pathogenic races of Fusarium have been used as agents against F. oxysporum (Postma and Luttikholt, 1996; Hervas et al., 1995), however, timing and dosage of the biocontrol agent were critical and in most cases effects did not persist.

Previously, the use of chitin-amendment compost for the control of substrate-borne disease showed promising results (Murphy et al., 1999). Crustacean shellfish waste was the chosen amendment as it is a rich source of chitin which has been shown to exert biological control through its promotion of antagonistic soil micro-organisms (Mitchell and Alexander, 1962). Chitin and its derivative chitosan (Evans, 1993; Ren and West, 1992; Akiyama et al., 1995; Gagnon and Ibrahim, 1996; Pearce et al., 1998) are also reported to elicit pathogenesis-related proteins, which play a role in disease resistance.

The objectives of this investigation were two fold; in the first instance to identify the causal agent of the disease. In the second instance to evaluate the potential of crustacean shellfish waste-amended peat to control the disease so that stocks of

Dianthus could be maintained in the long term in the glasshouse and avoid the catastrophic losses seen previously.

2. Materials and methods

2.1. Isolation of pathogen

Stem sections were taken at least 2 cm above soil level from Dianthus 'Mystère' glasshouse-grown plants showing advanced symptoms of wilt disease. In the laboratory, leaf material was trimmed from the stems using a scalpel and stem sections were thoroughly rinsed under tap water. In a laminar air-flow hood the stem sections were placed in 70% (v/v) aq. ethanol for 2 minutes, 10% (v/v) aq. Domestos (Diversey Levers, Northampton, NN3 8PD) for 15 minutes and rinsed three times in sterile distilled water. The stem sections were held in the final rinse of sterile distilled water for 5 minutes. Following surface sterilisation, the stem sections were cut into 1cm lengths. Each of these was placed in a petri dish containing 10ml of sterile distilled water. The sections were macerated aseptically. Serial dilutions of the macerate supernatant were made, using sterile distilled water as the dilutant. 1ml aliquots of each dilution were pipetted into petri dishes containing 50 ml of U1 medium (30 g l^{-1} sucrose, 2.15 g l^{-1} Murashige and Skoog (1962) basal salts, 1 mg l^{-1} of GA_3, 6 g l^{-1} agar, pH 5.8), VA medium (200 ml l^{-1} V8 vegetable juice (Campbell Ltd., King's Lynn, Norfolk, PE30 4HS, UK), 3.0 g l^{-1} calcium carbonate, 6.0 g l^{-1} agar-agar) or NA (1.0 g l^{-1} Lab Lemco Powder, 2.0 g l^{-1} Yeast extract, 5.0 g l^{-1} Peptone, 5.0 g l^{-1} sodium chloride, 6.0 g l^{-1} agar-agar) and spread evenly over the plate's surface using a sterile spreader. Petri dishes were sealed with parafilm

(American National, Chicago, USA) and placed in an incubator maintained at 24°C. Growth was examined after four days.

Control spore and hyphal cultures of the fungus, F. oxysporum f. sp. dianthi, were initiated on petri dishes of two media and grown at a range of temperatures (4, 18 or 37°C), in order to determine optimal conditions for growth. The media were VA (see above) or SA (D-glucose 5.0 g l^{-1}, L-asparagine, 1.0 g l^{-1}, magnesium sulphate, 5.0 g l^{-1}, sodium carbonate 1.04 g l^{-1}, di-potassium hydrogen orthophosphate 1.36 g l^{-1}, agar-agar 15.0 g l^{-1}. Cultures were examined after two weeks incubation in the dark at 24^{0}C. On a bi-weekly basis, the maintenance of pure cultures of F. oxysporum f. sp. dianthi was carried out by inoculating single spores or small mycelial segments onto the media, U1 and VA, cultures were incubated in the dark at 24°C. Cultures of the fungus were also stored at a temperature of -5°C.

2.2. Identification of F. oxysporum f. sp. dianthi

Using a needle, mycelia were scraped from the surface of petri dishes in which pure cultures of the isolate were growing. The mycelial scrapings were transferred to microscopic slides. The samples were carefully covered with a drop of sterile distilled water and air-dried. Samples were stained with lactophenol cotton blue. Excess stain was irrigated using sterile distilled water and the samples were again air-dried. A drop of immersion oil was placed over the slides, which were then examined using light microscopy under magnifications of 40x, 100x and 1000x. Morphological characteristics of the isolates were keyed out using Ellis (1985) and Barnett and Hunter (1972). Cultures of the pure isolate were sent for independent identification (CABI Bioscience, Egham, Surrey, TW20 9TY, UK).

2.3. Inoculation of in vivo Dianthus "Mystère" plants with pure cultures of Fusarium oxysporum f. sp. dianthi isolates

Fifteen symptomless, established three-month-old Dianthus 'Mystère' plants from aseptic in vitro cultures were established and grown on in a glasshouse in which *D.* 'Mystère' plants had never previously been cultivated after establishment. The plants were established in peat-based potting compost (Westland Horticulture, Dungannon, BT70 1NJ, N. Ireland, UK) and were potted up in 11.5 cm pots in a medium consisting of a 40:40:20 mix by weight of fine gravel, horticultural sand and potting compost. $1cm^2$ plugs of VA medium on which pure cultures of F. oxysporum f. sp. dianthi isolates were growing were inverted and placed on the soil surface of pots in which the plants were growing. One plug was placed in each pot. Plants were watered regularly, fertilised every two weeks using Miracle Grow™ (Miracle Garden Care Ltd., Godalming, Surrey, GU7 1XE, UK). Plants were sprayed regularly with a rotation of the insecticides Decisquick (AgrEvo Crop Protection) and Malthion (Hygeia Chemicals Ltd.) to maintain an aphid-free environment and observed on a daily basis for symptoms of wilt disease.

2.4. Inoculation of in vitro Dianthus 'Mystère' plants with pure cultures of Fusarium oxysporum f. sp. dianthi isolates

Twenty microplants, growing in vitro aseptic tissue culture, of each of five lines of D. 'Mystère' were inoculated with hyphal cultures of pure isolates of F. oxysporum f. sp. dianthi. Plantlets were grown in 120 ml plastic food tubs containing 50 ml of Dianthus medium (15 g l^{-1} sucrose, 2.15 g l^{-1} Murashige and Skoog (1962) basal salts, 1 mg l^{-1} GA_3, 6 g l^{-1} agar agar, pH 5.8), with four explants per tub. At the time of inoculation, microplants were four months old. Cultures

were placed in a growthroom under a regime of 23±2°C and 25-45 μmol m^{-2} s^{-1} light provided by white 65/80 W "Litegard" fluorescent tubes (Osram Ltd., Manchester, UK). Plantlets were observed on a daily basis for symptoms of disease occurrence and progression.

2.5. Investigation of the effectiveness of a chitin-amended compost in suppressing Fusarium wilt disease of Dianthus "Mystère" plants

Trials were set up in a Fusarium contaminated-glasshouse to ascertain whether Dianthus 'Mystère' microplants derived from tissue culture could be weaned and grown successfully in a peat-based compost formulated with crustacean shellfish waste (Suppressor™, Landtech Soils, Ltd., Co. Tipperary, Ireland). Four trials were designed to evaluate the efficacy of "Suppressor™" compost in controlling Fusarium wilt disease of D. 'Mystère' in glasshouse pot trials; (i) the control medium was that normally used for D. 'Mystère' cultivation, namely, a 40:40:20 mix by weight of fine gravel, horticultural sand and peat-based potting compost (Westland Horticulture, Dungannon, N. Ireland, BT70 1NJ, UK); (ii) a 3:1 mix of control compost and compost removed from infected D. 'Mystère' plants to provide a source of inoculum of Fusarium oxysporum f. sp. dianthi; (iii) a 3:1 mix of control compost substrate and "Suppressor™" compost; and (iv) a 2:1:1 mix of control compost, "Suppressor™" compost and contaminated compost from infected plants.

The D. "Mystère" in vitro-derived microplants used were two months-old in all cases. Each treatment consisted of four replicates of fifteen microplants (each weaning tray contained 15 modular sections). These microplants were weaned in modular trays covered with transparent plastic lids. Initially, high moisture levels within the covered modules were maintained by regular misting. Gradually, as the

microplants established, the moisture levels were reduced and eventually the lids were removed from the modules. Weaning took place in a glasshouse with a high background level of F. oxysporum f. sp. dianthi inoculum present. Data were recorded after six weeks.

2.6. SDS PAGE of pathogenesis related proteins

Dianthus tissue (1g tissue /ml buffer) was ground in Tris-HCl Buffer (100mM Tris-HCl buffer, pH7 containing 10mM 2-mercaptoethanol) using Agdia extraction bags and a ball-bearing grinder. The extract was passed through cheesecloth and filtered through Whatman No1 paper, centrifuged at 17,600g for 20 min at 4°C and stored at -20 °C. A standard curve of a 1mg/ml solution of bovine serum albumin was constructed by making up the following volumes to 5ml with Bradford Reagent (Alpha Technologies, Dublin 6, Ireland), 0, 0.125, 0.25, 0.5, 0.75, 1.0mg/ml. This was repeated for each sample and incubated for at least 2 min at room temperature. Optical density was read at (O.D.) 595nm. The BSA standard curve was used to calculate the protein content of the samples and was used to standardise the samples for gel electrophoresis.

Extracts were boiled for 10min with 1 vol. of sample denaturing buffer (125mM Tris base, pH adjusted to 6.8 with 3M HCl containing 0.4% (w/v) SDS, 10% (w/v) glycerol, 4% (v/v) 2 mercaptoethanol and 0.02% (w/v) bromophenol blue). Samples were loaded into precast 8-16% resolving gels (BIO-RAD, Alpha technologies, Dublin 6, Ireland). Gels were run in Running buffer (5x concentration, Tris base, 15g/l, glycine 72g/l, SDS 5g/l. Make up with distilled water and dilute to 1x concentration) for up to 45 min at 200v.

The gel was removed from the rig and fixed in 10% aq. acetic acid for 30min. The acid was poured off and kept for later. The gel was then washed in distilled water for 2 min x 3. Staining was carried out for 30min with gentle agitation using 2g silver nitrate, 3ml 37% formaldehyde made up in 2l with distilled water. The gel was washed very briefly in distilled water for 30s. It was then placed in developer (sodium carbonate 60g, 37% formaldehyde 3 ml, 400µl of sodium thiosulphate solution (10mg/ml), make up with 2l of distilled water) and rocked until the bands became visible. To prevent overstaining, 10% aq. acetic acid from previously was added. The gel was then washed in distilled water.

2.7. Enzyme extraction and assay

The procedure for extraction of enzymes from peat was based on Wirth and Wolf (1992). 5ml 0.5 M sodium acetate-acetic acid buffer pH 5 per 1g dry weight of soil, were mixed using a magnetic stirrer for 1h. The suspension was then centrifuged at 28,950g for 10min at 4°C and supernatant filtered through glass fibre filter paper. The supernatant was then stored at -20°C prior to analysis. Plant material was ground in liquid nitrogen and extracted as per Wirth & Wolf (1992) with 0.5 M sodium acetate - acetic acid buffer, pH5 (4ml/g tissue) centrifuged at 20,000g for 20mins (Mauch et al., 1988). Carboxymethyl-Chitin-Remazol Brilliant Violet (CM-chitin -RBV) and Carboxymethyl-Cellulose-Remazol Brilliant Blue (CM-cellulose-RBB) (Blue Substrates, Grisebachstraße 6, D-3400, Göttingen, Germany) were used as substrates to assay for endo-acting chitinase and endo-acting cellulase activity. Assays were performed in 96 well microtitre plates (Costar Europe, High Wycombe, UK; cat no. 3590). Each well contained the following, 50µl of substrate, 100 µl of extract, 50µl of buffer (0.2 M sodium acetate - acetic acid buffer, pH5). Control

wells contained no extract until after the acid addition. (4 control reps and 8 test reps were used). Incubation was carried out at 40°C for 3 hours. The reaction was stopped using 50µl of HCl (1N for CM-Chitin-RBV and 2N for CM-cellulose-RBB). Plates were cooled on ice for 10 min and centrifuged (1450g x 10mins). 175 µl of supernatants were transferred to a 96 well half size EIA plate (Costar, cat no 3690). Activity was read at 550nm for Chitin-RBV and at 600nm for Cellulose-RBB. Extracts with a reading > 0.1 were diluted down and assayed again as they were substrate limited. Calculation of enzyme activity was carried out using the following formula:

Absorbance x 1000 x min^{-1}

3. Results

3.1. Isolation and identification of Fusarium oxysporum f. sp. dianthi

Attempts to isolate the agent responsible for causing wilt disease of Dianthus 'Mystère' plants resulted in the growth of pure fungal colonies on the three growth media used in the procedure, U1, VA and NA. The surface fungal mycelium was white and had a cotton-like texture, while the mycelial mass in contact with the growth medium was purple-mauve in colour. When examined using light microscopy, the fungus displayed the characteristic morphological characteristics of Fusarium oxysporum as described by Ellis (1985) and Barnett and Hunter (1972). These traits included distinctive sickle-shaped macroconidia, simple characteristically shaped phiallides and the presence of chlamydospores. An independent identification by Dr. D. Brayford of CABI Bioscience confirmed the

isolate's identity as F. oxysporum. Since the fungus was isolated from a plant within the genus, Dianthus, the forma speciales of the organisms can be designated dianthi.

The number of colony forming units (CFUs) isolated per cubic cm of infected stem tissue was 5.5 x 10^4 for U1, 6.75 x 10^4 for VA and 7.5 x 10^4 for NA. Both spore and hyphal cultures of the fungus grew more successfully on VA that on SA, with cultures growing on SA appearing thinner and sparser than on VA. No pigmentation developed on cultures grown on SA medium. Cultures incubated at 4°C and 37°C failed to grow.

3.2. Inoculation of in vivo Dianthus 'Mystère' plants with pure cultures of Fusarium oxysporum f. sp. dianthi isolates

Four weeks after inoculating glasshouse-grown Dianthus "Mystère" plants with Fusarium oxysporum f. sp. dianthi, three of the fifteen treated plants displayed the symptoms of incipient Fusarium wilt, with a portion of their shoot tissue appearing chlorotic and flaccid and with purple-red anthocyanin blotches evident on their leaves. After a week, two of these three plants were dead. The lower stems of these plants were soft, with a slight brown discolouration of the vascular tissue visible. Two months after inoculation, only three of the 15 treated plants were still alive. However, all three of the surviving plants were in the latter stages of Fusarium wilt.

3.3. Inoculation of in vitro Dianthus Mystère' plants with pure cultures of Fusarium oxysporum f. sp. dianthi isolates

In vitro D. 'Mystère' microplants inoculated with F. oxysporum f. sp. dianthi displayed the same symptoms of infection as in vivo plants. Appearance of symptoms and disease progression was much more rapid, however. Within one

week of inoculation, microplants became chlorotic. One to two weeks after this, microplants were shrivelled. One week, post inoculation, F. oxysporum f. sp. dianthi was seen as cotton-like wisps enveloping the roots of plantlets. A week later, the fungus had become pigmented with its characteristic purple-mauve colour. At this stage, the medium began to discolour.

3.4. Investigation of the effectiveness of a chitin-amended compost in suppressing Fusarium wilt disease of Dianthus 'Mystère' plants

The results of the experiment carried out to investigate the effectiveness of shellfish waste "Suppressor™" substrate in controlling Fusarium wilt disease of D. 'Mystère' are given in Fig. 2. Fusarium was successfully isolated from the control-infected peat treatment as well as the Suppressor™-infected peat treatment. It was not isolated from either of the other treatments. In the absence of Fusarium inoculum, plant survival in Suppressor™ was 63%, which was over one, and a half times the survival of the controls (38%). In the presence of Fusarium inoculum 20% of the controls survived whereas 48% survived if weaned in Suppressor™. A chi-squared analysis showed a significant difference between these values at $p<0.001$.

3.5. PR Protein analyses

The banding pattern of the proteins separated by SDS PAGE is shown in Fig 3. When Fusarium is present there was an up-regulation of the protein between 37 and 50kD (~43kD) and a new band ~20kD (between 15 and 25kD) was present. When Suppressor™ was present a new band was present, just below the 25kD (~24kd) marker. It was still faintly present when both Suppressor™ and Fusarium were

present. Interestingly, there was a 50kD band present in the control that was not present in any of the other treatments.

3.6. Enzyme assays

The results of previous enzyme assays carried out on the peat and Suppressor™ are shown in Fig 4. These results show that the Suppressor™ has significantly elevated chitinase and cellulase compared to those present in ordinary peat. Chitinase levels are increased from 23 chitinase units to 411, while the cellulase units significantly changed from 49 to 2274.

The results of Dianthus extract chitinase assays are presented in Fig. 5 and the results of the cellulase assays are shown in Table 2. The chitinase levels are significantly higher than the control when Suppressor™ is present, 16.6 chitinase units and 132.6 chitinase units, respectively. Cellulase activity though much higher than chitinase were not significantly changed by the presence of Suppressor™, the Control and Suppressor™ being 17396, 20208 cellulase units, respectively. On introduction of infected peat into the compost no significant difference was seen in the cellulase activity though there were significant differences between the control and the control and infected treatments, 17395 cellulase units and 23020 cellulase units, respectively. Nevertheless, when infection was present in the controls chitinase activity did not change, however, the Suppressor™ chitinase activity remained significantly different from the control but no significant change occurred whether infection was present or not in the Suppressor™ treatments.

4. Discussion

Light microscopy examination of pure isolates from internal tissues of symptomatic plants showed that aetiological agent was Fusarium oxysporum f. sp. dianthi, an identification that was independently confirmed by Dr. D. Brayford of CABI Bioscience Identification Services. Both in vivo plants and in vitro microplants of D. 'Mystère' displayed the characteristic symptoms of Fusarium wilt when inoculated with pure cultures of the isolate, providing further confirmation that the causal agent was F. oxysporum f. sp. dianthi. Eleven discreet races of F. oxysporum f. sp. dianthi, are recognised, each with its own geographical distribution, host preference and morphological and genetic markers (Chiocchetti, et al, 1999). In order to assign a race to the agent isolated in this case, compatibility testing and molecular analysis would need to be carried out (Chiocchetti et al., 1999; Kalc-Wright et al., 1996; Manulis, 1994).

Chitin, which is a major component of the exoskeletons of crustaceans and of fungal cell walls (Campbell, 1996), and its derivatives, such as chitosan, are elicitors of plant defence responses (Hadwiger and Beckman, 1980; Walker-Simmons and Ryan, 1984). Such responses can confer protection on plants from attacks by pathogenic organisms (Bell et al, 1986; Dammann, et al., 1997; Titarenko et al., 1997). The presence of chitin in the plant's growth medium can serve to boost steady-state levels of the chemicals involved in the plant's defence response so that when it is challenged by the attack of a pathogenic organism, the plant is "immunised" (Bell et al., 1986; Berenbaum, 1995). The increased chitinase and cellulase levels seen in Suppressor ™ compared to the control peat corroborates this.

Previous research has reported the benefits of including chitin in the growth substrate for plants (Murphy et al., 1999). For example, the addition of shellfish

waste to the medium in which strawberry plants were grown was found to result in an increase in plant dry weight and in root length colonisation by mycorrhizal inoculants. With careful attention to timing the chitin-amended compost also reduced susceptibility to strawberry redcore (Murphy et al., 1999).

Here, enzyme analyses of the plants grown in Suppressor™ didn't show significant differences in cellulase units. However significantly higher levels of chitinase compared to plants gown in control compost were present in the Suppressor™ plants. These levels remained high whether infection was present or not and correlate to the disease survival (Fig. 2). The PR protein gel analysis (Fig. 3) showed differences in banding patterns when Suppressor™ was present, including an extra band of c. 24 kD. However, when infection was present the banding patterns were similar in the control and the Suppressor™ composts.

In summary, when chitin-amended compost was included in the substrate used in the cultivation of tissue culture-derived plantlets of D. 'Mystère', the survival rates were increased from 20.0% to 48.3% in the case of microplants inoculated with F. oxysporum f. sp. dianthi and from 38.3% to 63.3% in the case of microplants growing in a glasshouse where high levels of infection were recorded. In the absence of effective fungicides to control Fusarium wilt of Dianthus in the glasshouse, glasshouse sterilization followed by cultivation in chitin-amended compost may be an effective strategy to control disease development in long-term stock plant maintenance. Chitin-containing compost may function both by promoting soil-inhabiting fungal-antagonists and by eliciting host plant anti-fungal defences.

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Fig. 1. Top, a healthy *D.* "Mystère" plant. Bottom, plants, which have been lost to the disease. The tissue, which first showed signs of wilt, is straw coloured at this stage.

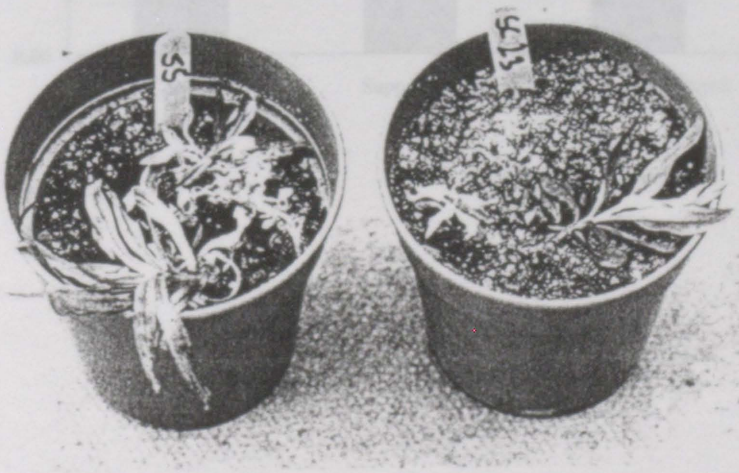

Fig. 2. The percentage survival of Dianthus "Mystère" in vitro-derived plantlets weaned in one of four separate media. The control condition involved the weaning of plantlets in an ordinary peat substrate. Suppresser™ medium contained shellfish waste, a source of chitin. Infected peat, derived from pots in which plants had succumbed to Fusarium wilt, served as an inoculum of F. oxysporum f. sp. dianthi.

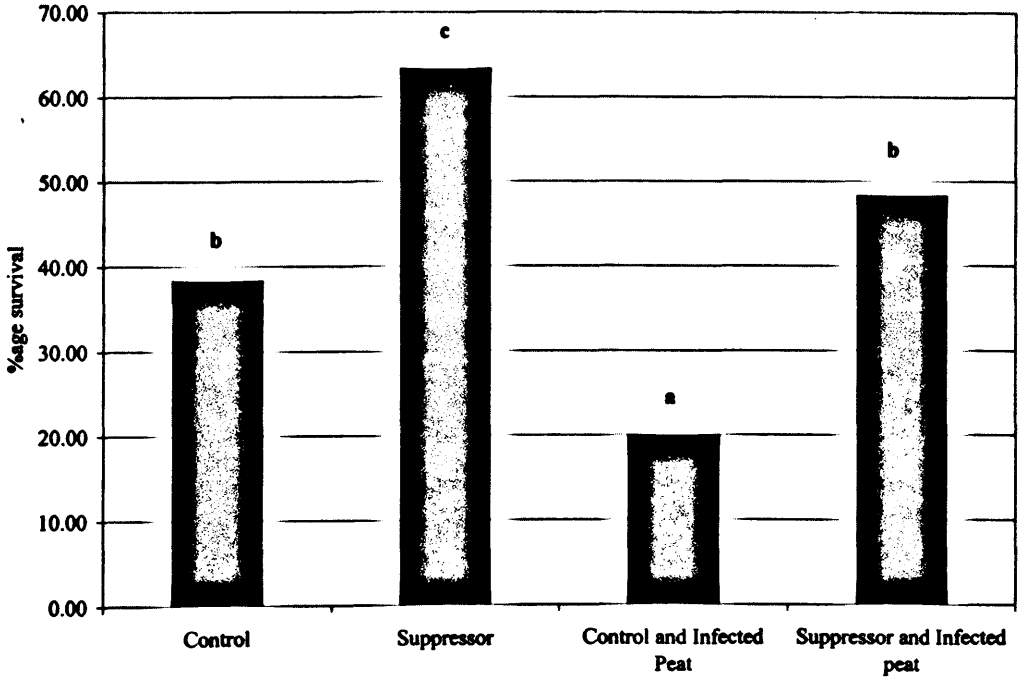

Fig. 3. SDS PAGE gel showing the banding pattern from plant extracts.

Lane 1: Marker

Lane 2: control plant extract

Lane 3: Suppresser™ plant extract

Lane 4: control Fusarium infected plant extract

Lane 5: Suppresser™ Fusarium infected plant extract

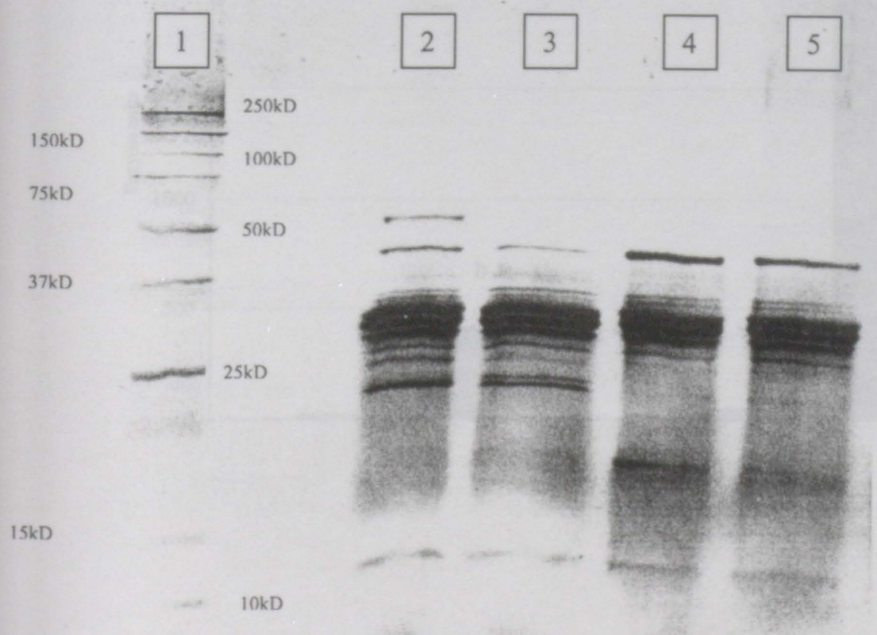

Fig. 4. Mean enzyme activities found in matured peats (approximately 1 year old)

Those values sharing a common letter are not significantly different ($P<0.001$)

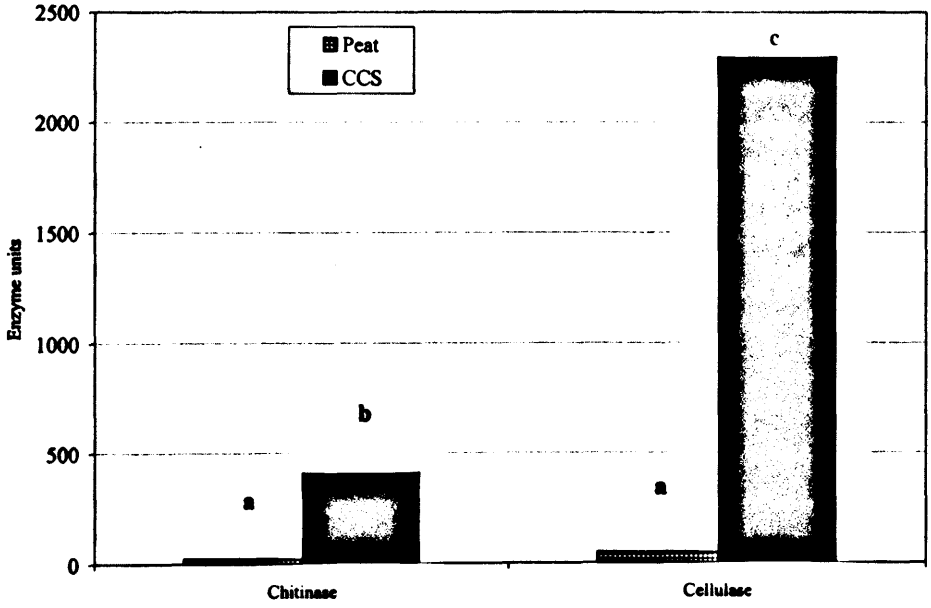

Fig. 5. Mean Chitinase Activity measured in Dianthus plant extracts. Those values sharing a common letter are not significantly different ($P<0.001$)

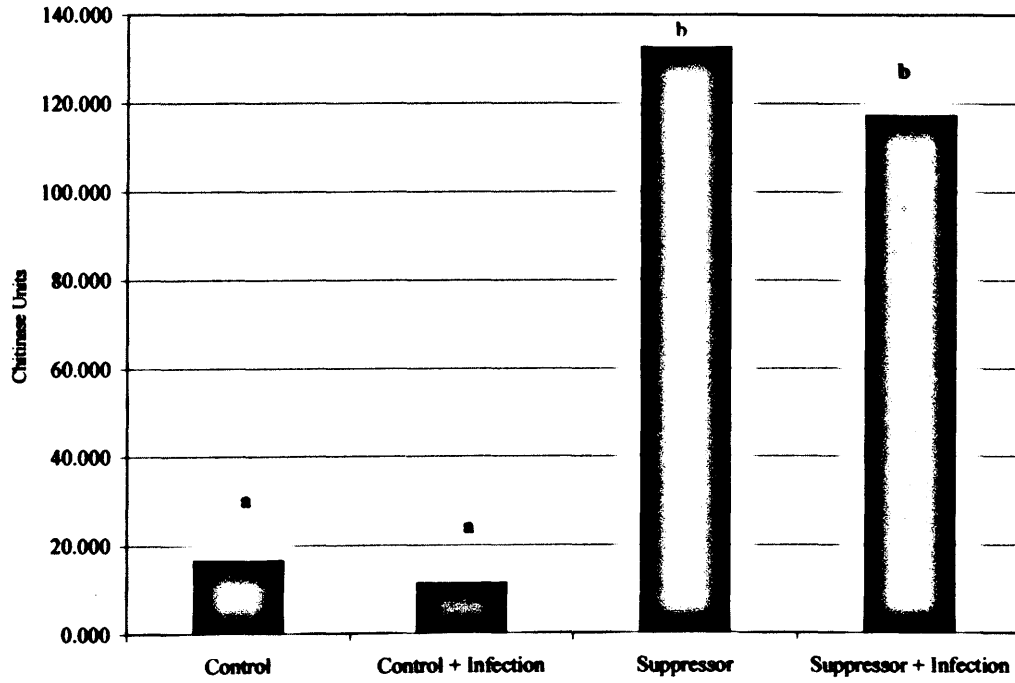

Table 1. The Approaches used in the control of Fusarium wilt disease.

Classification of Approaches	Brief Approach Description	Reference
Reduction of Fo inoculum levels	The use of hygiene/good sanitation in plant propagation	See Whealy, 1992
	Fumigation of the growth substrate	Ramirez et al., 1994
	Using raised beds for the cultivation of plants	See Whealy, 1992
	Application of fungicidal drenches to plants/cuttings	See Fletcher, 1984
	Soil pasteurisation of the growth substrate	Elena et al., 1997; Ramirez et al. 1994
	Soil solarisation	Elena & Tjamos, 1997; Elena et al., 1997
	Use of certified cuttings	See Whealy, 1992
Cultivation of plants under conditions unfavourable to Fo	Maintenance of low substrate pH, Ca^{2+} and N levels	Duijff et al., 1995; see Whealy, 1992
	Cultivation of plants at reduced temperatures	Ben-Yephet et al.; 1996, see Nelson, 1981
	Cultivation of plants under low relative humidity and low substrate water content	See Whealy, 1992
	Cultivation of plants under high light intensities	Ben-Yephet et al., 1996
Biological Control of Fo	'Immunisation' of plants using incompatible Fo races	Castillo et al.,. 1995; Migheli et al., 1996; Postma & Luttikholt, 1996; Rattink & Postma, 1996
	Inoculation of substrate with microbes suppressive of Fo	Elena & Tjamos 1997; Duijff et al., 1995; Vanpeer et al., 1991,1995; Carver et al., 1996
	Cultivation of plants undefined Fo-suppressive soils	Alabouvette, 1999
Fo = Fusarium oxyporum	Cultivation of plants in defined Fo-suppressive soils	Orlikowlki & Skrzypczak, 1997

Table 2. Cellulase analysis results of Dianthus plant extracts.

	Cellulase Activity (mean units)	Letters of significant difference
Control	17395.833	a
Control + Infection	23020.833	b
Suppressor	20208.330	a, b
Suppressor + Infection	21805.556	a, b

Data subjected to one way anova analysis using Prism™ software. Those values sharing a common letter are not significantly different ($P<0.05$).

Chapter Eight

Persistence and effects of human pathogens on aseptic plants *in vitro*

Section C: Investigation of persistence of enteric bacteria in/on plants

Preface to Chapter 8

This chapter was carried in collaboration with St. James' Hospital/Trinity College, Dublin. The work was presented as a poster at the International Society for Horticultural Science, International Symposium, August 1999, Cork, Ireland. After peer review it was published as a paper in the in Acta Horticulturae 2000, 530, 145-154.

PERSISTENCE OF HUMAN FOOD POISONING PATHOGENS IN A MICROPROAGATED VEGETABLE

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Abstract:

An increase in reports of disease outbreaks associated with fresh and ready-to-eat vegetables has prompted this study to review the risk of transmission of human food poisoning organisms in micropropagated vegetables. Surface sterilised seeds from lettuce, cabbage and carrot plants were germinated on an agar base inoculated with *E. coli* and *S. marcescens* respectively. Seedlings were then used for aseptic autotrophic tissue culture. The micropropagated plants were examined microbiologically by surface decontamination, and subsequently homogenisation of the plant material. The model strains were recovered both from direct culture and homogenate. Biochemical identification was carried out using the API system, and molecular typing was performed using pulsed field gel electrophoresis (PFGE). *E. coli* and *S. marcescens* were found to persist in autotrophic culture, indicating that the carbon sources required were acquired from plant exudates. After serial subcultures, inoculated bacteria were repeatedly re-isolated from the progeny plants though some plants were asymptomatic. In some cases the bacteria became pathogens *in vitro* in the latter subcultures.

Keywords: Clinical Isolate, Plant Tissue Culture, PFGE

1.0 Introduction

Micropropagation and hydroponic systems have become increasingly popular (Holdgate and Zandvoort 1997). Plant tissue culture and micropropagation is prone to contamination with human pathogens due to the 'hands-on' nature of the work (Leifert *et al.*, 1994). Weller (1997) stated that "the frequency of infections with common skin organisms of *Staphylococcus* and *Micrococcus* and the increasing percentage of infection with serial subculture implies contamination from human skin". It has also been reported that *T. interdigitale* was acquired from micropropagated plants by two horticulturists on separate occasions (Weller and Leifert 1996). The risk of human food pathogens being introduced into the food chain via the vegetable link has increased recently due promotion of the 'healthy' diet based on increased consumption of vegetables and the rapid expansion in sale of mixed root and haulm vegetables in prepacks. Consumption is projected to increase in the next few years with increased production of minimally processed convenience foods, development of value-added products e.g.: pre-washed prepared vegetable mixes, addition of sauces and meats etc. (Beuchat 1996, Rafferty and Cassells, 2000). There has been an increase in reports of disease outbreaks associated with fresh and ready-to-eat vegetables (WHO 1998, Beuchat 1996). These facts raise concern regarding transmission of food pathogens via infected micropropagated produce. A report in 1997 found that E.coli 0157:H7 could contaminate the edible parts of radishes after the seeds had been soaked in an E. coli 0157:H7 solution (Hara-Kudo *et al.*, 1997). There is a need to assess the potential health risks of the transmission of harmful bacteria via vegetables, which are eaten either raw or after minimal processing. This study has been undertaken to review the risk of transmission in micropropagated vegetables. The aim of this study is to inoculate at

low levels with selected human clinical strains and to then monitor whether these strains can survive in aseptic plant tissue culture conditions. If they can survive could they persist through serial subcultures?

2.0 Materials and Methods:

2.1 Strain Selection:

The following two strains were chosen for study: *Escherichia* coli (Clinical strain ref. no. 945.1 St James Hospital Dublin 8, Ireland) and *Serratia marcescens* (Clinical strain ref. no. 492.4 St James Hospital Dublin 8, Ireland)

The former was chosen as a non-pathogenic representative of food-poisoning *E.coli*, which was safe to use in the contained environment of *in vitro* work. *Serratia* is a well-known environmental organism (Holt 1985) and has been previously reported as a non-phytopathogenic endophyte of xylem in Citrus (Goto 1990). An outbreak of *Serratia marcescens* infections occurred in a university tertiary-care hospital (Vigeant *et al.*, 1998) and it was also noted as an opportunistic pathogen in St James Hospital Dublin (Fred Falkiner, St James hospital personal communication). All strains were provided by the Diagnostic Microbiology Laboratory, St. James's Hospital, Dublin 8, Ireland.

2.2 Plant Inoculation:

Strains were grown up to an OD of 0.4 at 600nm and diluted appropriately. The following series of dilutions were chosen. For *in vitro* work 10^{-7}, 10^{-8} and 10^{-9}. These dilutions were chosen as they represented, respectively, levels of bacteria that were detectable using conventional culture methods, levels below acceptable conventional plate count numbers and levels that could not be detected at all. 100µl

aliquots were plated onto water agar. These plates were used for germination of surface sterilised seeds for 8-10 days.

Seedlings were then used for aseptic nodal tissue culture. Control plants were indexed throughout by culturing on MacConkey plates overnight at 37°C

2.3 Autotrophic Tissue Culturing:

Brassica seed was surface sterilized in 80% aq. ethanol and immersed in 20% v/v aq. Domestos for 15-20min and washed in sterile distilled water (x3) in a laminar-flow cabinet prior to placing the seeds on plates of sterile water agar (6g Agar (Sigma-Aldrich Ireland Ltd) per L distilled water). There were 20 seeds per plate. Following germination the shoots were excised 8-10 days after inoculation and placed into Magenta GA-7 vessels (Sigma- Aldrich Ireland Ltd) each containing polyurethane foam (Plant Biotechnology (UCC) Cork) for support imbibed with half strength M+S mineral solution (Sigma) (Cassells and Walsh 1996). These were grown on in the growth room under the following standard conditions: 23±1 ^{0} C, 16 hour photoperiod (white 65/80 w litegaurd tubes, Osram Ltd., UK.) with PAR of 30 μmol m^{-2} s^{-1} at shelf height. These plants were bacterially indexed as previously described above to ensure asepsis of the plants.

In parallel, surface-sterilised seedlings were placed onto water agar plates that had been inoculated with 1x 10^{-7}, 1x 10^{-8} and 1x 10^{-9} dilutions of *Escherichia coli* and *Serratia marcescens*. This inoculum was prepared by growing cultures to an absorbency of 0.4 at 470nm. A standard plate count was carried out on MacConkey agar, the dilutions used contained the concentrations of bacteria as laid out in Graph 1.0. These seeds were germinated and transferred to magentas as above. When

plants were subcultured only the terminal node was used, which was the farthest from the point of inoculation.

Samples of spent media were taken at the end of the culture cycle and a dilution series was constructed to determine the number of bacteria present in the media during the 4-6 week growth period.

2.4 Plant Sampling:

Plants were harvested using a fresh pair of latex gloves per treatment to avoid cross-contamination. Each plant was packaged in a plastic bag (Glad Freezer Bags) shipped within 12hours from Plant Science Department in Cork to St. James Hospital in Dublin and microbiologically analysed within 12hours of receipt of delivery.

2.5 Preparation of the Plant Material:

All plant material was rinsed in sterile distilled water to remove excess surface dirt. Surface Sterilisation of the plant material proceeded by immersing the plants in 80% Ethanol (Ethanol absolute, Merck KgaA, Darmstadt, Germany) for 45 sec, then 2% Stericol (Stericol Hospital Disinfectant, Lever Industrial Ltd., Runcorn, Cheshire, UK) for 30 min, followed by washing in sterile distilled water (x3). Following sterilisation the plants were placed in 9ml Ringers (Oxoid Ltd., Basingstoke, Hampshire, UK) and 1ml Buffered Peptone Water solution (Oxoid Ltd., Basingstoke, Hampshire, UK), and homogenised using an Ultra Turrex T25 device (Janke & Kunkel Gmblt & Co KG, Staufen, Germany.). For the isolation of the Gram Negative Bacteria, *E.coli* and *S.marcescens*, the homogenate was plated on to

MaConkey agar (Oxoid Ltd., Basingstoke, Hampshire, UK) and incubated at 37°C for 24h.

2.6 Identification of Indicator Organisms:

Following incubation, all plates were examined morphologically for the presence of the indicator organisms. Additional identification tests such as the oxidase test were used, and Gram stains were also performed. All suspect colonies were cultured for purity on to the appropriate agar base and identified using the API 20E identification kit (Bio-Merieux SA, Montaleu, Vercieu, France). Confirmed isolates were cultured on to Columbia agar (Lab M, Bury, UK) supplemented with 7% horse blood, and frozen at -70°C on Protect beads (Technical Service Consultants Ltd., Lancashire, UK), until required.

2.7 Epidemiological Typing:

Bacteria were grown on Columbia agar, supplemented with 7% horse blood, incubated in air at 37°C for 48h. Cultures were harvested and suspended in 3ml SE buffer (5M NaCl, 0.5M EDTA). Cells were washed twice in fresh SE buffer and re-suspended to achieve a density equivalent to a Macfarland Standard No.4 (Bio Merieux SA, Marcy-l'Etoile, France). A 2% (w/v) low-gelling agarose (Sigma Chemical Co., St. Louis, MO, USA) was prepared in SE buffer, and dispensed into pre-warmed 1.5ml Eppendorf tubes (Sarstedt, Aktiengesellschaft & Co., Numbrecht, Germany). 220µl aliquots of the bacterial suspension were added to the tubes, mixed gently and transferred to the Block Mould (Bio-Rad Laboratories, Alfred Nobel Drive, Hercules, USA). Following refrigeration for at least 30 mins, the moulds were carefully transferred into labelled universals (Bibby Sterilin Ltd.,Tilling Drive,

Stone, Staffs, OSA, USA), containing 1ml lysis buffer (1M tris pH 8.0, 0.5M EDTA pH 8.0, lysozyme). The universals were incubated in a 37°C water bath (Grant Instruments (Cambridge) Ltd., Barrington, Cambridge, UK), for 2-3h and then transferred to newly labeled universals containing a 1% SDS and Proteinase K solution (SDS, TE Buffer, Proteinase K). These universals containing the blocks were then incubated at 50°C overnight. Blocks were washed in pre-warmed TE buffer, and the universals placed in a 50°C shaking water bath (Grant Instruments (Cambridge) Ltd., Barrington, Cambridge, UK). After 4 successive washes, the blocks were placed in fresh TE buffer and stored at 4°C overnight. A 2.5x5mm portion from each block was cut the next day, and placed into separate 1.5ml Eppendorf tubes containing 1ml of fresh TE buffer. The tubes were refrigerated for at minimum of 30 min. The slivers were then transferred to tubes containing 150µl of Reaction buffer (Promega Corporation, Woods Hollow Road, Madison, WI, USA) and refrigerated for at least 30 min. The enzyme *Xba* I mix (Promega Corporation, Woods Hollow Road, Madison, WI, USA) was prepared on ice and 50µl added to each tube. The tubes were incubated at 37°C for 3h by transferring the blocks in Modified TE buffer (1M Tris pH 7.6, 0.5M EDTA pH 8.0) at 4°C for 30 min.

2.8 Preparation of an agarose gel for PFGE:

As a general rule a gel concentration of 1.2% will give clear bands over a range of 1-2500kb. The gel size can be varied depending on the number of samples being processed. The slivers to be loaded were picked up using a sterile scalpel and placed against the leading edge of the well. The order of each block was recorded and a Molecular Weight Marker (Boehringer Mannheim Biochemica, GmbH, Germany) was also included. Once loaded, the wells were sealed with a sealing agarose and

allowed to set for 30 min at 4°C. 3L of cooled TBE (Tris base, Boric Acid, 0.5M EDTA pH 8.0) was poured into the Tank and allowed to equilibrate for 30 min. Once the gel was placed in the Tank, all equipment was switched on and parameters set as follows.

Pulsewave: Initial time: 5 sec Final time: 50 sec Run time: 22h.

Power Supply: Volts: 200 Run time: 22h.

When the run was complete the gel was stained with Ethidium Bromide (Sigma Chemical Co., St. Louis, MO, USA) to allow visualisation under UV light. The gel was placed in a suitably sized tray and covered with Ethidium Bromide and left at room temperature for 30 min. De-staining for 15 min followed staining. Waste Ethidium Bromide was placed in a waste container prior to decontamination. The gel was next photographed under UV light using a Polaroid MP+ Instant Camera System.

3.0 Results:

3.1 In Vitro Work

Experiments were carried out with *E.coli* and *S. marcescens*. Plants were grown for 4-6 week cycles in autotrophic systems. Serial subcultures were then carried out. Strains were recovered both endophytically and epiphytically (See **Table 1** and **2**). Physiological effects observed in detail during the first subculture were as follows:

Control plants were seen to grow up to 70 mm. The inoculated plants were stunted to about half that height and had fewer nodes. In all treated cases symptoms evident on the plant were blackening of the lower stem. The one exception to this was the treatment with *E.coli* 10^{-9} that didn't show evidence of basal stem rot and seemed

less stunted to about ¾ of the height of the Control plants. Similar results were recorded for the next subculture. Symptoms were observed about 2.5 weeks into culture as black/brown lesions at stem bases. After the third subculture the enteric strains became pathogenic to the plants *in vitro.*

3.2 Growth in inorganic M&S media

Samples of spent media were taken and a dilution series was constructed to determine the number of bacteria present in the media after 4 weeks the results are shown in **Table 3.** This shows that the inoculants multiplied in the autotrophic systems. Inorganic M&S is essentially a mineral salt solution which contains no carbon sources. For the bacteria to multiply they depended on plant leakage for nutrient supply

3.3 PFGE Results:

E. coli and *S. marcescens* were found to persist both epiphytically, and endophytically on all micropropagated plant material. PFGE banding patterns of the bacterial strains isolated, showed similar banding patterns to the original strains used in the study. Of all 39 *E. coli* strains typed, the resulting restriction patterns were indistinguishable from the original strains typed. The 58 *S.marcescens* isolates typed, showed the same banding patterns as the original strain.

4.0 Discussion:

Two bacterial strains of medical importance were chosen for this study. *E. coli* is a Gram-negative, lactose fermenting bacterium which is a normal part of the gut flora of mammals especially cattle and man. This bacteria has been associated in

causing infections in man and animals, many of the diarrhoeal type. The most noteworthy pathogenic sub-group is enterohemorrhagic *E. coli* (EHEC), of which the serotype 0157 is well known, is the causative agent of bloody diarrhoea. Outbreaks of *E. coli* 0157 have been reported world wide with several fatalities resulting (Bolton and Aird, 1998). *S. marcescens* is a Gram-negative, lactose fermenting organism implicated in causing a variety of nosocomial infections (Miranda *et al.*, 1996, Herra *et al.*, 1998). Its ability to survive in many different environments has highlighted it's ability to persist as a highly successful pathogen in clinical settings. *S. marcescens* has been isolated from medical equipment such as intravenous catheters and needles (Ashkenazi *et al.*, 1986), and blood transfusion bags (Parment *et al.*, 1993). The introduction of these pathogenic bacteria into the domain of growing vegetable plants, is indeed alien. These bacterial strains of clinical significance are typically not associated with plants and are not known plant pathogens. The subsequent re-isolation of *E. coli,* and *S. marcescens* from the chosen plant types, has proved to us that these human and food poisoning pathogens have the ability to survive on and within healthy micropropagated plants.

It was demonstrated that human pathogenic species, particularly *E. coli,* could survive *on* and *in* plants at very low concentrations. Strains were found to persist in autotrophic culture. This indicates that plant leakage supports growth of enteric bacteria. In the case of *Serratia* more growth was seen than that of *E. coli.* After serial subcultures inoculated bacteria were repeatedly re-isolated from the progeny plants though some plants were asymptomatic, but in some cases the bacteria became *vitro* pathogens in the latter subcultures. It is evident then that even dilutions as low as used here will still colonise those plants and persist via

serial subculture even in harsh bacterial environments (i.e. Inorganic Murashige & Skoog media)

Given this evidence, it would seem apparent then, that the potential risk factor associated with the consumption of plant food contaminated with human food poisoning bacteria should be more fully investigated.

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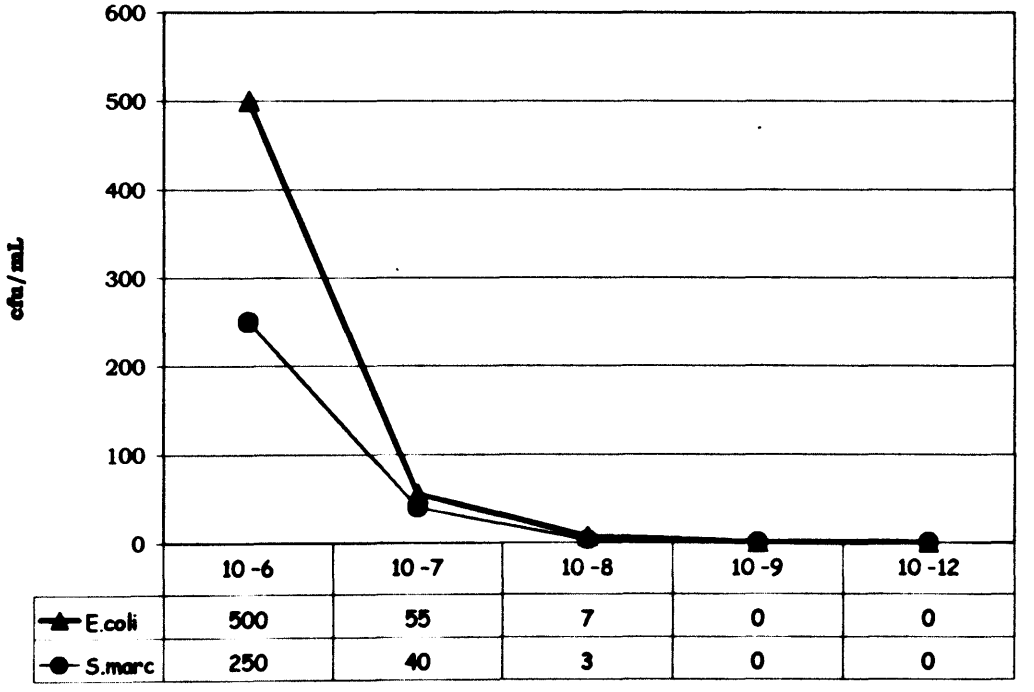
Graph 1 Inoculation Levels Used For *In Vitro* Work



	10 -6	10 -7	10 -8	10 -9	10 -12
E.coli	500	55	7	0	0
S.marc	250	40	3	0	0

Table 1 Presence Of *E. coli In Vitro.* At the 3rd subculture the inoculants became *vitro* pathogens

	Controls	E. coli 10^{-7}	E. coli 10^{-8}	E. coli 10^{-9}
4 weeks epiphytic	-	+	+	+
4 weeks endophytic	-	+	+	+
8 weeks epiphytic	-	+	+	+
8 Weeks endophytic	-	+	+	+

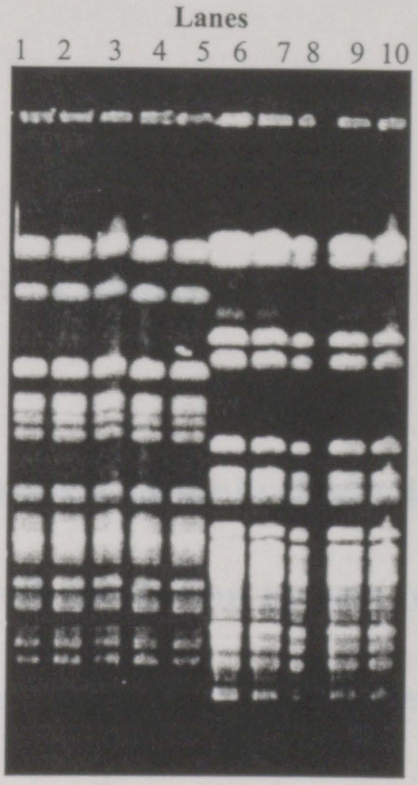
Table 2 Presence Of *S. marcescens In Vitro.* At the 3rd subculture the inoculants became *vitro* pathogens

	Controls	S. marcescens 10^{-7}	S. marcescens 10^{-8}	S. marcescens 10^{-9}
4 weeks epiphytic	-	+	+	+
4 weeks endophytic	-	+	+	+
8 weeks epiphytic	-	+	+	+
8 weeks endophytic	-	+	+	+

Table 3 Counts taken from autotrophic spent media

Treatment	Counts taken from spent media
Controls	0
E.coli 10^{-9}	8.67×10^{4} cfu/ml
E.coli 10^{-8}	4.6×10^{5} cfu/ml
E.coli 10^{-7}	4.77×10^{5} cfu/ml
S.marc 10^{-9}	5.81×10^{7} cfu/ml
S.marc 10^{-8}	1.54×10^{7} cfu/ml
S.marc 10^{-7}	1.08×10^{7} cfu/ml

Fig. 1 Gel showing *Xba*1 digestion patterns of *E.coli* (lanes 1-5) and *S.marcescens* (lanes 6-10)



Chapter Nine

Escherichia coli persists endophytically in cabbage and is associated with alteration in host protein and increased chitinase activity

Section C: Investigation of persistence of enteric bacteria in/on plants

Preface to Chapter 9

This work is a continuation of the work discussed in the last chapter (8). Sequencing of protein bands is as yet still underway in the Protein Facility in the University of Aberdeen and results were not ready for presentation at the time of submission. The style is that of the journal Acta Horticulturae.

ESCHERICHIA. COLI PERSISTS ENDOPHYTICALLY IN CABBAGE AND IS ASSOCIATED WITH ALTERATION IN HOST PROTEINS AND INCREASED CHITINASE ACTIVITY

Abstract:

Aseptic cabbage microplants were inoculated *in vitro* with *E. coli.* Established plants were grown in soilless culture and sampled using clinical pre-enrichment and selection techniques. An imunohistochemical *in situ* method detected *E. coli* endophytically in the microplants, however, only epiphytic *E. coli* could be recovered by the enrichment/selection method. At harvest, after 14 weeks in hydroponic culture, sampling was carried out again but the inoculant was detected infrequently and only epiphytically by the enrichment/selection method. Host proteins were extracted and separated by SDS-gel electrophoresis. There was a difference in protein banding in the region for putative pathogenesis-related proteins in *E. coli*-inoculated microplants. Chitinase levels were significantly higher in the latter. The results are discussed in relation to the microbial safety and potential allergenicity of raw salad vegetables.

Keywords: bacterial contamination, food poisoning, salad vegetables, immunohistochemistry, PAGE, pathogenesis-related (PR) proteins, plant tissue culture

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1.0 Introduction:

It is widely recognized by scientists, legislators, producers and consumers that there are increasing health risks associated with modern agricultural practices where the pressure to produce cheap food has led to intensification of production. (Beuchat, 1996; Little et al., 1997; Tauxe et al., 1997, Mahon et al., 1997). Globalisation of trade and intensification of agricultural production and practices such as organic (syn. biological, biodynamic, ecological) farming; land application of slurry and poultry waste; land drilling of abattoir waste; recycling of processing water and discharge of contaminated processing water are factors underlying the increase in biological pollution of the environment with human pathogenic bacteria (e.g. Koenraad et al., 1995). Supermarkets with their requirements for prolonged shelf-life and the rapidly expanding market for raw salad vegetable pre-packs and microwaveable vegetable pre-packs, are also increasing risk factors (Rafferty and Cassells, 2000). There is a need to assess the potential health risks of the transmission of harmful bacteria, applied as organic soil amendments to vegetables, which are eaten raw, or with minimal cooking e.g. microwave cooking. These risks are potentially two-fold; firstly from contamination with human-pathogenic bacteria; and secondly, from the effects of bacterial elicitation of pathogenesis-related proteins which are potential allergens (Neuhaus, 1999).

Here, aseptic cabbage plants were inoculated *in vitro* with a model strain of *E. coli* to establish gnotobiotic cultures. Microplants from these cultures were grown in soilless culture (grown hydroponically), were sampled using pre-enrichment and selective media for the epiphytic and endophytic persistence of *E. coli*. Interactions between *E. coli* and the host plant were investigated by analysing host tissues for pathogenesis-related proteins and chitinase activity. Pathogenesis-related proteins are induced in

pathogen-host interactions (van Loon, 1999). To distinguish between non-specific and pathogenesis-related protein changes, chitinases which are characteristic of pathogenesis-related protein induction, were assayed.

2.0 Materials and Methods:

2.1 Inoculation of aseptic seedlings:

Escherichia coli (clinical strain ref. no. 945.1; St James Hospital Dublin 8, Ireland) a non-pathogenic representative of food-poisoning *E. coli,* was selected as the model isolate. This isolate was grown in tryptone soya broth (Oxoid,Ltd., Basingstoke, Hampshire, England) to an OD of 0.4 at 470nm and diluted appropriately for use. The following series of dilutions were chosen: 10^{-12}, 10^{-9},10^{-6} and 10^{-4}. These dilutions were chosen as they represented levels of bacteria that were detectable using conventional culture methods (10^{-4}), levels below acceptable conventional plate count numbers (10^{-6}) and levels that could not be detected by conventional plating (10^{-9}, 10^{-12}) (Rafferty *et al.*, 2000). 100 µl aliquots were plated onto sterile water agar. These plates were used for germination of surface sterilised cabbage seeds (*Brassica oleracea* var. *capitata* L., F1 hybrid, 'Derby Day', suppliers: Tozer, Cobham, UK) for 8-10 days. Cabbage seed was surface sterilized in 80% (v/v) aq. ethanol and immersed in 20% (v/v) aq. Domestos (Lever Bros, Liverpool, UK) for 15-20min and washed in sterile distilled water (x3) in a laminar-flow cabinet prior to placing the seeds on plates of sterile water agar (6g l^{-1} agar, Sigma-Aldrich Ireland Ltd.). There were 20 seeds per plate. Nodes from the inoculated seedlings and non-inoculated controls were transferred to plant tissue culture medium and grown on for 5 weeks (see below).

2.2 Autotrophic tissue culture:

Following germination the nodes were excised 8-10 days after inoculation and placed in Magenta GA-7 vessels (Sigma- Aldrich Ireland Ltd) containing polyurethane foam (Plant Biotechnology (UCC) Cork) for tissue support, imbibed with half strength M+S mineral solution (cat. No M-5524, Sigma Chemical Co., Dublin, Ireland) (Cassells and Walsh 1996). These were grown on in a growth room under the following standard conditions: 23±1 ^{0} C, 16 hour photoperiod (white 65/80 w Liteguard tubes, Osram Ltd., UK.) with PPF of 30 μmol m^{-2} s^{-1} at shelf-height.

2.3 Soilless culture:

For soilless production of cabbages, small-scale hydroponic systems were set up (Fig. 1). Perlite and sand were sterilised by autoclaving on three consecutive days for 1h. The pots were filled with perlite and planted with 3-4 five week-old *in vitro* microplants per pot. There were ten pots per container. A top dressing of sand was used to prevent algal growth. Separate containers were used for the control plants and for each dilution to avoid cross-contamination. Half strength hydroponic culture medium (Hoaglands Solution; Sigma Chemical Co., Dublin, Ireland) was trickled though the pots for 15min every alternate 15 min throughout a sixteen-hour period. The pots were not fed during the dark period.

2.4 Monitoring *E. coli* in cabbage microplants in vitro and in and plants in soilless culture:

Microplants were monitored every 5 weeks after inoculation in gnotobiotic *in vitro* cultures. Plants were grown hydroponically for 15 weeks. The plants were sampled every 2-4 weeks (see 2.5 below) and the tissues analysed for surface and endophytic

bacterial contamination. After 8 weeks the medium was sampled for *E. coli*. Plant growth parameters were measured at week 9. Five plants were chosen at random and stem height and leaf widths were taken, plants were also monitored for any physical lesions or browning.

2.5 Bacterial indexing:

Microplants and established hydroponic plants were harvested using a fresh pair of latex gloves per treatment to avoid cross-contamination. Non-sterile plants were sampled by direct plating to MacConkey agar (Oxoid Ltd., Basingstoke, Hampshire, UK) and incubated at 37°C for 24h. MacConkey is a Gram-negative rod selective agar (York *et al.*, 2000). Presumptive colonies appear flat, dry and non-mucoid with a red to pink colour (York *et al.*, 2000, Oxoid manual, 2001). Isolates from this procedure were considered to be epiphytes. Whole plants were used when still small enough (up to 6 weeks in hydroponic culture), thereafter stem and leaf sections were used. In parallel, the plant material was surface sterilized by immersing the plants in 80% (v/v) aq. Absolute ethanol (Merck, Darmstadt, Germany) for 45 sec, then in 2% (v/v) aq. Stericol (Stericol Hospital Disinfectant, Lever Industrial Ltd., Runcorn, Cheshire, UK) for 30 min, followed by washing in sterile distilled water (x3). The Stericol surface sterilisation technique developed was devised to be stringent in order to ensure that results indicated true endophytic contamination (Rafferty *et al.*, 2000). These samples were incubated on MacConkey agar at 37°C for 24h. If no growth occurred the plants/tissues were placed in 9ml Ringers (Oxoid Ltd., Basingstoke, Hampshire, UK) and 1ml buffered peptone water solution (Oxoid Ltd., Basingstoke, Hampshire, UK), and homogenised by hand in a stomacher bag (Rafferty *et al.*, 2000.). The pre-enrichment step was used here to improve detection rates

(Blackburn and McCarthy, 2000). To select for *E. coli* the homogenate was plated on to MacConkey agar and incubated at 37°C for 24h. Isolates from this method were considered to be epiphytes.

2.6 Bacterial identification:

Following incubation, all plates were examined and colonies were complex streaked for purity on MacConkey agar overnight. Identification was carried out using the API 20E miniaturized biochemical identification kit (bioMerieux SA, Montaleu, Vercieu, France). This kit is specific to clinical enteric isolates and frequently used for isolate confirmation (Rhodes *et al.*, 1998, Brion *et al.*, 2000, Huys *et al.*, 2000, Turner *et al.*, 2000). Isolates confirmed by API characterisation were cultured onto Columbia agar (Lab M, Bury, UK) supplemented with 7% horse blood, and frozen at -70°C on Protect beads (Technical Service Consultants Ltd., Lancashire, UK).(Rafferty *et al.*, 2000).

2.7 Tissue preparation and sectioning:

Control and samples from gnotobiotic cultures were fixing with 4% (v/v) aq. paraformaldehyde (PFA) at pH 7.3. Tissues were fixed for 1h. Prior to dehydration in alcohol the tissue was washed 3 times in 10mMDTT (Dithiothreitol) made up in phosphate buffer (NaCl, 8g/l, KCl, 0.2g/l, $Na_2HPO_4.2H_2O$, 1.15g/l, pH7.3). Tissue was dehydrated in the following series of v/v aq. alcohol: 10%, 25%, 50%, 75%, 95%and 100%. Each step was carried out for 30 min at 4°C. The tissue was then placed into a small plastic cassette, positioned (to allow transverse sections to be made) and submerged in paraffin wax to a depth of approximately 1cm. The wax was then quickly cooled and allowed to harden overnight. These were then sectioned

on a microtome set to 5-7 μm. As the sections came off they were floated on water (set to 50°C) and the picked up on clean slides. These were allowed to set in an incubator for an hour. The sections were dewaxed and rehydrated by passing then through a histolene step and an alcohol series, each step took about 3min each. The slides were then ready for staining.

2.8 Immunohistochemical staining:

Initially a commercially available peroxidase-conjugated Rabbit Anti-*E. coli* antibody (Code PO361 by DAKO, Laboratory Instruments and Supplies Ltd., Co Meath, Ireland) was used with DAB (diaminobenzidine) as substrate. Subsequently, a double antibody sandwich method (DAS) was developed. For this, cultures of the model *E. coli* isolate were sent to the Biological Services Unit (National University of Ireland, Cork, Ireland) for development of polyclonal antibodies in rabbit. A commercial secondary anti -rabbit antibody (Code F0205 by DAKO) with an FITC tag was used to bind to this primary antibody. The dilution series for the direct antibody were 1/10, 1/100, 1/500, 1/1000. For the primary antibody in the DAS method the dilutions were, 1/100. 1/250 and 1/500 and the dilutions of the secondary antibody were 1/20 and 1/40. Incubation was carried out at 3-5 °C for 60 min and overnight with the direct antibody method, 30/60 min incubations were carried out for all dilutions of the primary and secondary antibodies in the DAS method as well as overnight incubations with the primary antibody. 30 and 60-min incubations were carried out at room temperature and overnight incubations were carried out in a moist chamber at 3-5 ° C.

2.9 SDS PAGE of cabbage proteins:

The extraction procedure was carried out as per Rahimi *et al.*, (1996). Cabbage tissue (1g tissue /ml buffer) was ground in Tris-HCl Buffer (100mM Tris-HCl buffer, pH7 containing 10mM 2-mercaptoethanol) using Agdia extraction bags (BioRad, Marnes-la-Coquette, France) and a ball-bearing grinder. The extract was passed through cheesecloth and filtered through Whatman No1 paper, centrifuged at 17,600g for 20 min at 4°C and stored at -20 °C. A standard curve of bovine serum albumin (BSA) was constructed by making up the following volumes to 5ml with Bradford Reagent (Alpha Technologies, Dublin 6, Ireland), 0, 0.125, 0.25, 0.5, 0.75, 1.0 mg ml^{-1} BSA. This was repeated for each sample and the dilutions were incubated for at least 2 min at room temperature. Optical density (OD) was read at 595nm. The BSA standard curve was used to calculate the protein content of the samples and was used to standardise the samples for gel electrophoresis.

Extracts were boiled for 10min with 2 vol. of sample denaturing buffer (125mM Tris base, pH adjusted to 6.8 with 3M HCl containing 0.4% (w/v) SDS, 10% (w/v) glycerol, 4% (v/v) 2 mercaptoethanol and 0.02% (w/v) bromophenol blue). Samples were loaded into precast 15% resolving gels (BIO-RAD, Alpha technologies, Dublin 6, Ireland). Gels were run in buffer (Tris base, 3g l^{-1}, glycine 14.4g l^{-1}, SDS 1g l^{-1}) for up to 45 min at 200v.

The gel was removed from the rig and fixed in 10% (v/v) aq. acetic acid for 30min. The acid was poured off and retained. The gel was then washed in distilled water for 2 min x 3. Staining was carried out overnight with gentle agitation using Coomassie Blue solution (500ml 100% ethanol, 160ml glacial acetic acid, 2 g Coomassie Blue diluted to 2l with distilled water). The staining solution was poured

off and several washes of destain (as staining solution but without the Coomassie Blue) were used over a 2-4hr period. The gel was then washed in distilled water.

2.10 Chitinase assay:

Plant extracts as used in PR protein analyses (see section 2.9) were assayed using the chitinase assay of Wirth and Wolf (1992). Carboxymethyl-Chitin-Remazol Brilliant Violet (CM-chitin -RBV) (Blue Substrates, Grisebachstraße 6, D-3400, Göttingen, Germany) was used as the substrate to assay for endo-acting chitinase. Assays were performed in 96 well microtitre plates (Costar Europe, High Wycombe, UK; cat no. 3590). Each well contained the following, 50µl of CM-chitin -RBV, 100 µl of extract, 50µl of buffer (0.2 M sodium acetate - acetic acid buffer, pH5). Control wells contained no extract until after the acid addition. (4 control replicates and 8 test replicates were used). Incubation was carried out at 40°C for 3 hours. The reaction was stopped using 50µl of 1N HCl. Plates were cooled on ice for 10 min and centrifuged (1450g x 10mins). 175 µl of supernatants were transferred to a 96 well half size EIA plate (Costar, cat no 3690). Activity was read at 550nm for Chitin-RBV. Extracts with a reading > 0.1 were diluted and assayed again to avoid errors due to substrate limitation. Calculation of 1 unit of enzyme activity was carried out using the following formula: Absorbance x 1000 x min^{-1}

3.0 Results:

3.1 Re-isolation of *E. coli* from inoculated cabbages:

No bacterial contaminants were detected in the *in vitro* non-inoculated microplants. Only *E. coli* was detected in *in vitro* inoculated microplants (Table 2). In the latter cultures, after 5 weeks *in vitro* epiphytic *E. coli* were isolated from the

lower inoculum dilutions 10^{-4} and 10^{-6}. No endophytic growth was detected (Table 2) by culture indexing (but see below).

When the plants from inoculated cultures were grown in soilless culture, after 6 weeks epiphytic *E. coli* was detected from the more concentrated inocula; it was also detected endophytically (10^{-4} and 10^{-6}). *E. coli* was not detected as an epiphyte in the lower dilutions but did appear endophytically (10^{-9} and 10^{-12}). At 8 weeks media from all hydroponic containers was analysed for bacterial contamination including *E. coli*. A positive ID for *E. coli* was only found in the 10^{-6} dilution treatment. The other isolates were not identifiable in the API Kit. After 10 weeks in hydroponic culture, no *E. coli* were isolated from any of the sampled tissues. Of the 13 epiphytic isolates none was found to be *E. coli*. At the end of the trial (15 weeks), none of the isolates detected internally or externally in the tissues gave a positive API identification for *E. coli* except for the plants in the 10^{-6} dilution treatment. Epiphytic *E. coli* were found on both stems and leaves.

3.2 Plant growth parameters:

Mid-way through the growth period, a series of measurements of the leaves and the stem heights were taken. Leaves and stems were chosen at random and 5 measurements of each were taken. The graphed results (Fig. 2) show that the *E. coli* had no adverse affect on the growth of inoculated cabbages. No lesions or wilting was observed on the plants that had been inoculated at germination with *E. coli*.

3.3 Immunohistochemical staining:

The initial immunohistochemical procedure used was based on a commercial peroxidase-conjugated rabbit anti-*E. coli* antibody. On microscopic examination of

sections stained using this antibody with DAB as substrate, it was seen that the antibody bound non-specifically to xylem vessel in non-inoculated controls. The technique was modified using specific polyclonal antibodies in a double antibody sandwich assay. In an effort to optimise the procedure, a variety of polyclonal antibody and secondary antibody dilutions were used as well as several incubation regimes. Best results were found if the following combination was used: primary antibody 1/100 dilution, secondary antibody 1/20 dilution with sequential 60 minute incubations at room temperature. No improvement was seen if the primary antibody was incubated overnight.

The results showed that while there was some background fluorescence of the xylem vessels, only in inoculated tissues did the tissue surrounding these fluoresce. Fig. 3 demonstrates the localization of *E. coli* within the tissues surrounding the vascular bundles of the stems of inoculated *in vitro* cabbages. The sections were from the control plants and the plants which had been inoculated with a 10^{-4} dilution of *E. coli*. No endophytes could be detected in these plants using clinical culturing methods (Table 2).

3.4 SDS-PAGE protein analyses:

An inverted image of the PR protein gel containing the protein extracted from control and inoculated cabbage plants is shown in Fig. 4. The cabbages were sampled after 15 weeks in the hydroponic system; *E. coli* was recovered culturally, as epiphytes, only from the stem and leaf of cabbages initially inoculated with dilution 10^{-6}. The gel used was specific for the resolution of proteins with molecular weights in the 20-50 kD range. A 37kD band is apparent in all samples but as the concentration of bacteria inoculum used increased, a new band appears just below

the 37Kd band at dilution 10^{-9} and is also visible in 10^{-6} and 10^{-4} inoculum dilutions. A new band appeared at the highest inoculum used at the 25kD. A band occurring in all treatments (between the 15kD and 25kD markers) appears more concentrated as the bacterial inoculum concentrations increase, i.e. from lanes 1 to 5.

3.5 Chitinase assays:

The results of the chitinase assays are shown in Fig 5. It can be seen that the extracts from control and those cabbages from 10^{-12} and 10^{-9} inoculum dilutions are not significantly different. However, those extracts from cabbages initially inoculated with 10^{-6} and 10^{-4} dilution of *E. coli* show significantly higher levels of chitinase activity. This may correlate with the increased expression of the ~20kD band seen on the PAGE gel (Fig 4).

4. Discussion

Bacterial endophytic colonisation of plants has been widely reported (Chanway, 1998) and previously it has been shown that *E. coli* may colonise plants endophytically (Cassells and Tahmatsidou, 1997) indicating that the internal tissues of plants may be relatively nutrient rich. The results from soilless culture substantiate concerns that routine cultural techniques for the detection of bacterial contamination of vegetables are not dependable in relation to endophytic bacteria. The latter pose human health risk, as endophytic bacteria are resistant to standard surface sterilization procedures. Similar concerns have been expressed regarding the escape of bacteria from surface sterilants by bacteria in biofilms (Costerton *et al.*, 1995). Here, in gnotobiotic cultures of cabbage and *E. coli*, *E. coli* was only detected in the culture medium and epiphytically but not endophytically in the plant tissues

when sampled with pre-enrichment and selective plating techniques. However, when examined by the DAS immunohistochemical technique it was found that *E. coli* was present endophytically in the tissues surrounding the vascular bundles (Fig. 3). Due to low titre, endophytic bacteria may not be expressed on selective agars within the traditional time limits used in testing, normally 24-48 h (Sata *et al.*, 2000, Yusof *et al.*, 2000).

Protein changes were detected by PAGE in the *E. coli*-colonised plants which were related to the inoculum concentration used (Fig.4). At the sampling period (15 weeks), the cabbages did not show symptoms of infection, had normal growth and *E. coli* was detected in only one dilution treatment and then as an epiphyte. The chitinase results (Fig. 5) corroborate what was observed by PAGE, as enzyme activity detected was significantly higher than in control, non-inoculated microplants. These results indicated a possible induction of host resistance following inoculation of aseptic cultures with *E. coli.*

The putative suppression of the *E. coli* within the plant may be due to host resistance induced by bacterial ethylene. Ethylene is a phytohormone, which is considered to be involved in the induction of pathogenesis-related proteins (Ohtsubo *et al.*, 1999). Some bacteria are known to produce ethylene e.g. *Pseudomonas*, *Ralstonia*, *Bacillus* (Weingart *et al.*, 1999, Bae and Kim 1998) and *Pseudomonas syringae* pathovars have also been shown to produce ethylene *in planta* (Weingart and Volksch, 1997). Though there are no reports in literature of ethylene production *in planta* by *E. coli,* however, in batch cultures *E. coli* has been shown to produce ethylene (Lloyd and Bunch, 1996). It is hypothesized that in the case of the cabbages inoculated with low levels of *E. coli* one of two responses may have occurred. Inoculation with *E. coli* may have elicited the plant ethylene-PR protein

pathway, or alternatively, ethylene production by *E. coli* may have induced PR-proteins including chitinases.

Chitinases have been previously reported in *Brassica* (Zhao and Chye, 1999) and are widely reported as components of induced resistance to plant pathogens (Hammond and Jones, 1996, van Loon 1999). They are also reported as having homology with proven human plant allergens (Yagami *et al.*, 1998, Neuhaus, 1999). Assuming that the chitinase molecular weight corresponds to that of the approx. 20K protein detected by PAGE (Fig. 4) then it may belong to the PR4 proteins, a class of pathogenesis-related proteins. These are usually endo-chitinases, which are made up of polypeptides of between 13-19kD. Hanninen *et al.*, (1999) previously showed that a PR4 protein from turnip (under stressed conditions) showed 70% homology to prohevin domains. This domain has found to be a major part of the protein that causes allergenicity to latex (Chen *et al.*, 1998). Pathogenesis-related proteins and phytoalexins are reported to affect consumer health as food allergens and teratogens (Moneret-Vautrin 1998, Gaffield & Keeler 1996). It is a cause of concern that *E. coli* taken up from the environment (Cassells and Tahmatsidou, 1997) from manures and contaminated water, may induce possible toxic substances in plants and also may pose a microbial threat to the so-called YOPI group (young, old, pregnant and the immunocompromised) as well as to the wider general public.

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Table 1. API confirmed re-isolations of *E. coli.* IV: Time *in vitro;* IH: Time in hydroponics; Sample numbers refer to the initial dilution of *E. coli* used to inoculated seeds.

	Sample	Location	Result
IV 5wks	-6	Epiphytic	*E. coli*
IV 5wks	-4	Epiphytic	*E. coli*
IH 6 wks	-12	Endophytic	*E. coli*
IH 6 wks	-9	Endophytic	*E. coli*
IH 6 wks	-6	Endophytic	*E. coli*
IH 6 wks	-6	Epiphytic	*E. coli*
IH 6 wks	-4	Endophytic	*E. coli*
IH 6 wks	-4	Epiphytic	*E. coli*
IH 8 wks	-6	Medium	*E. coli*
IH 15 wks	-6	Leaf, Epiphytic	*E. coli*
IH 15 wks	-6	Stem, Epiphytic	*E. coli*

Table 2. Expanded table of results for the *in vitro* sampling. IV: Time *in vitro;* IH: Time in hydroponics. Sample numbers refer to the initial dilution of *E. coli* used to inoculated seeds. NG: no growth; E. coli: isolated identity confirmed by API kit.

Sample label	Location	Result
Control	Endophytic	NG
Control	Epiphytic	NG
-12	Epiphytic	NG
-12	Endophytic	NG
-9	Epiphyte	NG
-9	Endophyte	NG
-6	Epiphyte	*E. coli*
-6	Endophyte	NG
-4	Endophyte	NG
-4	Epiphyte	*E. coli*

Table 3. Percentages. IV: Time *in vitro;* IH: Time in hydroponics.

	NG	N/I	*E.coli*
IV 5 weeks	80	0	20
IH 6 weeks	0	53	46
IH 10 weeks	54	46	0
IH 15 weeks	42	50	8
Total recovery %	44	37.25	18.5

Fig. 1. Plan and Front view of the hydroponic system used. A: Hydroponic plants,, B:, Pipe System for trickle feeding, C: Pots filled with Perlite, D: Nutrient Solution Pump. Arrows indicate the flow of the solution.

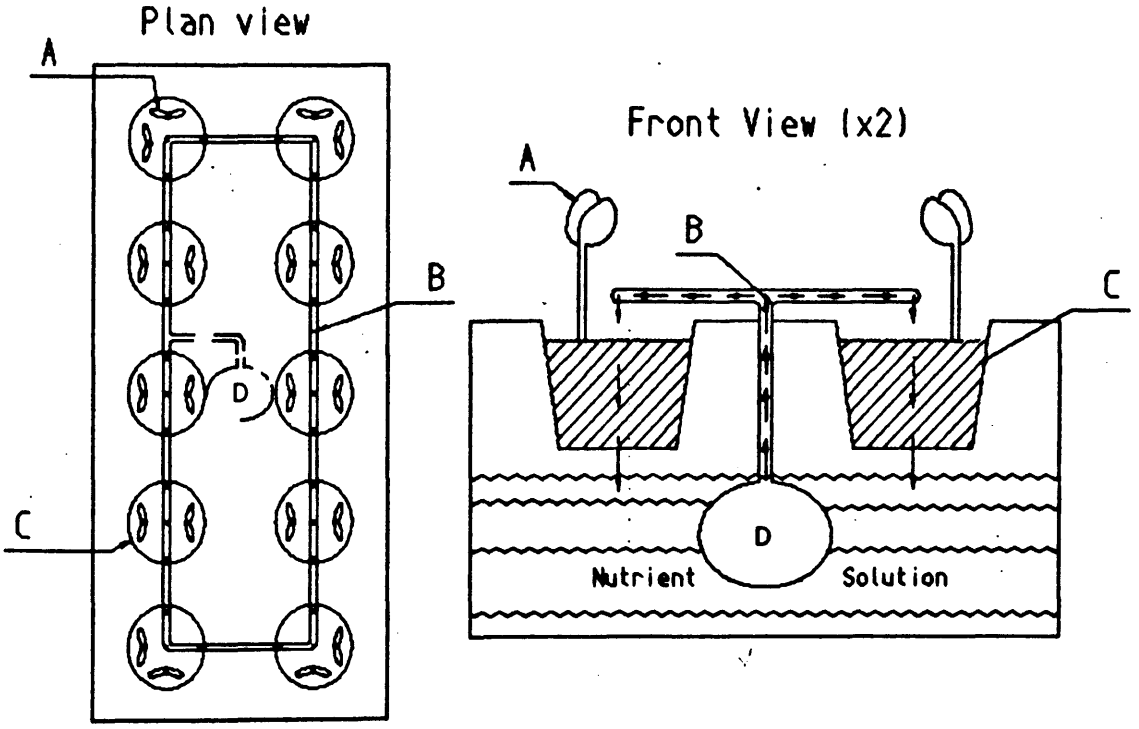
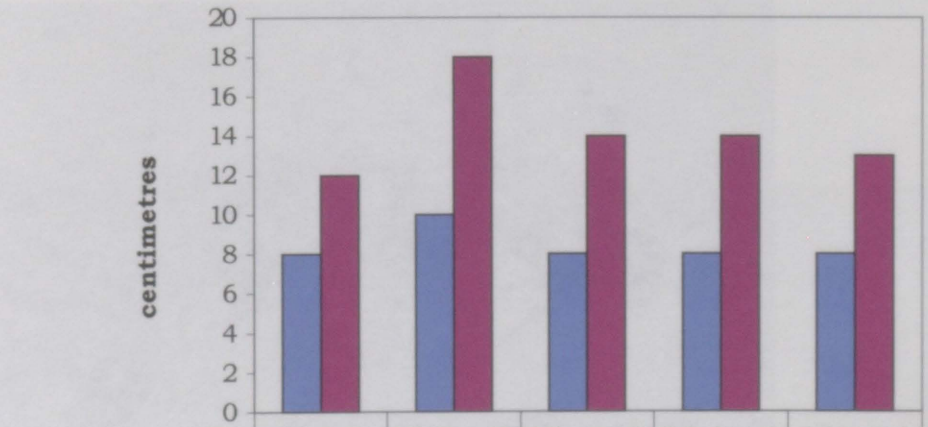


Fig. 2. Stem and leaf measurements of cabbages (after 9 weeks in hydroponics).



	Control	Dilution -12	Dilution -9	Dilution -6	Dilution -4
Median Stem Height	8	10	8	8	8
Median Leaf Width	12	18	14	14	13

Fig. 3. Top: Inverted image of a section of a control plant (*in vitro*, 5 weeks old). Bottom: Inverted image of a section of an inoculated plant (*in vitro*, 5 weeks old, inoculated at germination with a 10^{-4} dilution of *E. coli*)

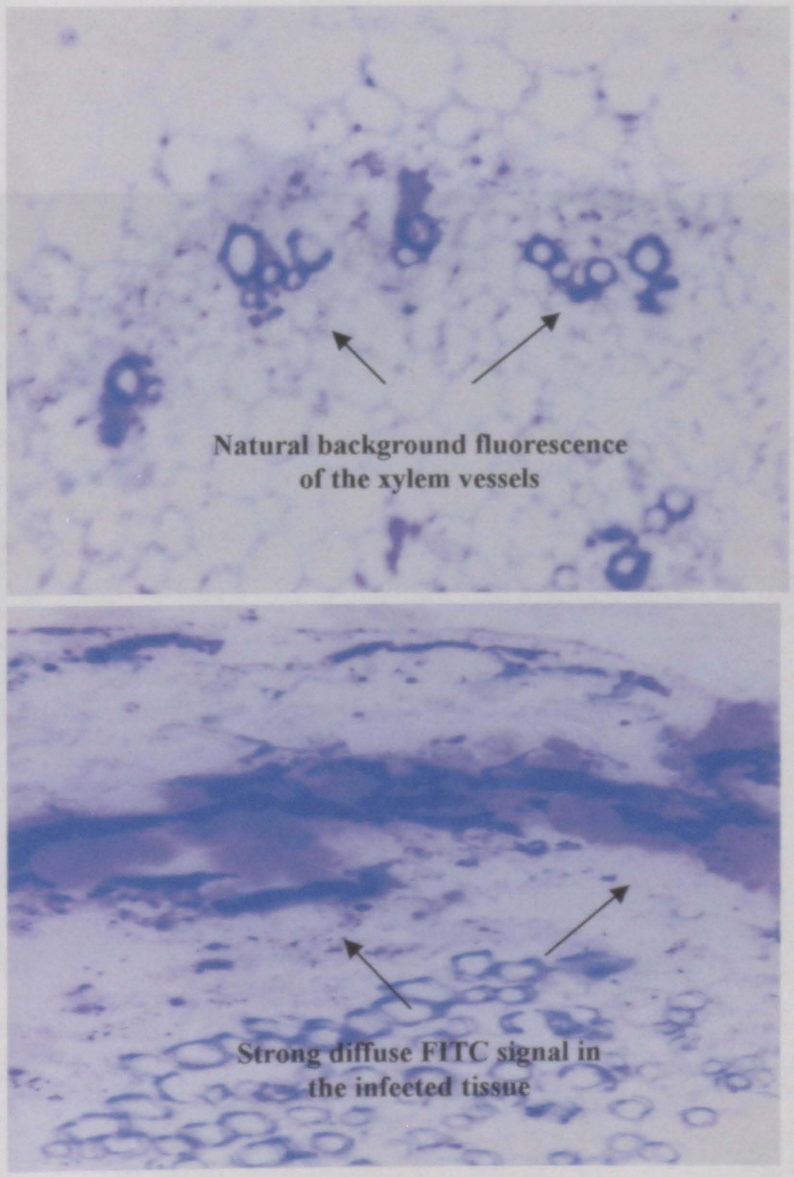


Fig. 4. PAGE gel of hydroponic cabbage protein extracts. The lanes (1 – 7, respectively) are as follows: plant inoculated with *E. coli* 10^{-4}; plant inoculated with *E. coli* 10^{-6}; plant inoculated with *E. coli* 10^{-9}; plant inoculated with *E. coli* 10^{-12}; control non-inoculated plant; DNA molecular weight markers; DNA markers repeated. Arrows indicate increase in banding intensity or appearance of a new band.

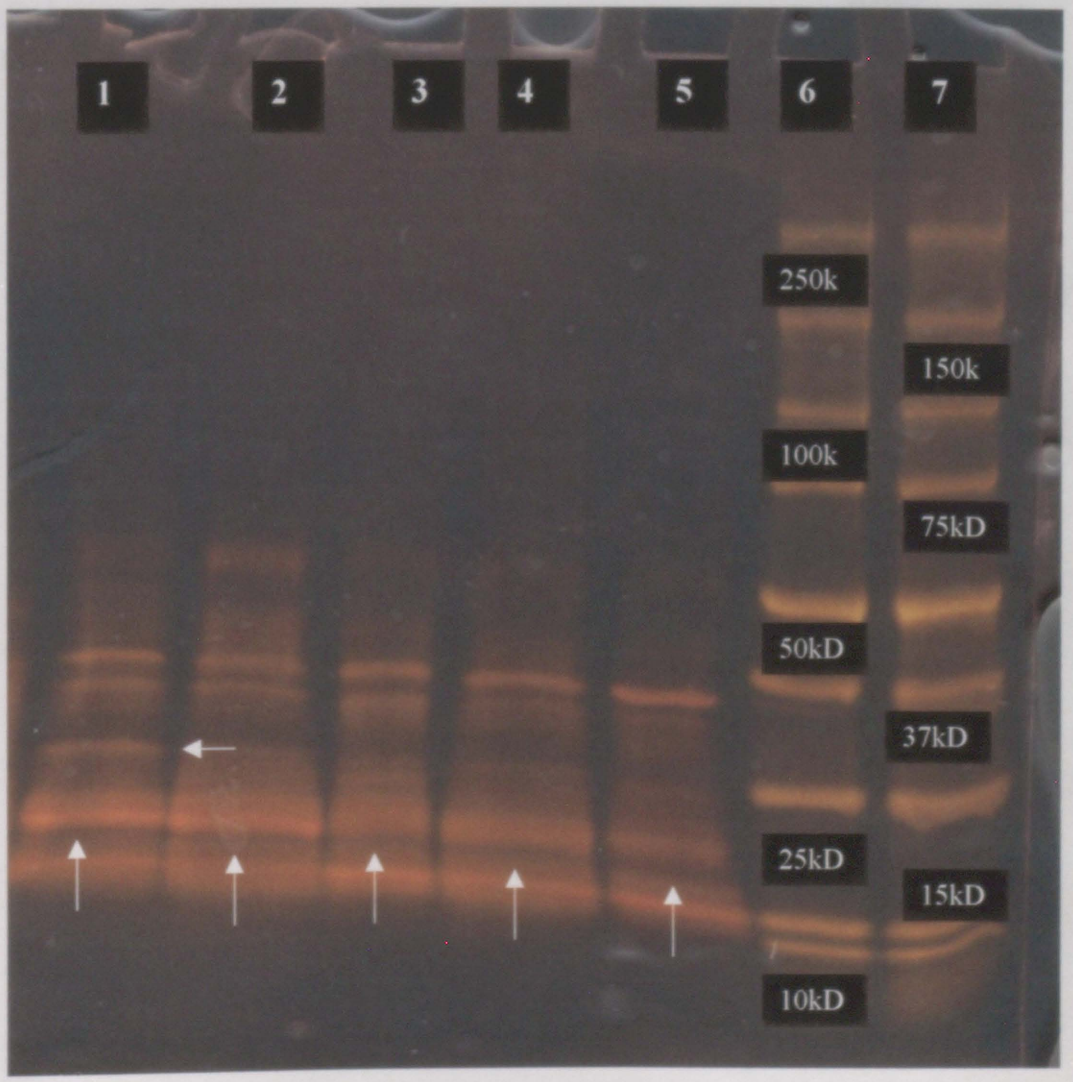
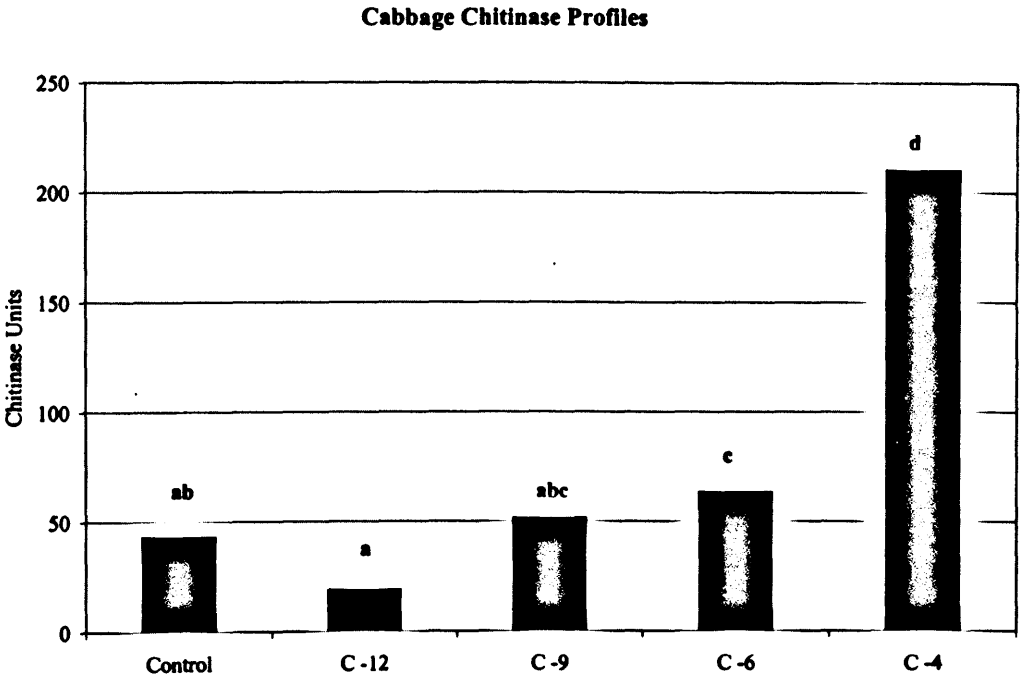


Fig. 5. Chitinase activity of hydroponic cabbage extracts.



Chapter Ten

General Discussion

Section D: Conclusions

General Discussion

Objectives of the work

The aims of this work were, firstly, to assess substrate amendment with crushed crustacean shellfish (CCS) waste as a method of biological control for soilborne disease; and secondly, to investigate the transmission of human pathogenic bacteria in raw salad vegetables. The overall object was to contribute to an understanding of the possible risks of sustainable crop production involving alternative disease control strategies where potential hazardous materials as here, are applied to crops, in the present model crushed crustacean shells (CCS). Crustacean shellfish is well documented to be a common source of human food poisoning pathogens. The material used, however, was not found to be contaminated with human pathogenic bacteria, a possible consequence of storage conditions and partial processing.

Model isolates of the human pathogenic bacteria, *E. coli* and *S. marcescens,* were deliberately inoculated into aseptic plants to follow their persistence in the salad vegetables in micropropagation and in hydroponic culture. Both in the case of the CCS and the model inoculants, efforts were made to elucidate the mechanisms involved in the interactions between the CCS, soil microorganisms (pathogens and antagonists) and the host plant, and between the model isolate and the host plant, respectively. The conclusions of the research are discussed below. Finally, HACCP guidelines for raw salad vegetable production are proposed based on results of both parts of the project (Chapter 11).

Section B: Investigation of the Biocontrol Properties of Chitin-Containing Crustacean Shellfish Waste

Biological Control using CCS as an amendment in the field

A problem with microbial inoculants is that they show strong host genotype-inoculum genotype-environment interaction. This necessitates expensive trials to optimise the inoculant for each host genotype/environment. Here an alternative approach to biological control was evaluated, namely, the use of an amendment with specificity for chinitolytic microorganisms. This strategy is potentially more durable as it can affect, with some selectivity, soil antagonists and its breakdown products may elicit host disease resistance (Chapter 5).

An objective of this work was to investigate the efficacy and mode of action of CCS in controlling *Sclerotinia* in Jerusalem Artichoke. CCS contains calcium and calcium is implicated in host resistance to *Sclerotinia* (Walsh, 1994), so a preliminary experiment was carried out to investigate the effects of calcium on *Sclerotinia* development in the field (Chapter 3). This was followed by a trial of CCS on disease development in the field and in store (Chapter 4). In the trials at Fota Island, Cork it was confirmed that increased calcium application reduced disease incidence without affecting yield. The costs of the high calcium treatment, found to cause the most disease reduction, were found to be easily absorbed if the market price was similar to that of seed potato prices. Currently the artichoke market is much smaller and the prices per tonne are extremely inflated due to short supply (€1270/tonne- Superquinn Supermarkets, personal communication, July 2001). However even assuming a drop to seed potato prices the crop would be able to absorb treatment costs.

Using CCS as an organic source of chitin and calcium the following was observed:

- CCS gave the greatest suppression of disease without significant effects on yield
- Stimulation of protease and chitinase producers in the soil was highest in the CCS treated plots
- Infected tubers were sensitised by CCS and showed significantly increased enzyme levels
- CCS formulated with peat (Suppressor™) was found to decrease spread of disease in store significantly

In conclusion, CCS soil amendment has some potential to reduce Sclerotinia disease development in the field by reduction of pathogen inoculum however, the stability of biological control strategies may be variable due to the strong interaction between the biocontrol agent, host genotype and soil environment (Boland, 1997).

The treatment may not be cost effective (€381/hectare) for all crops. Retail prices for Jerusalem artichokes are currently high in Ireland but demand for artichokes is low and so producers might not risk the additional cost of CCS soil amendment. The price of Artichokes traded in high volume could be similar to seed potato (€381per tonne) and if this was the case the price could be absorbed.

Storage of the crop is a problem as disease spreads throughout the crop store if present, particularly in our mild climate. Storage in Suppressor™ treatments may be economic where organic certification is required.

Evaluation in the glasshouse of Suppressor ™, a shellfish waste-containing compost

Trials with Suppressor™ in the glasshouse showed positive control effects, particularly in controlling wilt in the *Dianthus* microplants at weaning where almost

total loss of stock blocks occurred without treatment (chapter 7). However, it was found that strawberry microplants were not compatible with Suppressor™ at weaning (Chapter 6). Microplants inoculated with Vaminoc™ at weaning and then transferred to Suppressor™ compost 2 weeks post acclimatization, showed increased resistance to Redcore disease compared with plants maintained in non-amended potting compost. However, this additional repotting of plants would be labour intensive and combined with the inoculum and Suppressor™ costs, uneconomic except possibly in niche applications.

Chitinase producers and chitinase were found to be enhanced in the substrate by CCS amendment. Increases in chitinase produced *in planta* were also recorded (Chapters 5& 7). As discussed previously (Chapter 1), chitinase is an important factor implicated in biocontrol strategies. Promotion of extra-cellular enzymes in the substrate is a positive pathogen control factor as they can have long lasting effects surviving their microbial producers (Wirth and Wolf, 1992). Chitinase was also investigated *in planta* in the *Dianthus* trial. The *Dianthus* plants which survived infection by *Fusarium* wilt showed increased activity of this pathogenesis-induced enzyme. Electrophoresis of the *Dianthus* extracts also showed differential banding patterns when chitin and disease were present. Currently, the bands have been sent for sequencing to the Protein Facility, Dept. of Molecular and Cell Biology, University of Aberdeen, Scotland.

CCS substrate amendment protected micropropagated *Dianthus* plants where there was complete loss of the controls. When used in strawberry, in conjunction with mychorrizhae, some positive results were observed but there is a need to confirm that there are no negative host-substrate interactions and this would mean costly preliminary trials before applying the strategy to individual crop systems.

In summary, it has been demonstrated that there are strong correlations between incorporation of chitin in the substrate and suppression of *Sclerotinia*, *Phytophotora fragarie* and *Fusarium* wilt in Jerusalem artichoke, strawberry and *Dianthus* plants, respectively. However, there is evidence than that this control strategy may suffer from the host-pathogen specificity and environmental dependence that all biological control mechanisms are subject to. In addition, the cost of the treatment may, in general, be too high for ware crops but high value niche markets may be able to absorb the financial outlay.

Suggestions for future research on CCS as an amendment

Multi-locational and multi-annual trials are necessary to confirm the biological control potential of field amendment with CCS, as indigenous microbes will differ from area to area. It would be worth investigating if CCS supplemented with a nitrogen fertiliser improved disease suppression and yield. In addition, the effects should be investigated in long term trials (continuous trialling over 5-10 years) of CCS amendment in the field on antagonist populations to determine if the soil becomes pathogen suppressive.

Crop production systems for the future must be sustainable. While chemicals will play a role in the future, their adverse effects could be reduced if utilised in integrated pest management (IPM) strategies (Gullino *et al.*, 2000). As IPM is now accepted as the way forward, then further work with antagonist-promoting substrates and low doses of pesticides, combined with solarisation, would seem justified albeit having regard to cost effectiveness. For instance, combinations of alternative and conventional methods, with low levels of pesticide, may lead to the synergy seen in the control of peanut pathogens where soil solarisation used in combination with a

low dose of metham sodium resulted in the control of pod disease (Katan, 2000). This approach is less radical and looks at more cost effective ways of reducing chemical input and increasing more environmentally friendly input.

Here, the CCS was tested microbiologically for human pathogens. None were found in the sample tested but as this substrate comes from variable natural sources monitoring would need to be carried out on each batch before incorporation into soil or peat.

Section C: Investigation of Persistence of Enteric Bacteria in/on Plants

Persistence of enteric pathogens in planta

This section of the research dealt with the health risks associated with microbial contamination of raw salad vegetables and was based on inoculation with model strains of *E. coli* and *Serratia marcescens.*

As was demonstrated previously, ornamental plants can assimilate *E. coli* from manured soil (Cassells and Tahmatsidou, 1997). The work in Chapter 8 looked at the persistence of two model enteric bacteria *in planta.* Using clinical pre-enrichment and selective plating techniques, it was seen that *in vitro* plants, in a growth medium containing no carbon source, supported the growth and multiplication of the inoculants, which were found to be present on the plant surface and also *in planta.* During the sub-culturing process the inoculants were re-isolated despite many plants being asymptomatic. Three serial subcultures were carried out before the clinical isolates became *vitro* pathogens in this contained system. Further investigations with *E. coli* were carried out in mini-hydroponic systems (chapter 9)

The techniques used in this study involved clinical pre-enrichment and plating techniques, electrophoresis of pathogenesis related proteins, determination of chitinase activity as well as the development of a method for observing *E. coli in planta.*

- *E. coli* were observed in symptom-less *in vitro* cabbage plants *in planta* using the immunohistochemical technique developed
- Various inoculation rates did not adversely affect the growth of the cabbages
- At the end of the growth period, gel electrophoresis showed there was increased expression of proteins in a region that is associated with chitinase pathogenesis-related proteins
- Chitinase activity was increased with increased pathogen inoculum that corresponded to the altered PR protein-banding pattern.
- It was hypothesized that *E. coli* induced host resistance at a low level of inoculum as none of the plants showed any signs of necrosis

These results show parallels with inoculation of plants with biocontrol agents that induce the plant defense system (Hammond-Kosack and Jones, 1996) and raises food safety concerns in so far as PR proteins may be allergenic (Breiteneder and Ebner, 2000).

Suggestions for future research

The preliminary study confirms that *E. coli* and *S. marcescens* can be acquired by plants from the environment and that they can persist on/in the plant. These findings need to be confirmed for agricultural/horticultural production systems. The interaction between model isolates and the hosts also needs to be elucidated further. Of particular concern is the possibility that that the interaction may result in bacterial

suppression, leading to a greater risk of failure to detect contaminants. One aspect of the interaction and of the use of biocontrol strategies is the elicitation of PR proteins and possible increased allergenicity of plant produce.

Finally the persistence and transmission of potentially harmful bacteria as biofilms on raw or minimally processed salad vegetables should be further investigated.

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Chapter Eleven

Criteria for inclusion into HACCP plans for the safety of raw and minimally processed plant produce

Section D: Conclusions

Criteria for inclusion into HACCP plans for the safety of Raw and Minimally Processed Produce

Introduction to HACCP Concepts and Principles

HACCP stands for hazard analyses for critical control points. It was developed in the 1960s to ensure that food for NASA space missions was safe (Anon., 2000). It then became popular in the canned food industry and soon spread to most other food production systems (Kvenberg *et al.*, 2000).

HACCP is based on the following principles

- Analyses of potential food hazards in the system
- Identification of the points where these can occur
- Deciding on which points are critical to food safety
- Implementation of controls and monitoring of the CCP (Critical control points decided on in the previous step)
- Establishment of documentation and record protocols

Kvenberg *et al.*, (*loc. cit.*) reported that HACCP is regarded as the system of choice for food safety, as agencies such as the FDA and USDA describe it as a method that focused resources that can prevent hazards and errors. After initial development auditing and validation as well as regular reviews of the system implemented are extremely important and help to allow the system to be utilised safely for years or even decades after the original plan was implemented (Sperber, 1998)

For produce that is sold raw, the control lies with the producer and the packer. In the salad or sandwich industry the objective is to minimally process produce so consumer demand for raw-like or 'fresh' products would be met.

In each situation the risk must be known in order for it to be controlled. Snyder (2001) lists what is required for this:

1. Evidence of the hazard
2. Concentration at which normally healthy people get sick
3. Probability of a given concentration making people sick
4. Probability that a person will be immunocompromised and become sickened.

In addition to microbiological hazards, which are the main focus of this discussion, there are also two other classes of hazards that HACCP systems must take into consideration

1. Microbiological—due to pathogenic microorganisms and their toxins, includes marine animals as sources of toxic compounds, as with fish and shellfish.
2. Chemical—poisonous substances and foods that cause adverse food reactions.
3. Physical—hard foreign objects in the food and functional hazards. (Snyder, *loc. cit.*)

Critical limits are then set for each Critical Control Point (CCP). The critical limits are defined margins (maximum and/or minimum value/s) within which a parameter (any of the three listed above) must fall, so that the risk of a food safety hazard is eliminated, prevented or reduced to an acceptable level. Plans for *corrective action* must be drawn up in advance should a *deviation* occur. Collecting and reviewing all data (scientific and technical) generated to ensure that the system is operating in accordance with the HACCP plan is carried out continually as a method of *validation* (Anon., 2000b).

If all possible precautions have been taken by a producer/industry then records produced can show what is known as *'due diligence'*, which can be used in defence should a food borne outbreak occur (Synder, 2001). This means that management

must ensure that the HACCP system is applied and complied with during production and that all records are kept correctly.

Risks in the Fresh Produce System.

For almost a century produce contaminated in the field has been recognised as a source of human infection. Early in this century a 1912 Public Health Report called attention to the transmission of typhoid bacillus via fresh produce contaminated with human sewage (Creel, 1912). Many of the bacteria on vegetables, which have caused food poisoning, are derived from human faeces and can also be from animal faeces. The microbial load on fresh produce corresponds to those that are present in the environment during the growing season and at harvest time. In addition, microbes can contaminate postharvest, during storage or transport and temperature abuse during display could allow multiplication (Anon., 2000)

The United States Centre for Disease Control (CDC) reports that 77% of contamination in any food poisoning cases occur through cross contamination and the same is true of foodborne outbreaks associated with fresh cut produce (CDFA, 2001). While foodborne outbreaks associated with produce are low, they have doubled in the last ten years. Since 1987, the number of produce-associated outbreaks has doubled, raising concern among the produce industry, government agencies, and consumers. (Rangarajan *et al.*, 2000 a,b)

The CDC at present recommends that produce that will be consumed raw be washed thoroughly. They further recommend that the YOPI (Young, Old, Pregnant and Immunocompromised) group avoid eating alfalfa sprouts entirely as their safety cannot be assured, though methods to decontaminate alfalfa seeds and sprouts are under investigation (CDC, 2001).

HACCP plans are widely available and can be constructed for individual situations following the available guidelines, for example, the Fresh Produce Consortium in the UK have published guidelines (Anon 1999), as have the US based International Fresh Cut produce Association (Anon, 2000b). These guidelines are usually voluntary but due consideration of them is generally a legal requirement.

Prerequisites to establishing this system are that suppliers to processors utilise *Good Agricultural Practice* (GAP). This system is follows guideline set out by the appropriate authority for example in Canada, the Canadian Food Inspection Agency and in Ireland, An Bord Glas. These guidelines deal with land history and usage, types of fertilisers (organic and inorganic), quality of irrigation water, pesticide usage, hygiene regarding workers and farm animals, harvest and transportation. (Rangarajan *et al* b., 2000)

Sources of contamination

Contamination from animal and human faeces can occur directly or indirectly, at many points in the fresh-produce sequence (see Fig 1). Initially contamination can come from improperly composted manure spread as fertiliser, poor quality irrigation water, and faecal contamination from animals (wild or domestic) and from workers. During the harvest process, contamination may be caused by incorrectly cleaned harvesting machinery. Post harvest, contamination sources include dirty pallets, wash water and cross-contamination from other vegetables (Anon 2000a, Synder, 2001).

At the next stage, processing, storage, temperature regulation are important in controlling contamination, as are sanitation procedures throughout the factory. A primary concern is the wash water used on the vegetables as this can contaminate or

spread contamination to other produce. Infected workers as well as unsanitary cutting and shredding devices can be a core cause of in-plant contamination. Build up of *L. monocytogenes* on equipment can be a problem. (Anon., 2000a)

On the farm there are reasonable steps that a grower can take to reduce the risk that pathogens will contaminate the food produced. Good Agricultural Practices are advised (see earlier) and particular attention should be paid if manure or manure composts are being used.

Criteria for further investigation / Future Critical Limits?

Treatments for produce that is to be eaten raw are not reliable with respect to substantial reduction of the microbial load (Beuchat & Ryu, 1997). Risk elimination is not easy but careful management of these risks, usually based on identification and control of aspects of the chain between planting to plate, are relevant to contamination prevention and also to inhibition of microbial growth (Anon., 2000a).

The EU commission recognises that consumer confidence across Europe is generally low due to several food-related crises that have had an undermining effect. These include usage of illegal animal growth hormones, extensive use of nitrate and pesticides, use of artificial chemicals in food processing and the outbreaks of BSE and *E. coli* O157:H7 (Tent, 1999). Furthermore, the number of produce-associated outbreaks has doubled over the last 25 years, which has made the produce industry, government agencies, and consumers uneasy (Rangarajan *et al.*, 2001 b).

In response to this they aim to set up an infrastructure that will promote greater food safety and greater consumer confidence. This will be done by achievement of 5 objectives:

i. An adequate legislative structure

ii. Effective surveillance and inspection system

iii. Modern risk methodologies

iv. Responsible producers and industrialists

v. Education for the consumer

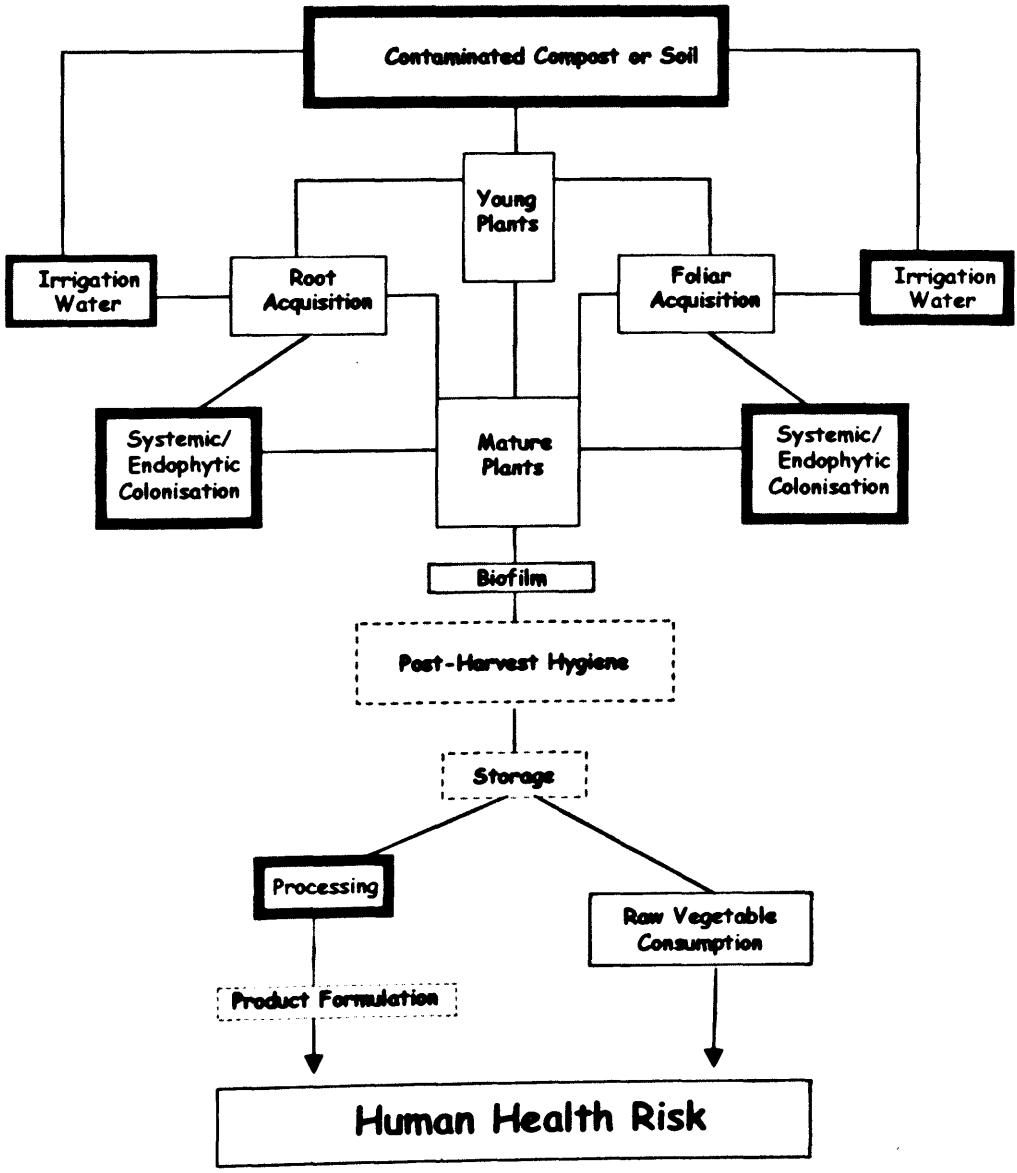
In an effort to fulfil these, the Program for Research and Technical Development would hope to fund among other priorities, improved understanding and control of contamination conditions, as well as new methodologies for assessing microbial chemical and allergenic risks (Tent, 1999). A full guarantee cannot be given that produce eaten is totally contamination free, however, risk reduction is feasible if due care on the farm is taken (Rangarajan et al a, 2000).

Regarding Fig. 1 HACCP on the farm should include risk analysis of the fertilisation methods used. Manure and recycled irrigation water can harbour enteric pathogens. It is best to reflect thoroughly on the system of fertilisation and irrigation for produce which is eaten raw (Brackett, 1994).

One of the potential risks associated with manure is *E. coli* O157: H7. Cattle are the primary reservoir of this pathogen along with sheep and pigs to a lesser degree (Jones, 1999). If using manure from cattle, it should be composted for an adequate amount of time before using for produce that would be eaten raw. Jones (*loc .cit*) reports that times of survival vary depending on the substrate and the temperature for *E. coli* O157:H7. It persists in soil for 60 days at 25°C but for a further 40 days if temperatures are down to 4°C and in aerated manure piles the pathogen can last for 2 months. Regular checking for pathogens, where samples would be sent for analyses to national testing centres, could monitor this point. Methods of controlling pathogen load in the manure include the composting procedures and making sure the length of time is over 3 months. In addition animal

husbandry methods, which reduce stress and hence faecal-shedding from cattle, can also be employed (Jones, 1999, Duffy *et al.*, 2000). Other methods of control of *E. coli* O157 on the farm include recommendations from the review by Teagasc (Duffy *et al*, 2000), alternative strategies include immunisation to reduce colonisation and thus prevalence of *E. coli* O157 in cattle and farm environs. Duffy *et al* (2000) also quote new departures into the use of *E. coli* O157:H7-specific bacteriophages and into the use of probiotic bacteria to out compete the pathogen. A control measure also advised by Jones (1999) is a ban on abattoir waste disposal on land. The author states that 26,000 tonnes of abattoir waste are spread on land in Scotland every year. Abattoirs generally do not have the capacity to store the waste for long periods and so it is usually spread untreated onto the land. As previously discussed *E. coli* 0157:H7 can survive long periods of time and the recommended 2 month cattle – clear period may not be long enough to ensure the decline of the pathogen. If this point is adequately controlled and monitored then the danger of pathogens entering as endophytes is also being controlled.

Fig. 1. Scheme highlighting areas that should be given careful consideration when conducting hazard analyses. Red boxes indicate steps that require careful monitoring and are sources of contamination or are areas that can be used to check for contamination. The lighter red box 'Biofilms' can be checked for, but control can only be carried out at previous steps. Dashed boxes are steps that are covered by conventional HACCP plans.



Irrigation water used for raw produce is recommend to be potable drinking water (Rangarajan *et al.*b, 2001). Any other water types should be regularly checked and again suitable control here regulates the endophyte population.

Further along the chain the problem for processors is the amount of material that would have to be checked and any protocols used to ensure contaminating microbes (be they epiphytes/endophytes) were not present would not be cost effective (Anon., 2000 b). The International Commission on Microbiological Specifications for Foods – ICMSF, (1996) does not recommend sampling and places the responsibility of control with the chain of hygiene and safety checks observed from producer to retailer.

However material at this point could be checked for allergenic substances, which would indicate that a harmful endophytic population was present. It must be said though that studies in the area of plant defence related proteins and allergen homology are only in their infancy and a lot more data would be required to implement any such step.

While biofilms can be checked for microscopically (Morris *et al.*, 1998) they are difficult to culture and are reported as 500 times more resistant than non biofilm bacteria to antibiotics and sanitisers (Nickel *et al.*, 1985). At this stage then control again goes back to ensuring that produce was grown stored and transported correctly which are part of any regular HACCP program if implemented correctly.

Studies undertaken on most minimally processed produce would take place on individual components (e.g. carrot shreds), but the more complex products now on the market, such as mixed salads, salads with cooked-meat/fish etc., have not been studied as an entity. It is now necessary to study these complex products which are new or in development for the market (Wiley, 1994). For example, a characteristic

of fresh vegetables that are consumed raw is that they have a high water content, generally with a neutral pH and are nutrient rich. This makes them capable of supporting the growth and/survival of almost any type of microorganism, any of the other components may harbour pathogens and these are then provided with a 'friendly' substrate for multiplication (Brackett, 1994).

Finally, studies by Beuchat and Ryu (1997) have shown that washes with chorine at the current permitted levels are not reliable enough to eliminate pathogens. They recommend that produce that is to be further processed and/or juiced would be better served if sanitised with a solvent that could remove the waxy cuticle and any microbes therein. However such sanitisers could not be used for fruits or vegetable required for immediate consumption as such solvents can have an unappealing effect on the appearance of the produce. This again underpins the need for prevention of the presence of high numbers of harmful bacteria on produce.

In summary, as food safety in this area runs from 'farm to fork', it determines that a team policy should be adapted in order to effectively employ any regulations or guidelines. Ideally for any the HACCP or hazard analyses system to run successfully key experts from all the pertinent areas such as agronomy/agriculture, plant physiology, microbiology, food sciences, packaging, engineering, distribution, marketing, and retail would need to be involved in a coherent manner. This is a varied and complex area of study. However, the concept of tracebility is not uncommon and applying it to the fresh produce industry would go a long way to ensuring safer food and boosting consumer confidence. There is no reason why such measures should not be taken with organic growing systems as these systems need control and validation also. The control of fresh-cut vegetables begins at the farm

level and as such a 'farm to fork' HACCP approach should be the foundation of control plans.

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Stimulation of wild strawberry (*Fragaria vesca*) arbuscular mycorrhizas by addition of shellfish waste to the growth substrate: interaction between mycorrhization, substrate amendment and susceptibility to red core (*Phytophthora fragariae*)

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Abstract

Wild strawberry (*Fragaria vesca*) microplants were inoculated at establishment in the glasshouse with the commercial inoculants Endorize IV, Vaminoc and *Glomus mosseae*. After 2 weeks, plants were transferred to control peat-based growth substrate and Suppressor®, a commercial peat substrate amended with chitin-containing shellfish waste. Percentage root length colonisation (%RLC) by Vaminoc and *G. mosseae*, but not Endorize IV, was stimulated significantly after 4 weeks growth in the amended substrate but there were no significant differences for any of the inoculants at 8 weeks. Runner production in Vaminoc-inoculated plants was unaffected by either growth substrate. Runner production was significantly reduced in Endorize IV and *G. mosseae* treatments in the control growth substrate, other growth parameters were not significantly affected.

Disease resistance to red core was increased by growth of the Vaminoc-inoculated plants for 4 weeks in Suppressor® before challenge in control compost. Neither Vaminoc inoculation nor growth in Suppressor® resulted in increased disease resistance.

Keywords: Chitin; Commercial mycorrhizal inoculants; Suppressor®; Red stele

1. Introduction

Inoculation of micropropagated plantlets with arbuscular mycorrhizal fungi (AMF) has been shown to increase establishment and to stimulate plant growth (Wang et al., 1993; Puthur et al., 1998). In general, when inoculating plants, consideration should be given to the interaction between host genotype, AMF isolate and growth substrate composition in order to optimise plant performance (Gianinazzi et al., 1990). Perrin et al. (1988) discussed the importance of characterising efficient AMF strains and the substrate receptiveness to mycorrhizal inoculum; this is described as the ability of a substrate to allow mycorrhizal association development on host plants from introduced inoculum. Azcón-Aguilar and Barea (1997) discussed the selection of growth substrates which favour the formation and functioning of mycorrhizae

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and the interaction between AMF and other components of the microbiota of the growth substrate, in relation to the biological control of root diseases. The complexity and variability of responses following the addition of organic amendments to the growth substrate is another factor which must be taken into consideration when examining plant-substrate–AMF interactions (Gryndler and Vosatka, 1996).

Here, the interactions are investigated between wild strawberry (*Fragaria vesca* L.), three commercial AMF inoculants and two peat-based substrates, one of which had been amended with shellfish waste, namely Suppressor®. The use of shellfish waste, an inexpensive source of chitin (Sugimoto et al., 1998), is based on well-established observations of biological control properties against soil fungi (*Fusarium solani* f. *phaseoli*) described by Mitchell and Alexander (1962) and due to the stimulatory effect reported towards AMF colonisation (Gryndler and Vosatka, 1996).

2. Materials and methods

2.1. Plant material and growth conditions

Aseptic seedlings of the outbreeding wild strawberry (*F. vesca* L.) were produced by aseptically germinating seeds (Chiltern Seeds, Ulverston, Cumbria, UK) for 12 days on water agar before transferring them for 4 weeks to half-strength Murashige and Skoog (1962) medium in vitro as described in Mark and Cassells (1996). The aseptic seedlings were acclimatised for 2 weeks (in plastic covered vented weaning trays) in a glasshouse in a peat vermiculite sand (PVS); [8:1:1 (v/v/v)] substrate which had been steam sterilised for 1 h at 121 C over three consecutive days and allowed to rest for a further week before use. The PVS was fertilised (NPK, 16:8:12) with 9 month Osmocote Plus® 1 g/l (Grace Sierra B.V. Herleen, The Netherlands) and limed (CaO, 5 g/l) to a pH of 6.2. For sterilised PVS the lime and osmocote were added after final autoclaving and cooling (Mark and Cassells, 1996). On acclimatisation, plants were inoculated with three commercial mycorrhizal inoculants; Vaminoc, *Glomus mosseae* (both from MicroBio Division, Herts, UK) and Endorize IV (Biorize, Dijon, France). The mycorrhizal inoculum was placed in the planting hole in direct contact with the plant root system, the amount of inoculum used was as recommended by the suppliers, i.e. 1 g of Vaminoc and *G. mosseae* inoculum per plant and 5% by volume (equivalent to 2.5 ml per 50 ml plug tray) for Endorize IV. The PVS substrate used for the acclimatisation stage was not amended with a chitin source as previous experimental work (unpublished) showed incompatibility with the chitin amended compost and microplants of *F. vesca* at acclimatisation.

Following acclimatisation mycorrhizal and control microplants were potted up in PVS substrate as described above (87 mm pots, Omnipot 9F, Congieton Plastic, Cheshire, UK) and in a PVS substrate which had been amended with a source of chitin (Suppressor®, Landtech Soils, Tipperary, Ireland) with a minimum of 16 plants per treatment. The treatments were randomly arranged in separated blocks on potting benches (which had been covered with plastic to prevent cross-contamination of the treatments) in a glasshouse at an ambient temperature of 15–25 C. Plants were grown with a 16 h photoperiod under high-pressure sodium lamps 400 W, 290/240 V (Thermoforce, Essex, UK).

2.2. Plant monitoring

Plants were assessed 4 weeks after potting up for early vegetative growth responses to AMF inoculation by counting the numbers of leaves per plant. Chlorophyll meter readings were taken weekly in order to assess the nutritional and health status of the plants using a Minolta Chlorophyll SPAD-502 meter (Minolta Camerak, Osaka, Japan). The percentage root length colonisation (%RLC) was assessed at 4 weeks and at 8 weeks after potting up following clearing in 10% (w/v) KOH and staining with 0.05% (w/v) aqueous trypan blue (Phillips and Hayman, 1970) and quantifying AMF presence using the magnified hairline intersect method of McGonigle et al. (1990) using a compound microscope at 100× magnification.

Vegetative growth responses were assessed by taking runner counts 4 weeks after potting up; these were mechanically removed and runner re-growth was quantified after a further 4 weeks. The number of crowns per plant and the percentage of shoot dry matter content were recorded at week 26. Flowering onset was monitored weekly in order to assess the

effects of mycorrhizal application and of the substrate amendment.

2.3. Infection with Phytophthora fragariae

A challenge with oospore inoculum of *Phytophthora fragariae* Hickman (from the Culture Collection, Department of Plant Pathology, National University of Ireland, Dublin, Ireland) was carried out on control plants and on plants which had been inoculated with Vaminoc on control and Suppressor® substrates. Plants which had been inoculated with Vaminoc and grown in Suppressor® for 4 weeks were divided into two batches, one of which was grown in Suppressor®; the other batch was repotted in non-amended substrate after 4 weeks. The plants were challenge inoculated with oospores at the end of this 8-week period.

The oospore inoculum was produced by inoculating acclimatised aseptically germinated seedlings of *F. vesca* with *P. fragariae* (from a culture which had been maintained on lima bean agar) in steam sterilised vermiculite and allowing the infection to develop as described in Mark and Cassells (1996). The oospore inoculum used was standardised by comminuting infected root material in an electric blender (Kenwood, Hants, UK), and had an estimated oospore concentration of 2.5×10^3 oospores per ml of inoculum. 5 ml of *P. fragariae* inoculum were used to inoculate each test plant in the disease challenge. After adding the *P. fragariae* inoculum to an inoculation hole made near the stem base of each plant being inoculated the plants were transferred to a controlled environment growth chamber and incubated for 2 weeks at 13–15 °C, 12 h photoperiod with PAR 9 μmol m^2 s^{-1}, after this period the temperature was reduced to 6°C and the vermiculite was allowed to dry out in order to induce oospore production (Mark and Cassells, 1996). Test samples were cleared and stained as for AMF detection (see above) and the response to the pathogen was assessed using disease severity indexes (DSI) as described by Milholland et al. (1989). This index is calculated by multiplying the number of oospores present per 1.0 cm root segment sampled by the percentage of root length infected and dividing by 100, any sample found to have a DSI of less than 1.0 is said to be resistant to *P. fragariae* whereas any value greater than 1.0 is considered susceptible. This method is an alternative to visual assessment which is viewed as being too subjective (Milholland and Daykin, 1993).

2.4. Statistical analysis

The Mann–Whitney (comparison of two treatments) and the multiple comparison Kruskal Wallis tests were used for non-parametric data which were analysed with the aid of Data Desk® 5.0 (Data Description, NY, USA). Median values were used to represent the central tendency in non-normal data.

3. Results

3.1. The effects of shellfish waste amendment on mycorrhizal colonisation

Growth of microplants in Suppressor®-amended-PVS resulted in increased %RLC of *F. vesca* by all three AMF isolates; this increase was significant for Vaminoc and *G. mosseae* (Table 1) 4 weeks after potting up. There were no differences detected in Suppressor® at week 8; this indicates that the acceleration of colonisation induced by substrate amendment occurred within 4 weeks of transfer to this medium. Vaminoc-associated colonisation reached a plateau by week 4 without further increase at week 8. The same result was obtained for *F. ananassa* cv. Tenira (data not shown).

3.2. The interaction between substrate amendment and mycorrhization on plant growth

Table 2 shows that significant plant growth effects occurred in Suppressor®-amended-PVS. The number of runner plants was significantly lower in uninoculated plants, plants inoculated with Endorize IV and with *G. mosseae*. The depressive effect of the substrate amendment on runner production was not observed with Vaminoc-inoculated plants. The runner counts recorded at week 8 show a similar pattern. This indicates that a depression rather than a delay in runner production occurs as a result of the substrate amendment. Other growth parameters monitored, namely, leaf number, chlorophyll content, percentage of shoot dry matter and crown count showed no significant

Table 1
Effect of shellfish amendment of the growth substrate on median percent root length colonisation (ϕ %RLC) at 4 and 8 weeks for *F. vesca*

Treatment[a]	(ϕ %RLC)	95% CI[b]	Treatment[c]	(ϕ %RLC)	95% CI[b]	Effect
Endorize IV Ch$^-$	8.5	[5–17]	Endorize IV Ch$^+$	12.5	[5–17]	NS[d]
Vaminoc Ch$^-$	17.5	[11.7–25.6]	Vaminoc Ch$^+$	37.0	[11–50]	S[e]
G. mosseae Ch$^-$	5.0	[3–15]	*G. mosseae* Ch$^+$	18.7	[3–86]	S[e]
Endorize IV Ch$^-$	25.0	[14–43]	Endorize IV Ch$^+$	30.0	[16–47]	NS[d]
Vaminoc Ch$^-$	24.5	[13–48]	Vaminoc Ch$^+$	37.0	[11–50]	NS[d]
G. mosseae Ch$^-$	24.5	[9–46]	*G. mosseae* Ch$^+$	30.5	[12–50]	NS[d]

[a] Ch$^-$: control PVS growth substrate, without chitin amendment (8 plants per treatment).
[b] Confidence intervals.
[c] Ch$^+$: Suppressor®-amended substrate, with chitin amendment (8 plants per treatment).
[d] Not significant.
[e] Significant ($p<0.05$, Mann–Whitney *U*-test).

differences, except for Endorize IV inoculated plants which produced significantly more runners independently of growth substrate composition.

A slight reduction occurs in the percentage of flowering in the non-mycorrhizal plant population, but not significantly so, the differences are also not significant between any of the AMF treatments (Fig. 1). *G. mosseae* plants grown in Suppressor® had a higher percentage of flowering, this is not significantly higher.

3.3. The effect of substrate amendment and mycorrhization on the severity of redcore

The Vaminoc inoculant was used here as it had shown the highest positive response in the mycorrhizal inoculum — substrate amendment trial above. DSI for all six treatments studied, namely, Vaminoc, plus and minus substrate amendment, at 4 and 8 weeks, are shown in Table 3. The treatments are ranked in increasing disease severity, mean values are included for clarity. The lowest DSI is observed for Vaminoc-inoculated plants which were grown in Suppressor® for 4 weeks before transfer to non-amended substrate (plants were transferred as the stimulatory effect of amended substrate on %RLC reached a plateau at 4 weeks; see Section 3.1). This is the only treatment which results in a DSI of less than 1.0 which is under the resistance threshold, this value differs significantly from the median DSI value for similar plants which were grown on in Suppressor®. Vaminoc and Suppressor® separately are seen to reduce disease severity but not significantly from their respective control treatments. Interaction analysis of variance (ANOVA) confirms that a significant interaction occurs between growth substrate type and AMF inoculation.

Table 2
Effect of shellfish amended growth substrate on the vegetative growth response in *F. vesca*, median (ϕ) runner count data 4 and 8 weeks after potting up (codes as Table 1)[a]

Treatment	Week 4		Week 8	
	Median	95% CI[b]	Median	95% CI[b]
Control Ch$^-$	7.5 b	[3–10]	6.0 b	[3–9]
Control Ch$^+$	2.0 a	[1–4]	1.5 a	[0–4]
Endorize IV Ch$^-$	1.0 a	[0–2]	1.0 a	[0–2]
Endorize IV Ch$^+$	3.0 a	[0–5]	2.0 a	[0–8]
Vaminoc Ch$^-$	7.0 b	[5–10]	7.0 b	[5–9]
Vaminoc Ch$^+$	6.5 b	[4–10]	6.5 b	[4–9]
G. mosseae Ch$^-$	2.0 a	[0–4]	1.5 a	[1–3]
G. mosseae Ch$^+$	0.0 a	[0–2]	0.0 a	[0–3]

[a] Median runner count values followed by the same letter (horizontally) are not significantly different ($p<0.05$, 15 plants per treatment).
[b] Confidence intervals.

4. Discussion

Vestberg (1992) found that of six AMF strains used to inoculate commercial strawberry, three were found to be highly efficient and the three others were less efficient. Here, the vegetative response of *F. vesca* to AMF inoculants containing different isolates was shown to vary confirming previous results with this species (Mark and Cassells, 1996). Suppressor®, the shellfish waste amended growth substrate used here

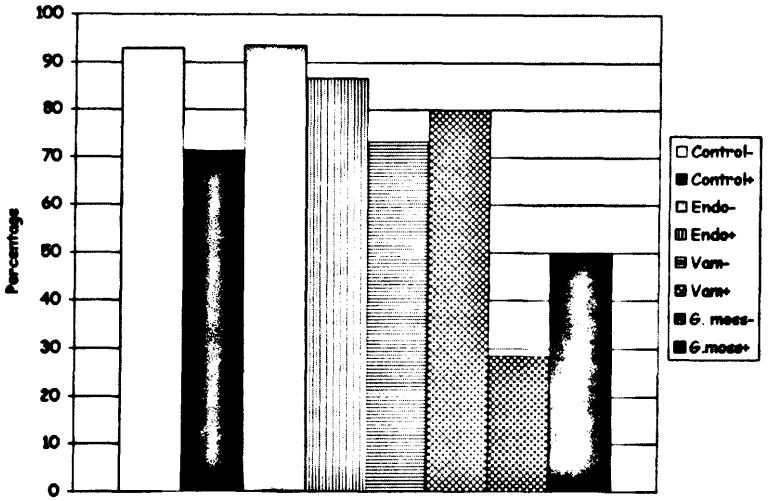

Fig. 1. Percentage of plants in each treatment which had flowered by week 26. Ch^-, control PVS growth substrate; Ch^+, PVS substrate plus Suppressor®; Endo−, in PVS; Endo+, in PVS containing Suppressor®; similarly for Vam−, Vam+, *G. moss*− and *G. moss*+.

was found to increase the percentage of root length colonisation confirming the findings of Gryndler and Vosatka (1996). Stimulation of mycorrhizal colonisation, however, was not associated with significant growth increases or earlier flowering (Fig. 1), as reported by Wang et al. (1993). A depression of runner plant production was seen to be associated with the inoculant–Suppressor® interaction, except for Vaminoc. This may be due to a genotype-dependent interaction of the AMF inoculant with the substrate. The lack of variation in the other growth parameters monitored such as early leaf count and crown numbers (Table 2) and in the percentage of dry matter content, indicate that the quality of the mycorrhized plant material in control and shellfish waste amended growth substrate is not generally adversely affected. The shellfish waste amendment did not alter the nitrogen content of the host plant to a level detectable with the chlorophyll meter. This also agrees with the findings of Gryndler and Vosatka (1996). This parameter is important as nitrogen affects root colonisation by AMF and nitrogen stress, like phosphorus stress, promotes root colonisation by AMF (Sylvia and Neal, 1990).

Caron (1989) recommended environmental manipulation in order to trigger and enhance the activities

Table 3
DSI following challenge with *P. fragariae* inoculum at week 8 of Vaminoc-inoculated plants grown in control substrate, and shellfish amended substrate and in amended substrate for 4 weeks followed by return to control compost for 4 weeks before challenge[a]

Rank	Treatment	(Mean DSI)	Median DSI	95% Confidence limits
1	Vam^+ Ch^+ (4 week)	(0.91)	0.47 a	[0.08–3.85]
2	Vam^+ Ch^-	(3.62)	3.48 ab	[0.21–9.49]
3	$Vain^-$ Ch^+ (4 week)	(3.68)	3.49 ab	[0.48–9.2]
4	Vam^- Ch^+ (8 week)	(4.45)	3.46 ab	[0.21–12.6]
5	Vam^- Ch^-	(4.79)	3.47 b	[0.66–13.42]
6	Vam^+ Ch^+ (8 week)	(12.48)	9.33 b	[1.12–29.55]

[a] Median values followed by a different letter (horizontally) were found to differ significantly following the Kruskal–Wallis test. ANOVA significant interaction between Chitin and Vaminoc ($H=7.43>\chi^2=5.99$; $p<0.05$).

of biocontrol agents. The interaction of the host genotype-AMF–growth substrate composition with the root disease *P. fragariae* (Table 3) indicates that manipulation of the growth substrate composition may result in a significant reduction in disease severity. Azcón-Aguilar and Barea (1997) reported that enhancement of root resistance or tolerance to pathogen attack is not expressed in all substrates. The variation in DSI (Table 3) seen here confirms the latter. An important factor is seen to be the timing of inoculum interaction with the amended growth substrate, which interact significantly.

The shellfish waste amendment is also seen to accelerate as well as stimulate AMF colonisation by Vaminoc, exploitation of the shellfish waste amendment is only possible 2 weeks after acclimatisation (due to toxicity to the young microplant) by which time early AMF infection has taken place (<10% data not presented). The most effective protection against *P. fragariae* occurs when Vaminoc-inoculated plants were grown in Suppressor® for 4 weeks and then transferred to a non-amended substrate. In conclusion, positive interactions between the host plant, mycorrhizal inoculant and shellfish waste amended growth substrate and resistance to *P. fragariae* have been demonstrated. However, the complexity of this interaction is such that commercial exploitation of this tripartite relationship would appear difficult, especially when confronted with the biological diversity of soils.

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PERSISTENCE IN *IN VITRO* CULTURES OF CABBAGE (*BRASSICA OLERACEA* VAR *CAPITATA* L.) OF HUMAN FOOD POISONING PATHOGENS: *ESCHERICHIA COLI* AND *SERRATIA MARCESCENS*

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Abstract

An increase in reports of disease outbreaks associated with fresh and ready-to-eat vegetables prompted this study to evaluate the risk of transmission of human food poisoning organisms in micropropagated vegetables. Here, cabbage is used as a model plant and *Escherichia coli* and *Serratia marcescens* as model human pathogens. Surface sterilised cabbage seeds were germinated on water agar and co- inoculated with *E. coli* or *S. marcescens*. Nodal explants were then used to establish autotrophic tissue cultures. The culture medium and micropropagated plants were examined microbiologically at each subculture. The latter were surface sterilised and the tissues homogenised prior to bacterial screening. Both model strains were recovered from the culture medium and tissue homogenate. Biochemical identification was carried out using the API system, and epidemiological typing was performed using pulsed field gel electrophoresis (PFGE). *E. coli* and *S. marcescens* were found to persist in autotrophic (aseptic microhydroponic) culture, indicating that the carbon sources required for growth were acquired from microplant exudates. *E. coli* and *S. marcescens* were repeatedly re-isolated from the progeny microplants after serial subcultures. Some microplants were asymptomatic in the first subculture; both isolates became pathogenic *in vitro* in the third subcultures.

1. Introduction

Plant tissue culture is prone to contamination with human pathogens due to the manual nature of the work (Leifert *et al.*, 1994). Weller (1997) stated "the frequency of infections with common skin organisms of *Staphylococcus* and *Micrococcus* and the increasing percentage of infection with serial subculture implies contamination from human skin". It has also been reported that *Trichophyton interdigitale* was acquired from micropropagated plants by two horticulturists on separate occasions (Weller and Leifert, 1996). The risk of human food pathogens being introduced into the food chain via vegetable materials has increased recently due to promotion of the 'healthy' diet based on increased consumption of vegetables and the rapid expansion of sales of mixed root and haulm vegetables in prepacks. Consumption is projected to increase in the next few years with increased production of minimally processed convenience foods, development of value-added products e.g. pre- washed prepared vegetable mixes, addition of sauces and meats etc. (Beuchat, 1996; Rafferty *et al.*, 1999). There has been an increase in reports of disease outbreaks associated with fresh and ready-to-eat vegetables (WHO, 1998; Beuchat, 1996). These data raise concern regarding transmission of food pathogens via infected micropropagated produce. A study in 1997 found that *E. coli* 0157:H7 could contaminate the edible tissues of radish after the seeds had been soaked in an *E. coli* 0157:H7 solution (Hara- Kudo *et al.*, 1997). There is a need to assess the potential health risks of the transmission of harmful bacteria via vegetables, which are eaten either raw or after minimal processing. This study has been undertaken to review the risk of transmission in micropropagated vegetables. The aim of this investigation is to monitor

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whether human pathogenic bacteria can persist in aseptic plant tissue culture through serial subcultures and thus pose a risk to the health of the production workers and upstream, to consumers. This is a general study involving a number of clinical bacterial strains and vegetable host. Here, cabbage is used as the model plant and *E. coli* and *S. marcescens* as the model pathogenic strains.

2. Materials and methods

2.1. Bacterial strain selection

The *Escherichia coli* strain used was a clinical isolate (Clinical strain ref. no. 945.1 St James's Hospital Dublin 8, Ireland). The former was chosen as a representative of food- poisoning *E. coli* which was safe to use in the contained environment of *in vitro* culture. *Serratia marcescens* (Clinical strain ref. no. 492.4 St James's Hospital Dublin 8, Ireland) is a common environmental organism (Holt 1985). An outbreak of *Serratia marcescens* infection occurred in a university tertiary- care hospital (Vigeant *et al.*, 1998) and it has been also recorded as an opportunistic pathogen in St James's Hospital Dublin (Falkiner, unpublished) All strains were provided by the Diagnostic Microbiology Laboratory, St. James's Hospital, Dublin 8, Ireland.

2.2. Plant inoculation

Both model strains were grown up to an optical density of 0.4 at 470 nm and diluted appropriately. The following series of dilutions were chosen for *in vitro* work: 10^{-7}, 10^{-8} and 10^{-9}. These dilutions were chosen as they represented, respectively, levels of bacteria that were detectable using conventional culture methods, levels below acceptable conventional plate count numbers and levels that could not be detected (Fig. 1). Aliquots (100 µl) were plated on to water agar (6 g l^{-1}) when the seeds were being plated. Inoculated and non- inoculated (control) plates were used for germination of surface sterilised seeds for 8- 10 days. These seedlings were used as a source of nodal explants for tissue culture. Bacterial screening of control plants was carried out as described previously (Barrett and Cassells, 1994). Microplants were screened throughout the study by culturing tissue homogenates on MacConkey plates (Oxoid Ltd., Basingstoke, Hampshire, UK) overnight at 37 °C.

2.3. Autotrophic tissue culture

Cabbage seed was surface sterilized in 80 % (v:v) aq. ethanol and immersed in 20 % (v:v) aq. commercial hypochlorite solution (Domestos; Lever Bros, Liverpool, UK) for 15- 20 min and washed in sterile distilled water (x 3) in a laminar-flow cabinet prior to placing the seeds on plates of sterile water agar (6 g l^{-1} agar) which had been inoculated as above with diluted bacterial suspension (inoculated) or non-inoculated (controls). There were 20 seeds per plate. After 8- 10 days, seedlings were transferred to Magenta GA-7 vessels (Sigma-Aldrich Ireland Ltd, Dublin) each containing polyurethane foam (Plant Biotechnology (UCC) Cork) imbibed with half strength Murashige and Skoog (1962) mineral salts solution (Sigma) (Cassells and Walsh, 1996). These were placed in a growth room under the following conditions: 23 ± 1°C, 16 hour photoperiod (white 65/80 w Liteguard tubes, Osram Ltd., UK.) with PAR of 30 µmol m^{-2} s^{-1} at shelf height. These plants were screened for bacteria as below. Nodes were excised from the microplants at 4 - 6 week intervals for subculture on to the same medium. Three subcultures were carried out.

2.4. Bacterial screening of cultures and plant tissues

Samples of spent media were taken at the end of each culture cycle and a dilution series was constructed to determine the amount of bacteria present in the media during the 4- 6 week growth period.

Sampling of plant material involved surface sterilisation by immersing the microplants in 80 % ethanol (ethanol absolute, Merck, Darmstadt, Germany) for 45 sec, then in 2 % Stericol (Stericol Hospital Disinfectant, Lever Industrial Ltd., Runcorn, Cheshire, UK) for 30 min, followed by washing in sterile distilled water (x 3). Following sterilisation the microplants were placed in 9 ml Ringers solution (Oxoid Ltd., Basingstoke, Hampshire, UK) and 1 ml buffered peptone water solution (Oxoid Ltd., Basingstoke, Hampshire, UK), and homogenised using an Ultra Turrex T25 (Janke & Kunkel Gmblt & Co KG, Staufen, Germany.). For the isolation of the Gram- negative bacteria, *E. coli* and *S. marcescens*, the homogenate was plated on to MaConkey agar (Oxoid Ltd., Basingstoke, Hampshire, UK) and incubated at 37° C for 24 h.

2.5. Biochemical identification of bacterial isolates

Following incubation, all plates were examined morphologically for the presence of bacteria. Where present, the oxidase and Gram stains were also performed. All suspect colonies were cultured for purity on the appropriate agar medium and identified using the API 20E identification kit (bioMerieux SA, Montaleu, Vercieu, France). Confirmed isolates were cultured on Columbia agar (Lab M, Bury, UK) supplemented with 7 % horse blood, and frozen at - 70° C on Protect beads (Technical Service Consultants Ltd., Lancashire, UK), until required.

2.6. Epidemiological typing:

Bacteria were grown on Columbia agar, supplemented with 7 % horse blood, incubated in air at 37 °C for 48 h. Cultures were harvested and suspended in 3 ml SE buffer (5 M NaCl, 0.5 M EDTA). Cells were washed twice in fresh SE buffer and re-suspended to achieve a density equivalent to a MacFarland Standard No.4 (Bio Merieux SA, Marcy- l'Etoile, France). A 2 % (w/v) low-gelling agarose (Sigma Chemical Co., St. Louis, MO, USA) was prepared in SE buffer, and dispensed into pre-warmed 1.5 ml Eppendorf tubes (Sarstedt, Aktiengesellschaft & Co., Numbrecht, Germany). 220 µl aliquots of the bacterial suspension were added to the tubes, mixed gently and transferred to the block mould (Bio-Rad Laboratories, Alfred Nobel Drive, Hercules, USA). Following refrigeration for at least 30 min, the moulds were carefully transferred into labelled universals (Bibby Sterilin Ltd.,Tilling Drive, Stone, Staffs, OSA, USA), containing 1ml lysis buffer (1 M tris pH 8.0, 0.5 M EDTA pH 8.0, lysozyme). The universals were incubated in a 37 °C water bath (Grant Instruments (Cambridge) Ltd., Barrington, Cambridge, UK), for 2 - 3 h and then transferred to newly labeled universals containing a 1 % SDS and Proteinase K solution (SDS, TE Buffer, Proteinase K). These universals containing the blocks were then incubated at 50 °C overnight. Blocks were washed in pre- warmed TE buffer (1 M Tris pH 7.6, 0.5 M EDTA pH 8.0), and the universals placed in a 50 °C shaking water bath (Grant Instruments (Cambridge) Ltd., Barrington, Cambridge, UK). After 4 successive washes, the blocks were placed in fresh TE buffer and stored at 4 °C overnight. A 2.5 x 5 mm portion from each block was cut the next day, and placed in separate 1.5 ml Eppendorf tubes containing 1 ml of fresh TE buffer. The tubes were refrigerated for a minimum of 30 min. The slivers were then transferred to tubes containing 150 µl of reaction buffer (Promega Corporation, Woods Hollow Road, Madison, WI, USA) and refrigerated for at least 30 min. The enzyme *Xba* I mix (Promega Corporation, Woods Hollow Road, Madison, WI, USA) was prepared on ice and 50 µl added to each tube. The tubes were incubated at 37 °C for 3h by transferring the blocks to TE buffer at 4 °C for 30 min.

2.7. Pulse field gel electrophoresis (PFGE):

As a general rule a gel concentration of 1.2 % will give clear bands over a range of 1- 2500 kb. The slivers to be loaded were picked up using a sterile scalpel and placed against the leading edge of the well. The order of each block was recorded and a molecular weight marker (Boehringer Mannheim Biochemica, GmbH, Germany) was also included. Once loaded, the wells were sealed with a sealing agarose and allowed to set for 30 min at 4° C. Cooled TBE (3 l) (Tris base, Boric Acid, 0.5 M EDTA pH 8.0) was poured into the tank and allowed to equilibrate for 30 min. The run parameters were: pulsewave: initial time: 5 sec; final time: 50 sec; run time: 22 h; power supply: 200 Volts.

When the run was complete the gel was stained with ethidium bromide (Sigma Chemical Co., St. Louis, MO, USA) at room temperature for 30 min. Following staining, the gels were de- stained for 15 min. The gel was photographed under UV light using a Polaroid MP+ Instant Camera System.

3. Results

3.1. Growth of *E. coli* and *S. marcescens* in autotrophic microplant culture

Samples of spent media were taken and a dilution series was constructed to determine the amount of bacteria present in the media after 4 weeks; the results are shown in Table 1. This shows that both model strains multiplied in the autotrophic systems. Murashige and Skoog (1962) mineral salt solution, used as the medium in autotrophic culture contains no carbon sources and does not support the growth of *E. coli* or *S. marcescens*. However, both grew on basal salt medium, the only carbon supplementation coming from microplant exudate.

3.2. Symptom expression in the inoculated microplants

E. coli. strain 945 and *S. marcescens* strain 492.2 were recovered from the spent medium and surface sterilised plant tissues at each subculture (Tables 2 and 3). No bacterial contamination was detected in the non-inoculated control microplants and no bacterial isolates, other than the model strains, were isolated from the inoculated control microplants. At the end of the first subculture the control microplants were on average 70 mm in height. The inoculated microplants were stunted to approx. half that height and had fewer nodes. In all microplants from inoculated cultures, symptoms were evident on the plant as blackening of the lower stem (Fig. 2). An exception to this was the treatment with *E.coli* at 10^{-9} that did not show evidence of basal stem rot and was less stunted to approx. 70 % of the height of the control microplants. Similar results were recorded for the second subculture. Symptoms were observed after 16 days in culture and were expressed as black/brown lesions at stem bases. After the third subculture both model strains became pathogenic to the plants *in vitro*.

3.3. PFGE results

Both *E. coli* strain 945 and *S. marcescens* strain 492.4 were found to persist in the culture medium in the presence of plant tissues and in homogenates of surface sterilised microplant tissues. PFGE banding patterns of the bacterial strains isolated, showed identical banding patterns to the original strain used as inoculum (Fig. 3). Of 39 *E. coli* isolates typed, the resulting restriction patterns were indistinguishable from the original strain typed (data not presented).

4. Discussion

Bacterial strains of medical significance were chosen for this study. *E. coli* is a Gram-negative, lactose fermenting bacillus which is a member of the gut flora of mammals especially cattle and man. This organism has long been recognised as a cause of a wide range of human infections many of the diarrhoeal type. The most noteworthy pathogenic sub- group enterohaemorrhagic *E. coli* (EHEC), of which the serotype 0157 is well known, is the causative agent of bloody diarrhoea. Outbreaks of *E. coli* 0157 have been reported worldwide (mostly from the developed nations) with several fatalities resulting (Bolton *et al.*, 1998). *S. marcescens* is a Gram-negative, lactose fermenting organism implicated in causing a variety of nosocomial infections (Miranda *et al.*, 1996; Herra *et al.*, 1998). Its ability to survive in many different environments accounts for its potential to act as an opportunistic pathogen in clinical settings. *S. marcescens* has been isolated from medical equipment such as intravenous catheters and needles (Ashkenazi *et al.*, 1986), and blood transfusion bags (Parment *et al.*, 1993). Typically these bacterial strains of clinical significance are not associated with plants and are not known plant pathogens though both may be widely encountered in the environment. The subsequent re- isolation of *E. coli*, and *S. marcescens* from the model plant types, demonstrates that these human and food poisoning pathogens have the ability to survive on and possibly within healthy micropropagated plants.

It was shown that human pathogenic species, particularly *E. coli*, could survive *on* and *in* plants at very low concentrations. Strains were found to persist in autotrophic culture. This indicates that plant leakage supports growth of enteric bacteria. It was observed that *Serratia* grew to a higher cell count in the cultures than *E. coli*. After serial subcultures inoculated bacteria were re-isolated from the progeny microplants though some microplants were asymptomatic; in other cases the bacteria became *vitro*- pathogens in the later subcultures. It is evident then that these bacteria, even at dilutions as low as used here, can still colonise microplants and persist in serial subculture even in harsh bacterial environments, namely, Murashige & Skoog (1962) mineral salts medium. Given these results, the potential risk factors associated with micropropagation and with microplants for human consumption should be more fully investigated.

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Tables

1. Bacterial cell counts in spent media from autotrophic cultures

Treatment	Counts from spent media
Controls	0
E.coli 10^{-9}	8.67×10^{4} cfu/ml
E.coli 10^{-8}	4.6×10^{5} cfu/ml
E.coli 10^{-7}	4.77×10^{5} cfu/ml
S.marc 10^{-9}	5.81×10^{7} cfu/ml
S.marc 10^{-8}	1.54×10^{7} cfu/ml
S.marc 10^{-7}	1.08×10^{7} cfu/ml

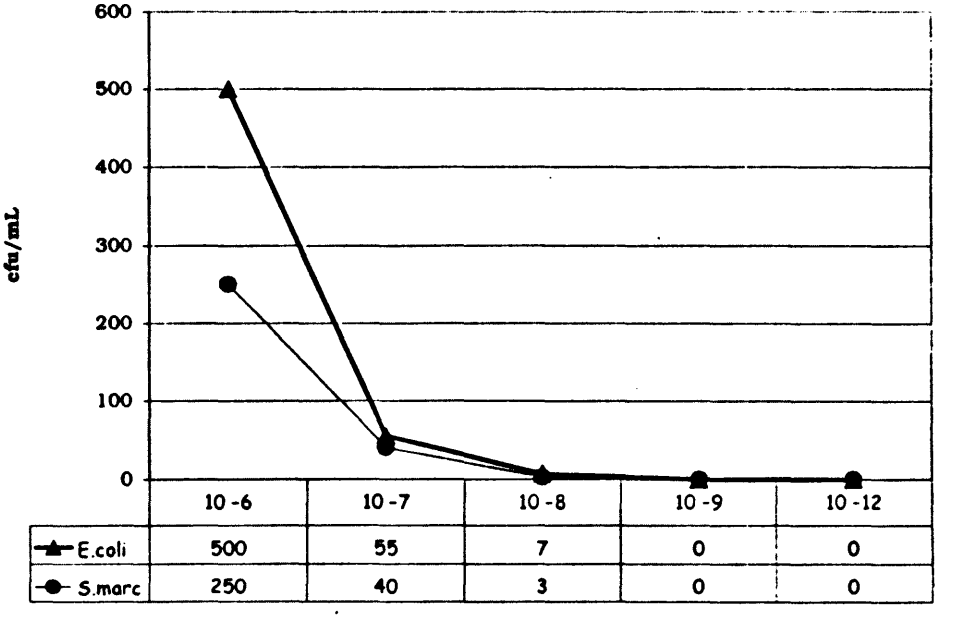
2. Persistence of *E. coli in vitro.*

Location	Controls	E. coli 10^{-7}	E. coli 10^{-8}	E. coli 10^{-9}
4 weeks epiphytic	-	+	+	+
4 weeks endophytic	-	+	+	+
8 weeks epiphytic	-	+	+	+
8 weeks endophytic	-	+	+	+

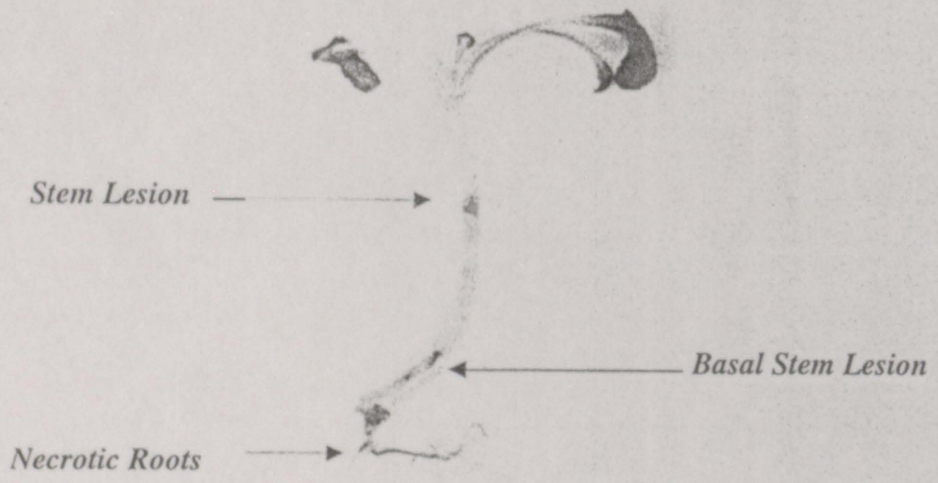
3. Persistence of *S. marcescens in vitro*

	Controls	S. marcescens 10^{-7}	S. marcescens 10^{-8}	S. marcescens 10^{-9}
4 weeks epiphytic	-	+	+	+
4 weeks endophytic	-	+	+	+
8 weeks epiphytic	-	+	+	+
8 weeks endophytic	-	+	+	+

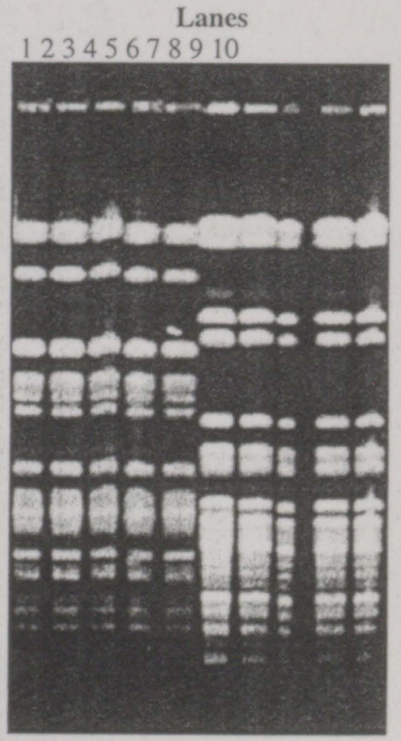
Figures



1. Inoculation levels used for *in vitro* studies.



2. Cabbage microplant inoculated with *E. coli* strain 945 showing stem and basal stem lesions



3. PFGE gel showing *Xba* digestion patterns *E. coli* (lanes 1- 5) and *S. marcescens* (lanes 6- 10)

Human Food Poisoning Pathogens Associated with Plant Produce

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Introduction

In recent years there has been an increase in food poisoning associated with fresh produce (1). Contributing factors include an increased rate in consumption of produce per capita, intensification of agricultural production, modern processing techniques, and globalisation of the market (2).

Sources of Contamination

Primary sources of bacterial contamination in food production are contaminated soil, water, feed and manure resulting in contaminated raw ingredients/raw materials (e.g. packaging) (3). Listeria monocytogenes, Clostridium botulinum, and Bacillus cereus can be naturally present in soils. Campylobacter jejuni, Escherichia coli O157:H7, Salmonella and Vibrio cholerae are more likely to contaminate produce through vehicles such as improperly composted manure or irrigation/wash water containing untreated sewage. Secondary contamination in the processing industry may occur from unhygienic employees/surfaces, dirty process water, faulty air handling systems, and others (3). Wild or domestic animals are another source of contamination. Taken together, primary and secondary contamination provide a potential basis for contamination from farm to fork (4). Investigators have long been concerned with the threat posed from faeces-fertilised produce. A 1912 Public Health Report called attention to the transmission of typhoid bacillus via fresh produce contaminated with human sewage. (cited in ref. 5). Recently several foodborne disease outbreaks have been linked to vegetables (see ref. 6). Such reports have enhanced speculation that pathogens present in agricultural manure would pose a threat if applied to crops (5).

Plant Transmission

Bacteria survive in association with plants in a variety of ways. They are commonly found as epiphytes, but they also have more specialised methods of association.

1. Endophytic Survival

A method of avoiding the exterior stresses on a plant is to live within the tissue, which affords protection. Common endophytic isolates from plants include Beijerinckia, Azotobacter, Erwinia, Klebsiella, Enterobacter, Bacillus (7) and Clavibacter (8). Endophytes have been shown to survive in the following plant tissues: vascular tissue, (9) roots (10, 11), stems and cotyledons/leaves (12, 13). Endophytic presence in aseptic tissue culture has also been noted (14), and this may have implications for vegetable crops raised from microplants and transplants. Systemic colonisation can afford protection for the bacterial endophyte from competition and environmental stresses such as washing and surface sterilisation procedures (15).

2. Biofilms

Various investigators have reported biofilms in the marine environs, implanted medical equipment, and water distribution systems (16). Costerton (17) defines

biofilms as "Matrix enclosed bacterial populations adherent to each other and/or to surfaces or interfaces. The definition includes aggregates and flocculates and also adherent population within the pore spaces of porous media." It was noted that biofilm cells are at least 500 times more resistant to antibacterial agents than their planktonic counterparts. The control of biofilm bacteria has been the focus of vast amounts of applied and medical research. Why biofilm bacteria are less susceptible to usual lethal treatments is still unclear (17). Morris et al. (18) observed biofilms directly on the leaf. The plant species chosen were all vegetables that are eaten raw (spinach, lettuce, Chinese cabbage, celery, leeks, basil, parsley and broad-leafed endive). Recovered biofilms using leaf washings and agar impressions revealed that they contained multiple species (19). Costerton (17) quotes studies on depth of biofilms, one homogeneous biofilm studied was made up of Vibrio parahaemolyticus, a well-known food poisoning agent. This would indicate that food poisoning agents could survive in this form.

Water Transmission

Use of contaminated irrigation water or inadequately treated water has been quoted as a vehicle of transmission for various food poisoning agents (20, 21). A major American producer of fresh-cut carrots now includes testing of irrigation and processing water for total coliforms and *E. coli* (3). Many plant pathogens are spread in irrigation water, for example potato brown rot disease. The causal agent is Pseudomonas solanacearum/Ralstonia solanacearum biovar 2A. The bacterium has been found in most infected countries in surface water (22), ditch water (23), and the weed Solanum dulcamara growing along waterways (24). The pathogen can overwinter successfully in the roots (25), from which it can spread to potato crops when associated water is used for irrigation (26). It may be possible for human pathogens to follow this transmission route.

Emerging Pathogens

Various factors contribute to emerging pathogens including the globalisation of the food supply (3) as well as changing microbial populations (27). Increasingly since the late 1980s, Campylobacter infection has risen to and surpassed that of Salmonella and campylobacteriosis is more common across the world (28). The Super family VI includes the genera Campylobacter and Helicobacter. These microorganisms are gram negative, motile by means of flagella, spiral shaped, and microaerophilic (29).

1. Campylobacter

During the past decade *Campylobacter* has emerged as a major cause of human enteritis (4, 30–33). Patients excreting the organism and healthy carriers such as poultry and pigs provide a constant flow of the bacterium into the environment. The application of natural or untreated water for irrigation of farmlands is a route of direct contamination. Waterborne outbreaks of Campylobacteriosis have been reported in Sweden, the U.S., Canada, England, Yugoslavia and Norway as cited in ref. (21). Koenraad draws attention to the possible presence of Campylobacter species in water in a viable but noncultivable (VBNC) form (30). Campylobacter have been isolated from fresh market produce; 3.8% of the samples were positive for Campylobacter (21). Harris et al. cite Doyle et al. (1986) as having isolated Campylobacter jejuni from a small percentage of commercial mushrooms (1.5%). Despite many

investigations, the sources of the majority of sporadic cases of human campylobacteriosis remains unconfirmed. However, the major sources for Campylobacter in produce include untreated waters and soil and manure. Poultry may have an important role in human infection, but other sources cannot be ignored (31).

2. Helicobacter

H. pylori is the most common chronic infection in humans and is the major etiological agent for chronic active gastritis (29, 35). It is often present in ulcer disease and atrophic gastritis (36); it is being actively explored as a risk factor for gastric carcinoma. H. pylori is fastidious and requires 3 or more days for isolation; microaerophilic conditions must be constantly maintained (29). Little is known about environmental sources of H. pylori, though the faecal oral route has long been suspected (35). That produce may be a vehicle in H. pylori transmission is based on serosurveys. A study in Chile showed a significantly higher prevalence in lower socio-economic groups. Since a key factor in enteric pathogen transmission in Chile is the use of sewage-contaminated irrigation water on produce, it was suggested that this might also be a route of transmission for H. pylori (Hopkins, 1993, cited in ref. 35). Helicobacter has been associated with waterborne transmission (37) probably in a VBNC (38). It is possible Helicobacter may not have been directly isolated from produce because of the difficulty in culturability and/or detection.

Conclusions

Considering that bacteria are known to survive on salad vegetables as biofilms and as endophytes, this presents us with a risk. Whether human pathogens can survive on fresh produce requires further examination. Prevention of the transmission of human pathogens in the food industry involves taking action at all stages in the chain from farm to fork. Properly composted manure and irrigation water from a clean source should be used on growing crops. All processing should include sanitary-designed processing facilities, highly evolved hazard analysis carried out for critical control points plans, sanitation regimens, good management practice, employee training and monitoring in basic hygiene, and perhaps inclusion of irradiation as a final precautionary step (3). The latter should not be used on its own or to process poorer-quality raw materials. Research is necessary to understand more fully the survival mechanisms of pathogenic bacteria on fresh and minimally processed produce (3).

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