# The distribution of *Phytophthora* in *Citrus* orchards on the West Bank - A plant microbiological study

by

ABDUL-HADI YUSEF AHMAD HAMDAN

Department of Genetics and Microbiology

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## ABSTRACT

The aim of this study was to examine the distribution and extent of variation in *Phytophthora* species in orchards of the West Bank of the River Jordan.

The survey was conducted on two main sites contrasting in their geography, topography and *Citrus* host, which were sampled fortnightly over a period of nine months. In addition twelve other sites distributed throughout the West Bank were sampled once only.

Only two species were isolated from all sites namely *Ph.citrophthora* (Smith & Smith) Leonian and *Ph.nicotianae* van Breda de Haan.

The extent of variation between the two species was assessed using morphological and electrophoretic parameters. Diversity within the more numerous species , *Ph.citrophthora* was examined using these methods and also pathogenicity parameters.

The two species were readily distinguishable on morphological and electrophoretic grounds (including zymograms and protein profiles). Genotype - environment analysis of the two species revealed some diversity, namely that around 20 % of isolates differed from the remainder. There was no significant variation among the *Ph.citrophthora* isolates regarding virulence towards leaf discs obtained from detached middle age *Citrus* leaves of 5 *Citrus* hosts. However, the hosts could be ranked as they differed in their resistance to *Ph.citrophthora* .

No marked variation was detectable within 63 *Ph.citrophthora* isolates by studying total protein extracts separated electrophoretically and stained for nine enzymes. However, denatured protein extracts showed marked variation. The isolates could not be grouped (using principal component analysis) whether in respect of site or isolation method.

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To

my dear wife

**Khitam**

For her endless devotion, encouragement and support

## Abbreviations

| | |
|---|---|
| AK | Alkaline phosphatase |
| AP | Acid phosphatase |
| Bis | N,N', Methylene-bis-'acrylamide |
| CA | Carrot agar |
| CMA | Corn meal agar |
| CV8A | Cleared V8 agar |
| CV8CMOA | Cleared V8 corn meal orange agar |
| DI | Direct inspection |
| DR | Double Ribiero fluid medium |
| DW | Distilled water |
| HEPES | N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid monosodium salt |
| G6PD | Glucose 6 phosphate dehydrogenase |
| LAP | Leucine amino peptidase |
| LDH | Lactate dehydrogenase |
| MDH | Malate dehydrogenase |
| ME | Malic enzyme |
| MTT | [3-(4,5,-Dimethyl thiazole-2-yl)2,5-diphenyl tetrazolium bromide] |
| $NAD^+$ | α-nicotine amide adenine dinucleotide |
| $NADH^+$ | α-nicotine amide adenine dinucleotide (Reduced form) |
| NADP | α-nicotine amide adenine dinucleotide phosphate |
| NBT | Nitro blue tetrazolium |
| PA | garden Peas agar |
| PDA | Potato dextrose agar |
| PGI | Glucophospho isomerase |
| PMS | N-Methyl phenazonium methosulphate |
| RER | Radial extension rate |
| RSM | Ribiero's synthetic medium |
| SDW | Sterile distilled water |
| SDS | Sodium dedocyl sulphate (Sodium lauryl sulphate) |
| SSE | Sterile soil extract |
| TEMED | N,N,N',N'-Tetramethyl ethylene diamine |
| TO | Tetrazolium oxidase |
| V8 | Cleared V8 liquid medium |
| WA | Water agar |
| XDH | Xanthine dehydrogenase |

## TABLE OF CONTENTS

# CHAPTER ONE

## Diversity in pathogen populations

### General Introduction

Variation is the rule rather than the exception in most fungi, and "there is no such thing as typical or atypical, but merely variability of living things" (Leonian, 1934). Two basic facts are well documented in the literature regarding fungal populations, namely the extreme diversity of fungal species in most communities and the second is the presence of habitat specificity for individuals or related species (Christensen, 1981).

The genus *Phytophthora* de Bary is of considerable world importance because it contains many major plant pathogens, affecting many economically important crops ( e.g. potato, cocoa, *Citrus* ). Whilst the disease syndromes may vary, and become complicated by further attack by other microbes, the overall result of *Phytophthora* disease is severe yield losses ( Gregory, 1983).

This genus includes around 70 species distributed world wide, with well documented variability within many of these species (Erwin, 1983). Comprehending the nature and causes of this variability within species is of extreme importance in that it may lead to greater understanding of pathogenicity, mechanisms of host resistance and to improved methods of control.

Different populations or species may differ in their frequency of variants and such frequency can be used to indicate the genetic identity or distance between populations (Barrett, 1986). However, very often a latent capacity for variation in terms of phenotypic plasticity can be seen in all organisms, and can often be detected if deliberate changes in environmental factors are made during growth. Thus, phenotype is the

product of interactions between genotype and environment (Talbot, 1971), and it is often difficult to ascribe definitive explanations of variation in fungi uniquely to one or the other source.

Variation in the genotype might be a result of mutation, heterokaryosis, sexual or asexual mechanisms of genetic recombination, plasmogamy or nuclear reassortment (Erwin, 1983). Moreover these mechanisms may be influenced by environmental factors ( Flentje, 1970; Parmeter, 1970).

Variation in fungal populations may be studied by a range of traditional means of assessment of sexual and asexual fructifications, physiological parameters, genetic studies, virulence or pathogenicity, or more modern techniques including protein profiles, zymograms, nucleic acid mapping or serology.

The morphological features of sexual and asexual fructifications form the major characters used in fungal taxonomy and can also be used to assess variation within species. (Clare *et al* ., 1968; Beaks & Ford, 1983; Seviour *et al*., 1985). However, problems with obtaining and manipulating the sexual state in *Phytophthora* are commonly reported in the laboratory. Moreover, some species (e.g. *Ph.citrophthora* ) do not have a well defined sexual state. Asexual characteristics of isolates often show considerable variability, with extreme variation in sporangia dimensions within *Phytophthora* species (e.g. Leonian, 1934). However the sporangial characteristics were used as basis for taxonomic keys (Waterhouse, 1963, 1970), but later the sporangial dimensions were suggested to be of value to differentiate some species (e.g. *Ph.citrophthora* ) from others (e.g. *Ph.drechsleri* ) avoiding the extreme sizes (Waterhouse *et al.*, 1983).

Temperature and nutrition are extremely important factors that affect the size of morphological structures of fungi and mycelial growth (e.g. Cowling & Kelman, 1964; Raabe, 1967; Bagga & Salley, 1974 ; Barr

1986). Thus, species or isolates within species may show variations resulting from genotype-environment interactions (Fripp,1972). Temperature is a critical determinant of performance both between and within species. *Phytophthora* species have been divided into three groups according to optimum temperature for growth with temperature varying from (30-32 °C) for *Ph.parasitica* to (15-20 °C) for *Ph.phaseoli* and *Ph.primulae* and (24-28 °C) for *Ph.citrophthora* (Waterhouse *et al.*, 1983). Nutrition, which includes carbon source(Christie, 1958), nitrogen source (Cameron & Milbrath, 1965), and vitamins (Cameron, 1966) may play a role in showing the variability within and between *Phytophthora* species. They may affect the colonial morphology, radial extension rate and fructification production.

Variation in virulence among isolates of plant pathogenic fungi has been known to exist for a long time (Erwin, 1983). The interaction between the virulence genes of fungi and resistant genes of hosts expresses itself as variation in pathogenicity. Resistance is the dominant factor in the host and virulence is a recessive in the pathogen and in some instances are inherited by single major genes (Christ *et al* ., 1987) . However, resistance and avirulence alleles may also fuction as conditional genes (Vanderplank, 1982). Variation in pathogenicity of *Phytophthora* has been of interest since the 19th century when *Ph.infestans* was first investigated (Gallegly, 1983). The extent of this variation within species can be assessed using a variety of methods including inoculation of the trunk of large trees ( Rosettie, 1947), seedlings ( Perlberger, 1936; Middleton, 1985), fruits or leaves ( Ann, 1984).

Analysis of electrophoretic profiles of soluble proteins and enzymatic patterns have been found to show variation between and within species. These profiles are the expression of the genetic constitution of the fungus (Burdon & Marchall, 1981; Itavaara, 1988; Carder, 1989). Proteins coded by

different alleles on separate genetic loci will possess different electrophoretic mobilities, due to variations in amino acid content. Thus, the protein extracts from the fungal mycelia stained for specific enzymes or total protein may be postulated to reveal clear variations between and within species (e.g. Rice & Crowden, 1987; Seviour *et al*., 1985). These more recent techniques have been used in the assessment of variation within and between species in addition to the traditional use of morphological, physiological methods and pathological parameters (e.g. Vallavieille & Erselius, 1984; Hansen *et al* ., 1986) to form a stronger basis for the analysis of variation and diversity in fungi.

This thesis reports on a detailed study of the diversity, distribution and abundance of *Phytophthora* species on the West Bank of the River Jordan. *Citrus* orchards were systematically sampled over 9 months in a range of sites and were then examined in the laboratory (Chapter 3). Inter-population variation, initially at a species level, and then at the subspecific level was assessed in three ways:

i) Growth (as extension rates) and isolate-environment interactions (Chapter 4)

ii) Host pathogen interactions (Chapter 5)

iii) Protein electrophoresis (Chapter 6).

## CHAPTER TWO

### Species identification

## 2.1 Introduction

The process of identification is the determination of the identity of an unknown specimen, relating observed characteristics of the unknown to known criteria of identified specimens and thus concluding that one specimen is the same or different from others (Walker, 1975).

The basis of identification for the species concept in *Phytophthora* has been morphological characters since the earliest times (de Bary,1876) when sporangia and sporangiophores were the standard characters of identification. Later, the use of other morphological and physiological characters was added to these criteria, e.g.: sexual characters (Tucker, 1931), colony morphology and temperature requirements for growth (Reichert & Littauer, 1931; Tucker, 1931; Leonian, 1934; Schiffmann, 1951).

A short cut procedure for identification to species level was suggested by Waterhouse and Blackwell (1954) by concentrating on specific traits such as abundance of chlamydospore production for *Ph.parasitica* or specificity towards a particular host group as a means for delimiting procedures. After classification, taxonomic keys were prepared (Waterhouse, 1963; 1970; Newhook *et al* ., 1978; Ho, 1981; 1982; Waterhouse *et al* ., 1983) by which species can be identified separately and identified in groups sharing certain taxonomic features.

Serological methods involving antibodies and molecular methods using DNA probes may give rise to identification but they are still in their infancy, restricted to experimental systems at present (e.g. Goodwin *et al* ., 1989; Panabieres *et al* ., 1989), and might be costly to run.

Twelve species of *Phytophthora* have been related to disease

symptoms in *Citrus* around the globe, but the isolates from the area east of the Meditterranean comprise only *Ph.citrophthora* (Reichert, 1932; Hartmann, 1969; Cinar *et al* ., 1976; Hassan *et al* ., 1989) or *Ph.citrophthora* and *Ph. parasitica* (Reichert & Littauer, 1931; Perlberger, 1936; Mansoori & Fassihiani, 1984; Sneh & Katz, 1988). However, Schiffmann(1951) recovered a third species (*Ph. hibernalis* Carne) in addition to these two which can be easily differentiated by its semipapillate obovoid sporangia with tapered base and inability to grow above 23-26°C (Newhook *et al* ., 1978). The maximum temperature is near 25°C (Waterhouse and Waterson, 1964,a).

In this chapter, the basis of identification for *Ph.citrophthora* and *Ph.nicotianae* will be recalled as these are the two species isolated from this area and compared with 12 putative isolates of each species isolated during this study. The cultural characteristics including colonial morphology and effect of temperature, sporangial morphology and gametangia production will be discussed as reported in the literature and compared with those of randomly chosen isolates from the present study. The inability to recover any isolates of *Ph.hibernalis* will be argued and the use of the name *Ph.nicotianae* rather than *Ph.nicotianae* var. *parasitica* or *Ph.parasitica* will be justified.

## 2.1.1 Cultural characteristics

### Colony morphology

Colony morphology is an expression of hyphal branching pattern resulting from hyphal extension rate, branching morphology, branch angle and branch diameter (Waterhouse, 1970; Prosser, 1983) which differs between species and to a slight extent within species (Kellam and Zentmyer, 1986).

It is customary to describe the colony morphology of an isolate or a group of isolates as an aid placing the isolate in a specific category (Valvielle & Erselius, 1984). It was suggested by Ho (1978) that the specific characters of the peripheral branching of a colony might be useful in identification.

*Ph.citrophthora* has been shown to produce finely radiate colonies on CMA (Waterhouse *et al* ., 1983; Kellam & Zentmyer, 1986) with "flame-like" sectors (Waterhouse & Waterson, 1964), and few or no aerial mycelia (Tucker, 1931). The hyphae are appressed (Ho, 1981) taking a star shape in old cultures (Perlberger, 1936) with regular margins (Reichert & Littauer, 1931).

Cultures of *Ph.nicotianae* on CMA are reported as variable, but commonly diaphanously fluffy in an irregular rosette pattern (Waterhouse, 1963; Waterhouse & Waterson, 1964, c) distinctly irregular and tufted (Ho, 1981) or uniform without obvious pattern (Waterhouse, 1983), terminating in irregular margins (Reichert & Littauer, 1931).

**Effect of temperature**

The effect of temperature on the radial extension rate is of great differential value between *Ph.citrophthora* and *Ph.nicotianae*. The latter species will grow at 36-37 °C (Waterhouse & Waterson, 1964, c)., whilst the former will cease growth at 2-4°C below this temperature (Perlberger, 1936; Waterhouse & Waterson, 1964, b). Table 2.1 shows the maximum and optimal temperature for *Ph.citrophthora* and *Ph.nicotianae* as reported in the literature. Most workers agree to within $\pm$2°C in the temperature requirements for these two species.

Table 2.1. Optimum and maximum temperatures (°C) of *Ph.citrophthora* and *Ph. nicotianae* as reported in literature.

| Reference | *Ph.citrophthora* | | *Ph.nicotianae* | |
|---|---|---|---|---|
| | optimum | maximum | optimum | maximum |
| Tucker (1931) | 25-27.5°C | 35°C | 30°C | 37.5°C |
| Perlberger (1936) | - | 32.5 | - | 35 |
| Schiffmann (1951) | 25 | 30 | 32 | - |
| Waterhouse (1963) | 24-28 | 32 | 30-32 | 37 |
| Waterhouse & Waterson (1964, b, c ) | " | " | " | " |
| Hartman & Nienhaus (1974 a ) | 24-28 | 31 | - | - |
| Boccas & Lavielle (1978) | - | - | 30-32 | - |
| Newhook *et al* .,(1978) | - | - | - | >35 |
| Feld *et al* .,(1979) | 24-28 | - | 30-32 | - |
| Ho (1981) | - | 30-35 | - | >35 |
| Vanderwegen (1982) | 25-27.5 | 32 | 28-30 | 37 |
| Waterhouse (1983) | 24-28 | - | 30-32 | - |
| Ho *et al* .,(1984) | 25-28 | 34-35 | - | - |
| Valvielle & Erselius (1984) | - | 32 | - | >35 |
| Mansoori & Fassihiani (1985) | 25 | 32 | 30 | 37 |
| Kellam & Zentmyer (1986) | 24 | <36 | | |

### 2.1.2 Sporangia

Sporangial size is one taxonomic feature commonly used in the identification of some *Phytophthora* species, but is only useful in delimiting those with sporangial length less than 45 µm from those more than 75 µm (Waterhouse *et al.*, 1983). Environmental conditions such as light, darkness, substrate, presence of free water and age of culture may have an effect on sporangial shape too. However, extreme individual sporangia should be ignored when considering shape and size of sporangia (Newhook *et al.*, 1978).

Sporangia of *Ph.citrophthora* are highly variable in shape and size. The sporangia can be spherical, oval, obpyriform, limoniform or elongated (Smith & Smith, 1906; Waterhouse, 1970) but always with conspicuous papillae. Table 2.2 shows the range of length, breadth and their ratio which have been reported, in which mean length for example, ranges from 37-63.4 µm.

*Ph.nicotianae* sporangia are typically prominantly papillate, broadly ovate, turbinate or nearly spherical with a beak like apex (Waterhouse & Waterson, 1964 , c). Table 2.3 shows the variation in length and breadth of sporangia of *Ph.nicotianae* as reported in the literature.

In spite of the fact that *Ph.citrophthora* falls in the group which produces sporangia longer than 75 µm (Waterhouse, 1983) there seems to be no clear distinction between this species and *Ph.nicotianae* regarding sporangial dimensions as illustrated in Figure 2.1, which presents literature values of length and breadth graphically. It can thus be concluded that sporangial dimensions do not distinguish these species very readily.

Table 2.2 Sporangial dimensions in *Ph.citrophthora* as reported in the literature.

| Reference | Length µm | Breadth µm | l:b |
|---|---|---|---|
| Leonion (1925) | 30-(5)-90 | 20-(35)-60 | 1.4 |
| Tucker (1931) | 37 | 24 | 1.5 |
| Waterhouse (1964) | 40-90 | 27-60 | 1.5 |
| Rebiero (1977) | 45-90 | 27-60 | 1.5 |
| Boccas and Lavielle (1978) | 45-90 | 26-60 | 1.6 |
| Ho (1983) | 30-50 | - | 1.6 |
| Vanderwegen (1983) | 26.9-(35.9)-90 | 21-(28.9)-60 | 1.24 |
| Fechtenberger *et al.*, (1984) | 55 | 35 | 1.6 |
| Mansoori & Fassihiani (1985) | 49 | 36 | 1.4 |
| Kellam and Zentmyer (1986) | 63.7 ± 7 | 35.2 ± 2 | 1.87 ± .2 |

**Table 2.3** Sporangial dimensions in *Ph.nicotianae* as reported in the literature

| Reference | Length μ m | Breadth μ m | l:b |
|---|---|---|---|
| Ashby (1928) | 30 | 25 | >1.4 |
| Tucker (1931) | 22-(40)-63 | 17-(29)-41 | 1.4 |
| Waterhouse (1963) | 38-50 | 30-40 | 1.4 |
| Waterhouse & Waterson (1964) | 38-50 | 30-40 | 1.4 |
| Waterhouse (1974) | 18-(38)-50 | 18(30)-40 | 1.3 |
| Boccas and Lavielle (1978) | 38-50 | 30-40 | 1.3 |
| Ho (1983) | 30-50 | - | 1.6 |
| Vanderwegen (1983) | 28-(43.3)-64 | 16-(26.8)-34 | 1.62 |
| Mansoori & Fassihiani (1985) | 35.5 | 32 | 1.1 |
| Ho & Jong (1989) | 37-(42.5)-63 | | |

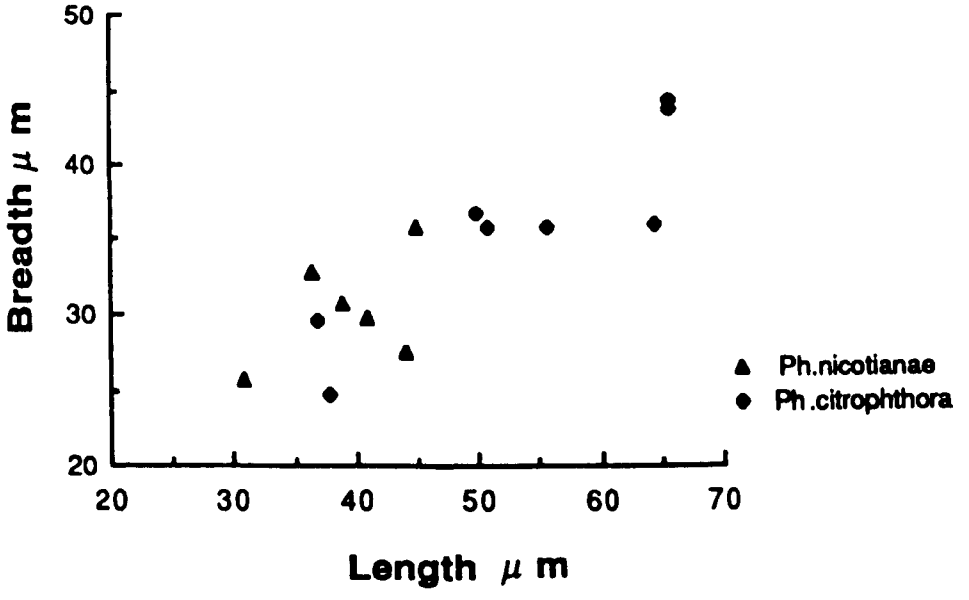



**Figure 2.1.** Sporangial dimensions of *Ph.citrophthora* and *Ph.nicotianae* from the data reported in the literature

### 2.1.3 Gametangia production

*Phytophthora* species may be heterothallic or homothallic. Homothallic species are those which produce oospores in single isolate cultures, whereas heterothallic ones display contrasting compatibility types which if paired in culture can produce oospores. However, if stimulated by an opposite external stimulus these isolates may also be induced to produce oospores by selfing (Stamps, 1953; Zentmyer, 1975; Brassier, 1971; 1972; 1975a; 1975b; Reeves & Jackson, 1974). There is still considerable discussion over the nature and control of sexual reproduction in *Phytophthora* (Ann & Ko, 1988; Ko, 1988) from the taxonomic viewpoint, the presence, sizes and shapes of oogonia, antheridia and oospores are essential tools of identification (Waterhouse *et al.*, 1983). *Ph.citrophthora* and *Ph.nicotianae* are both heterothallic species (Ho, 1981; Savage *et al.*, 1968).

*Ph.citrophthora* was regarded by most investigators as incapable of producing gametangia in single or dual cultures (Tucker, 1931; Perlberger, 1936; Waterhouse, 1963; 1970; Waterhouse & Waterson, 1964; Newhook *et al.*, 1978; Feichtenberger *et al.*, 1984; Valvieilli & Erselius, 1984; Sneh & Katz, 1988). However, a few reports have been published claiming the production of gametangia in dual cultures with other *Phytophthora* species (i.e. *Ph.palmivora* or *Ph.parasitica* (Laviola & Gallegaly, 1967; Savage *et al.*, 1968; Vanderwegen, 1983). Most of the oospore progeny in these studies did not germinate. Average sizes of oogonia were 31 µm in diameter, oospores 23.8 µm and the amphigynous antheridia were 14.2 µm long by 14.4 µm wide (Savage *et al* ., 1968).

While *Ph.nicotianae* is generally considered to be heterothallic, certain

isolates may produce gametangia by selfing, (Waterhouse & Waterson, 1964,c) especially after long incubation periods of up to several months (Ashby, 1928; Tucker, 1931; Savage *et al.*, 1968; Tsao *et al.*, 1980), but pairing with intraspecific or interspecific compatible isolates of A1 and A2 mating types can induce gametangia if the selfing fails (Waterhouse & Blackwell, 1954; Waterhouse & Waterson, 1964,c; Boccas & Zentmyer, 1976; Ho, 1981; Vanderwegen, 1982; Ko *et al*., 1986; Ho & Jong, 1989). However, selfing ability can be lost after 4-5 weekly transfers, or choosing the inoculum from an oospore free section of the plate (Savage *et al*., 1968; Brasier, 1972; Tsao *et al.*, 1980). Table 2.4 shows gametangial dimensions as reported in the literature.

### 2.1.4 *Ph. nicotianae* var. *nicotianae* and *Ph. nicotianae* var. *parasitica*

The species *Ph.nicotianae* Breda de Haan (Van Breda de Haan, 1896) has been subdivided into two varieties (var. *parasitica* and var. *nicotianae*) by several workers and the var. *parasitica* has been raised to species status by some workers (Waterhouse, 1963) as it has priority over *nicotianae*. There is considerable confusion in the literature over the distinction between the status of these varieties (Waterhouse *et al.*,1983; Gallegly, 1983; Ho & Jong, 1989). Cultural characteristics do not seem to be easy to apply since the descriptions of the varieties initially distinguished as diaphanously fluffy, or irregularly fluffy (Waterhouse & Waterson, 1964c, d) have been reported for isolates from tobacco (Apple, 1957) and isolates from a variety of host plants (Ho & Jong 1989). Serological studies by Morton and Dukes (1967) indicated that both varieties were very similar while it was found (Ho & Jong, 1989) that 35 isolates from different

plant hosts failed to differentiate into two morphological varieties. However, Erselius and Valvieille (1984) used the name *Ph.nicotianae* for isolates from hosts other than *Nicotiana tabacum* and used the varietal name only for isolates from the latter.

Throughout this work, isolates from *Citrus* orchards in the West Bank of the River Jordan will hence be called *Ph.nicotianae* without a varietal epithet because of this continuing uncertainty.

### 2.1.5 Species differentiation

Colony morphology and temperature requirements clearly play a major distinctive role in separating *Ph.citrophthora* and *Ph.nicotianae* species. However, sporangial dimensions do not seem to be of a great differential importance, but the phenomenon of readily producing gametangia by *Ph.nicotianae* is obviously of use in differentiating these species. Representative isolates chosen randomly from those recovered during this survey will be examined for colony morphology, temperature requirements, sporangial dimensions and gametangia production since they are so important.

## 2.2. Materials and Methods

### 2.2.1 Cultural characteristics

Twelve isolates of *Ph.citrophthora* from the collection and 12 isolates of *Ph.nicotianae* chosen randomly (Table 2.5) were compared. One authentic isolate, IMI *Ph.citrophthora* 129906 supplied by the Commonwealth Mycological Institute was also included. After growing the

Table 2.4. Mean gametangial dimensions of *Ph.nicotianae* var *parasitica* or *Ph.parasitica* as reported in the literature.

| Reference | Oogonium Diameter μ m | Oospore Diameter μm | Antheridium l x b* |
|---|---|---|---|
| Tucker (1931) | 21.5-(26.4)-30.4 | 16.9-(22.8)-27.1 | - |
| Waterhouse & Blackwell (1954) | 18-30 | 13-25 | - |
| Waterhouse (1963) | 24-26 (Max 31) | 18-20 | 16 x 12 |
| Waterhouse & Waterson (1964) | " | " | " |
| Savage *et al.*, (1968) | ± 22 | ± 17 | - |
| Waterhouse (1974) | 24-26 | 20 | 12 x 10 |
| Newhook *et al.*, (1978) | <28 | <20 | - |
| Boccas & Lavielle (1978) | - | 20-30 | - |
| Ho (1981) | >35 | - | - |
| Vanderweyen (1983) | 26-(27.8)-31.6 | - | - |
| Ho & Jong (1989) | 29 ± 2 | 23 ± 2 | 10 x 12 |

*l : length μ m

b : breadth μ m

cultures on PDA for 3 days, 5mm agar plugs were subcultured from actively growing margin of the colonies on to the centre of 9 cm plastic petri dishes containing PDA, CA, CV8A, PA or CMA prepared as shown in Appendix 2. Three replicates of each isolate on each medium were incubated in the dark at 25°C for 3 days. The colony radial extension rate was measured as mm $h^{-1}$, the colony morphology assessed visually and representative isolates were photographed.

### 2.2.2 Effect of temperature

Four isolates of each of *Ph.citrophthora* (IC130018, IA14002, IIC130012, IIE12003) and *Ph.nicotianae* (ID7002, IA7007, IIC130025, IIB7005) were chosen randomly for assessment. The isolates were removed from under paraffin oil and subcultured on PDA for 3 days. Agar blocks were cut from the growing margins of the colonies using a sterile cork borer, 5 mm in diameter, transferred on to the centres of 9 cm plastic petridishes containing RSM. Subsequently, two replicates of each isolate were incubated at 25, 30, 32, 35, 37 and 45°C for a period of 5 days in the dark. Plates on which no growth was noticed were all transferred to 25°C for a further period of 5 days.

Measurements of radial extension for each plate were taken at 72, 96 and 120 $h^{-1}$. Data were expressed as radial extension rate per 24 $h^{-1}$.

### 2.2.3 Sporangial characteristics

Five isolates were chosen randomly from each of the *Ph.citrophthora* and *Ph.nicotianae* collections (*Ph.citrophthora* IB7005, IB8002, IE5002, IIA2005, IIE2009; *Ph.nicotianae* IB13004, IC8001, ID11003, IID3006, IIA10004) to grow at 25°C on PDA for 3 days. Using a cork borer, 5 mm blocks of agar were subcultured on to CA and incubated for 3 days.

Table 2.5. Isolates used in the assessment of cultural characteristics of *Ph.citrophthora* and *Ph.nicotianae*

| No. | *Ph.citrophthora* | *Ph.nicotianae* |
|---|---|---|
| 1. | IB7005 | IA7003 |
| 2. | IB13001 | IB13004 |
| 3. | IE2006 | ID7001 |
| 4. | IB8002 | ID11003 |
| 5. | IB120012 | IC8001 |
| 6. | IE5002 | IC14001 |
| 7. | IIE12001 | IIA30012 |
| 8. | IIE5002 | IIE80012 |
| 9. | IIA13003 | IID3006 |
| 10. | IIE7004 | IIB7006 |
| 11. | IIA2005 | IIC130026 |
| 12. | IIE8004 | IIA10004 |
| 13. | IMI129906 | |

Three square blocks of agar 4 $cm^2$ in size were cut from the growing margins of the colonies using a sterile scalpel, transferred to sterile plastic petri dishes containing 7-10 autoclaved hempseeds. The plates were flooded with sterile soil extract prepared as in Appendix 2 and incubated under fluorescent light to initiate sporulation. Three days later, the extending hyphal mats were examined under a stereoscope for sporangia. A piece of sporangium-bearing mycelia was cut with a sterile scalpel and a coverslide (sterilized by passing through the flame of a bunsen burner) inserted under the cut mycelium. The coverslide was removed from the petridish and a microslide was inverted on top. A drop of lactophenol cotton blue was added to one side of the mount and using a filterpaper from the opposite side, the water was replaced by the stain. The slide was gently heated over a flame to remove any trapped air bubbles. After blotting any extra stain, the mount was sealed with nail varnish to prevent evaporation. The slide was examined within 48h. Dimensions of the length, breadth, pore and papilla for 100 sporangia of each isolate were measured using a micrometer attached to the 10X occular of a Vickers microscope, with 40X objective. The relationship between the occular micrometer and a stage micrometer was later determined and the dimensions were corrected to micrometres.

### 2.2.4 Production of chlamydospores

Mounts prepared from the five isolates assessed for sporangia production (section 2.2.3) of *Ph.citrophthora* and *Ph.nicotianae* were assessed at the same time for chlamydospores production. Dimensions of fifty chlamydospores and wall thickness were measured from the isolates which were capable of producing them using the method in section 2.2.3, and the results were recorded in micrometres.

### 2.2.5 Production of gametangia

#### a. Pairing procedure

Blocks of agar 5 mm in diameter were cut using a cork borer, from the actively growing margins of 3 day old cultures on PDA plates and placed either in the centre of fresh plates or paired with a second isolate, placing each block in the centre of one half of the plate. The plates were incubated in the dark at 25°C for 12 days, then inspected from underneath using 10X followed by 40X objectives. Gametangia production was graded from (-) for none to (++++) for gametangia distributed all over the plate. Isolates which failed to produce gametangia within this time were further incubated for upto 4 weeks more before discarding as incapable of producing gametangia.

Thirteen isolates of *Ph.citrophthora* and 12 of *Ph.nicotianae* (Table 2.5) were assessed individualy on CMA, PA, CV8A, CA and CV8CMOA. In addition, each isolate was paired with all the other 26 isolates. The pairings were made on all five media.

The remaining 36 isolates of *Ph.nicotianae* obtained during the orchard survey were assessed singly on a later occasion.

Isolates which produced gametangia on any media (sometimes more than one medium) were reassessed in duplicate on the most suitable medium. This time the agar plugs were cut from the gametangia producing area on the original test plate. Neither CV8CM0A nor PA were used in this assessment due to the production of a dense mycelial mat.

#### b. Production of gametangia in single zoospore cultures

Sporangia were produced as described in section 2.2.3 using 5 mm agar plugs from the gametangia producing areas of 4 *Ph.nicotianae* isolates

(IC140010, IID3007, IIB13001, IIC130027) chosen randomly from the gametangia producing isolates. The zoospores were released, counted, and the zoospore suspension was diluted to contain 50-80 $cm^{-3}$. Finally 0.5 $cm^3$ were spread aseptically over the surface of 1% WA or CMA. The zoospores were allowed to encyst, then located under a stereoscope. The tip of a bent pasteur pipette was used to draw a circle around each zoospore to mark its position. The plates were incubated at 25°C for 2h, then the germinating zoospores were located again. With the help of the fine tip of a sterile scalpel, 20 germlings were transferred with the surrounding minute circular plug into the centres of petridishes containing freshly prepared media. The media were selected as most conducive to gametangia production by each isolate in the past. Twenty more germlings were plated in tetrads evenly separated in five petridishes.

Growth was examined after 12 days incubation at 25°C in the dark and the production of gametangia was assessed in both single and tetrad cultures. The location of the gametangia was then related to the position of the germlings.

**c. Measurement of gametangia dimensions:**

To measure the dimensions of the gametangia in *Ph.nicotianae*, 5 isolates (IC14001, IC8001, IB13004, IID3007, IIB13001) were chosen randomly from the 16 isolates which were able to produce these organs. A block of agar containing gametangia-producing mycelium was mounted on a microslide, lactophenol cotton blue added and a coverslip laid carefully on top. The mount was then heated over a flame to dissolve the agar and remove air bubbles, then sealed with nail varnish. The diameter of 50 oogonia, oospores including their wall thickness and the dimensions of their amphigynous antheridia were measured as described for sporangia in

section 2.2.3.

Oogonia could be differentiated by their shape, being spherical to pyriform, smooth, hyaline to yellowish, enclosing an oospore, which after fertilization develops into a single, smooth or rough, spherical, hyaline to yellowish oospore, nearly filling the interior of the oogonium.

## 2.3. Results

### 2.3.1 Cultural characteristics

***Ph. citrophthora***

Plate 1 shows the growth pattern of representatives from the 12 isolates compared with the authentic *Ph.citrophthora* culture on CA, CMA, CVBA, PA and PDA. The pattern of the colony was essentially the same on all media, being star to rosette or chrysanthemum shaped with a few aerial hyphae .

The radial extension rate for the isolates ranged from 0.18 mm $h^{-1}$ to 0.37 mm $h^{-1}$ depending on the growth medium, which is shown in Table 2.6 as the mean growth rate for the groups of survey isolates, with the authentic isolate shown individually.

***Ph. nicotianae***

Plate 2 shows the growth pattern of representative isolates on the five media. The colony pattern of all isolates on these media was again very similar, irregularly fluffy, with no zones or striations. However, a rose like

Plate 1. Growth pattern of *Ph.citrophthora* on five media

A. PDA B. CA C. CV8A D. PA E. CMA

Top : Representatives isolates

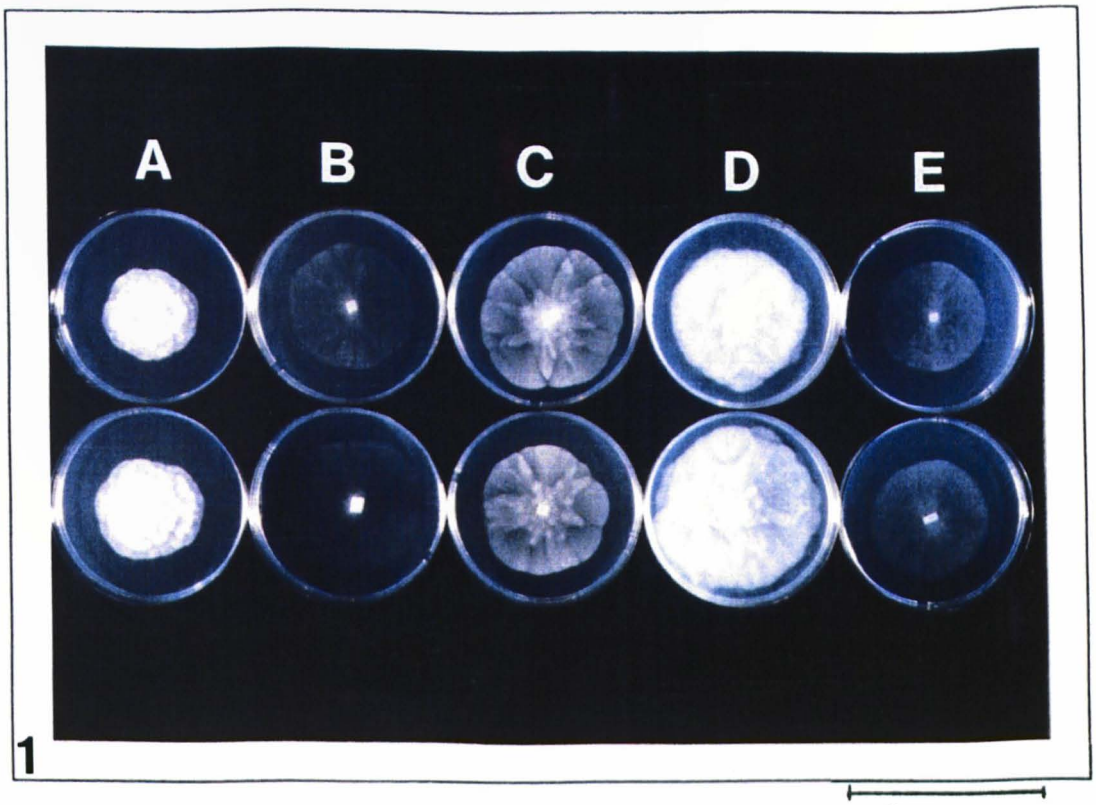
Bottom : Authentic isolate IMI 129906

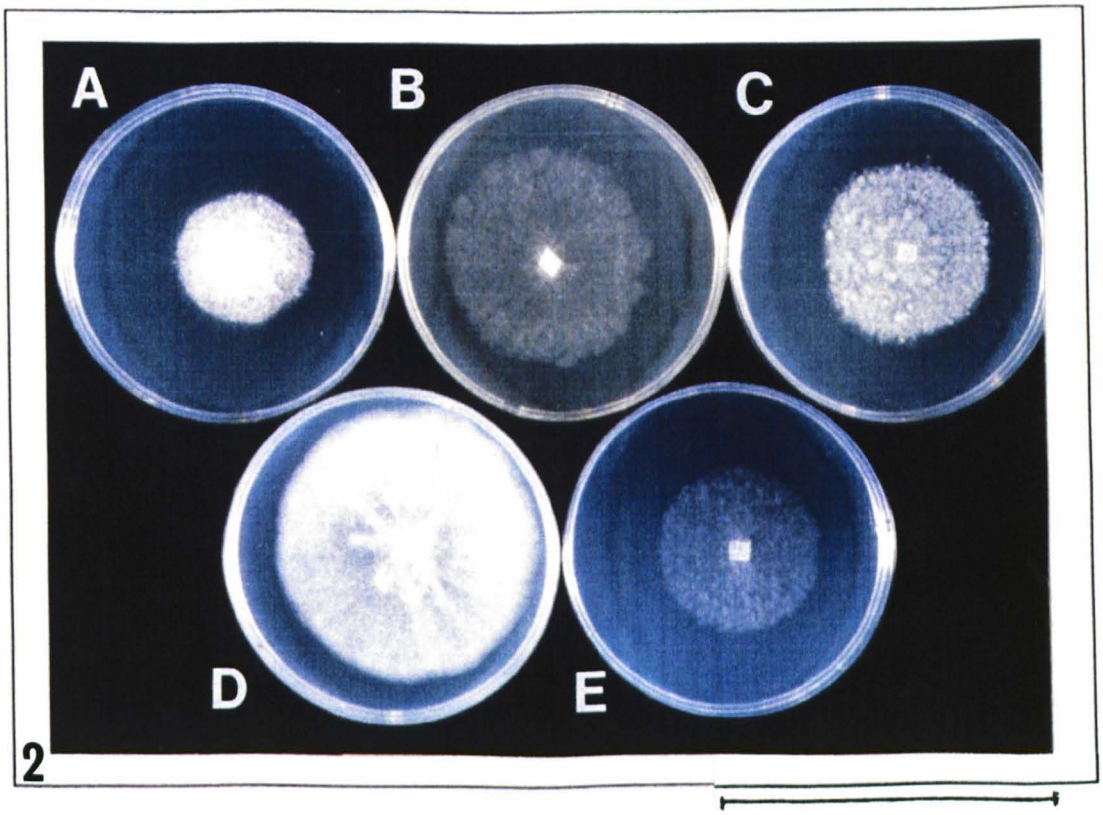
The isolates were incubated at 25°C for a period of 3 days

Plate 2. Growth pattern of *Ph.nicotianae* on five media

A. PDA B. CA C. CV8A D. PA E. CMA

The isolates were incubated at 25°C for a period of 3 days


A
B
C
D
E
1
9 cm


A
B
C
D
E
2
9 cm

pattern was seen in the centre of the CA colonies.

Measurement of radial extension rate for the isolates indicated a range between 0.11 mm $h^{-1}$ and 0.41 mm $h^{-1}$ depending on the growth medium. The mean growth of the isolates on each medium is shown in Table 2.6. Figure 2.2 shows a comparison between RER of *Ph.citrophthora* and *Ph.nicotianae* on the 5 media.

### 2.3.2 Effects of temperature

Figure 2.3 shows the mean RER for *Ph.citrophthora* and *Ph.nicotianae* over the temperature range of 25-45°C. The optimum temperature for *Ph.citrophthora* was 25°C. The RER declined gradually at higher temperatures leading to no or negligible growth at 35°C and above.

**Table 2.6.** Radial extension rate $mm.h^{-1}$ on 5 media, of *Ph.citrophthora* and *Ph.nicotianae* grown in the dark at 20 °C for 3 days.

| *Phytophthora* | Origin | PDA | CMA | CV8A | CA | PA |
|---|---|---|---|---|---|---|
| *Ph.citrophthora* | survey* | 0.21± 0.01 | 0.25± 0.02 | 0.26± 0.03 | 0.27± 0.03 | 0.33± 0.03 |
| *Ph.citrophthora* | IMI | 0.21 | 0.25 | 0.30 | 0.28 | 0.32 |
| *Ph.nicotianae* | survey | 0.16± 0.02 | 0.20± 0.01 | 0.21± 0.02 | 0.29± 0.03 | 0.36± 0.02 |

* Mean of 12 isolates.

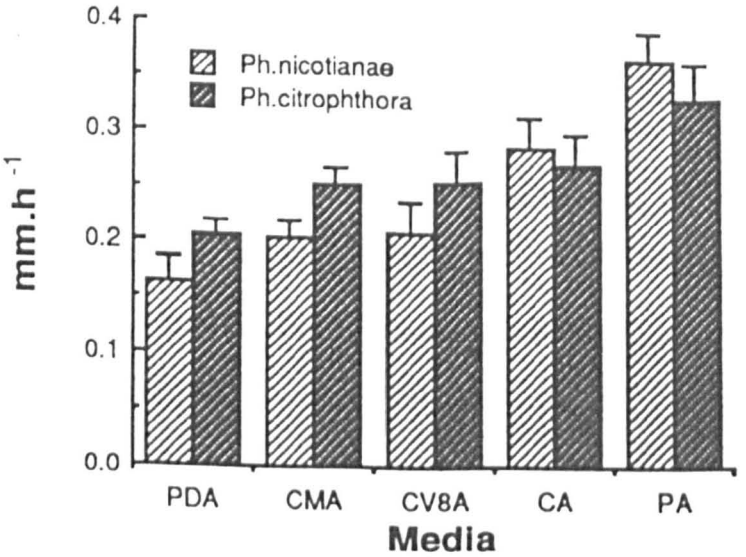



**Figure 2.2.** Comparative growth of *Ph.citrophthora* and *Ph.nicotianae* taken as the average radial extension rate of 12 isolates from each species on 5 media, each replicated thrice. Bars indicate 2 X SE.

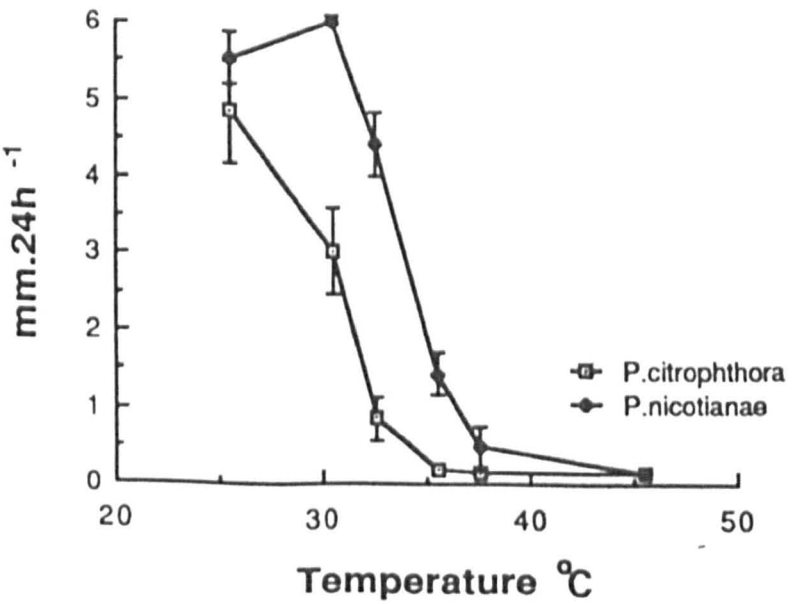



**Figure 2.3.** Radial extension rate / day of 4 isolates from each of *Ph.citrophthora* and *Ph.nicotianae* at varying temperature. Bars indicate 2 X SE.

The optimum temperature for *Ph.nicotianae* was slightly higher than that of *Ph. citrophthora* being 30°C. All four isolates continued to grow at 35°C. While growth was slight at 37°C for three of the isolates, the fourth (IA7007) failed to grow at this temperature.

None of the isolates from either species was able to grow at 45°C. Isolates were killed by exposure to this temperature; subsequent transfer of plates to 25°C for 5 more days indicated no resumption of growth.

### 2.3.3 Sporangial characteristics

***Ph. citrophthora***

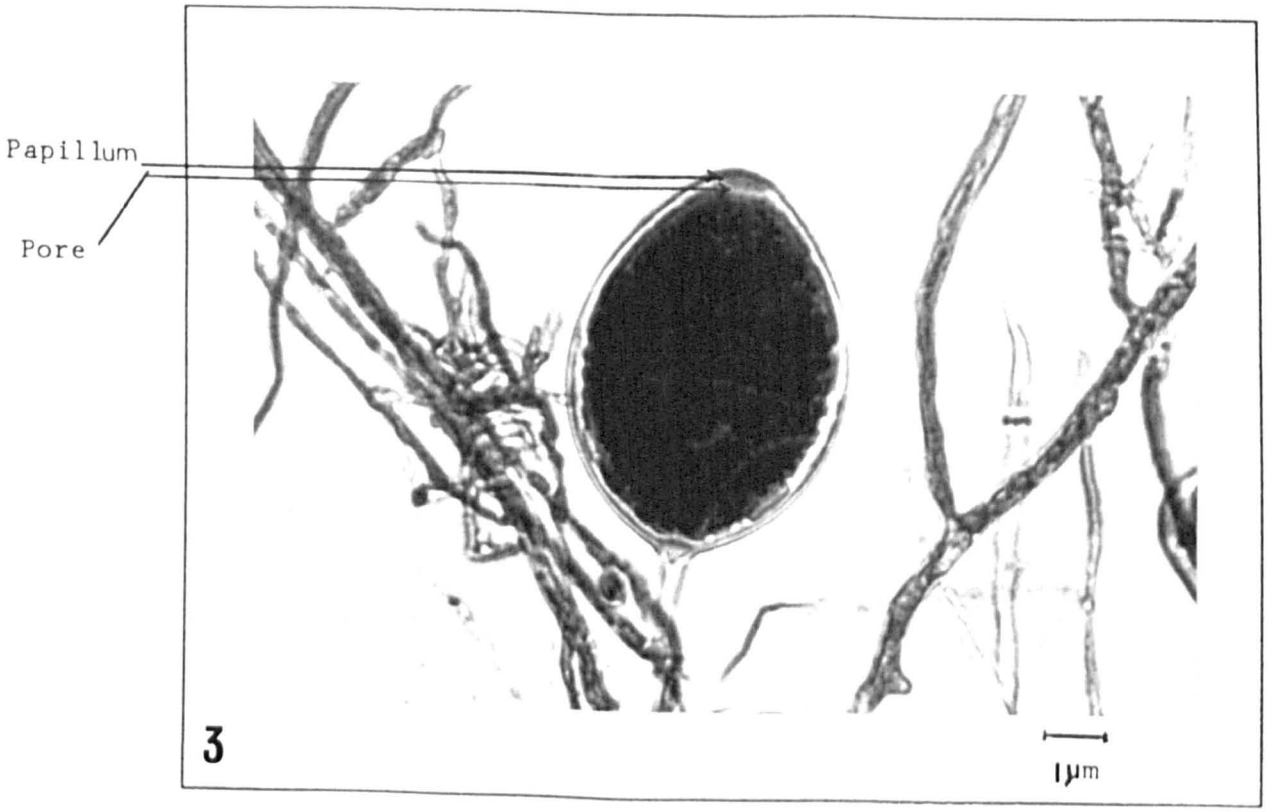
The sporangia were carried on sporangiophores of irregular length and branching. Sporangia were variable in shape between limoniform and obpyriform (Plate 3). Table 2.7 shows that sporangia varied from 35-83 µm in length, with an average of 51.4 ± 3.6 µm, with pore size of 3.9-9 µm (average 6.9 ± 0.3 µm) and prominent papillae measuring between 1.29-5.16 µm long with a mean of 2.77 ± 1.13 µm. Sporangial breadth was from 23.3-43.9 µm with an average of 33.7 ± 2.6 µm. The length to breadth ratio was 1.5.

***Ph. nicotianae***

These sporangia were also carried on irregularly long and branching sporangiophores. Sporangia as shown in Plate 4 were broadly ovoid to spherical with beak like papillae. Table 2.8 shows that the length of the sporangia varied between 38.7 and 65.8 µm with an average of 49.8 ± 3.4 µm, while the breadth varied between 25.8 and 47.7 µm with an average of

**Plate 3.** Sporangium of *Ph.citrophthora*

**Plate 4.** Sporangium of *Ph.nicotianae*


Papillum
Pore
3
1µm

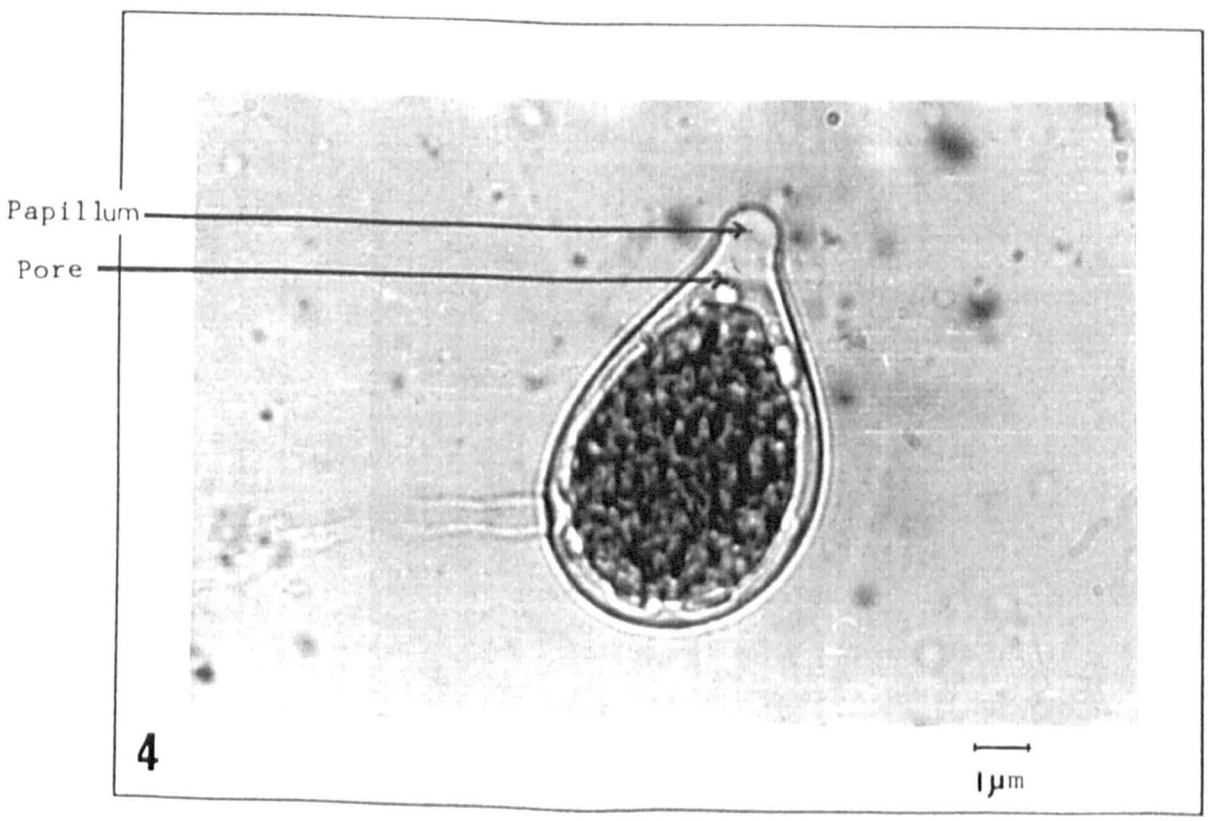

Papillum
Pore
4
1µm

**Table 2.7** Sporangial dimensions of *Ph.citrophthora* (Mean of 100 sporangia from each isolate)

| Isolate number | Sporangial length µm | Sporangial breadth µm | l:b | Pore size µm | Papilla size µm |
|---|---|---|---|---|---|
| IB7005 | 36.1-(52.9 ± 9.2)-83.9 | 25.8-(32.8 ± 4.1)-37.4 | 1.6 | 3.87-(7.1 ± 1.14)-9.03 | 1.29-(3.6 ± .85)-5.16 |
| IB8002 | 37.4-(51.4 ± 8.1)-64.5 | 24.5-(32.3 ± 3.0)-38.7 | 1.6 | 5.16-(6.37 ± .3)-6.45 | 1.29-(1.57 ± .6)-3.87 |
| IE5002 | 34.8-(45.9) ± 6.0)-64.5 | 25.8-(31.3 ± 2.4)-36.1 | 1.5 | 6.45-(7.12 ± .65)-7.74 | 1.29-(1.37 ± .31)-2.58 |
| IIA2005 | 34.8-(53.7 ± 6.4)-67.1 | 25.8-(38 ± 3.5)-43.9 | 1.4 | 5.16-(6.8 ± 1.07)-9.03 | 1.29-(1.96 ± .79)-3.87 |
| IIE2009 | 34.8-(55.3 ± 7.6)-69.7 | 23.2-(33.1 ± 4.7)-40 | 1.7 | 5.16-(7 ± 1.15)-9.03 | 1.29-(2.24 ± .73)-3.87 |
| Mean | 34.8-(51.5 ± 3.6)-83.9 | 23.2-(33.7 ± 2.6)-43.9 | 1.5 | 3.87-(6.88 ± .31)-9.03 | 1.29-(2.77 ± 1.13)-5.16 |

**Table 2.8 Sporangial dimension of *Ph.nicotianae* from (Mean of 100 sporangia from each isolate)**

| Isolate number | Sporangial length µm | Sporangial breadth µm | l:b | Pore size µm | Papilla size µm |
|---|---|---|---|---|---|
| IB13004 | 38.7-(50.9 ± 6.6)-65.8 | 27.1-(37.3 ± 5.4)-47.7 | 1.36 | 5.16-(6.86 ± .96)-9.03 | 1.29-(2.9 ± 1.0)-5.16 |
| IC8001 | 46.0-(47.6 ± 4.6)-50.1 | 25.8-(33.9 ± 3.7)-40 | 1.4 | 5.16(6.8 ± .74)-7.74 | 1.29-(3.3 ± 1.35)-5.16 |
| ID11003 | 41.0-(47.5 ± 3.9)-59 | 31.0-(36.2 ± 3.4)-45 | 1.3 | 6.45-(7.46 ± .6)-9.03 | 1.29-(3.4 ± 1.1)-5.16 |
| ID3006 | 41.3-(47.7 ± 3.4)-54.2 | 31.0-(36.3 ± 3.5)-44 | 1.3 | 6.45-(7.33 ± .61)-7.74 | 1.29-(2.8 ± .8)-6.45 |
| IIA10004 | 43.9-(55.2 ± 5.6)-65.8 | 31.0-(37.4 ± 3.8)-44 | 1.5 | 5.16-(6.71 ± 1.19)-9.03 | 1.29-(3.1 ± .64)-5.16 |
| Mean | 38.7-(49.8 ± 3.4)-65.8 | 25.8-(36.2 ± 1.4)-44.7 | 1.4 | 5.16-(7.03 ± .34)-9.03 | 1.29-(4.0 ± .25)-6.45 |

36.3 ± 1.4 μm. Beak like papillae varied in size between 1.3-6.5 μm with an average of 4.0 ±0.3 μm. The length to breadth ratio was 1.38.

### 2.3.4 Chlamydospores

***Ph. citrophthora***

No chlamydospores were produced by any of the *Ph.citrophthora* isolates.

***Ph. nicotianae***

Chlamydospores were formed abundantly within the period which allowed sporangia production (3 days). They were circular in shape, intercalary with a diameter that varied from 31-61μm, averaging at 47.29 ± 3.08 μm, and a wall thickness of 2-4 μm (average 3.54 ± .29 μm). Table 2.9 shows the average dimensions of 50 chlamydospores for 5 *Ph.nicotianae* isolates.

### 2.3.5 Production of gametangia

**a. Pairing procedure**

***Ph. citrophthora***

None of the *Ph.citrophthora* isolates tested was able to produce gametangia in single isolate culture or in dual cultures with other isolates of the same species or any of the *Ph.nicotianae* isolates .

Table 2.9. Chlamydospore dimensions of *Ph.nicotianae* ( Mean of 50 chlamydospores measurement of 5 isolates)

| Isolate number | Diameter µm | Wall thickness µm |
|---|---|---|
| IB13004 | 31-(44.1±6.29)-57 | 2-(3.3±0.83)-4.0 |
| IC8001 | 36-(50.4± 6.32)-59 | 2-(3.8± 0.56)-4.5 |
| ID11003 | 35-(44.5± 4.87)-59 | 2-(3.3± 0.61)-4..0 |
| ID3006 | 33-(46.9± 5.63)-61 | 2-(3.4± 0.69)-4.5 |
| IIA10004 | 33-(50.5± 5.24)-59 | 2-(3.9± 0.32)-4.5 |
| Average | 31-(47.3± 3.08)-61 | 2-(3.54± 0.29)-4.5 |

*Ph. nicotianae*

The results of gametangia production are summarized in Table 2.10. Of the 48 *Ph. nicotianae* isolates, 14 were able to produce gametangia in single isolate cultures, frequently on more than one of the media by the end of 12 days. A further two isolates had produced gametangia at the end of 6 weeks. The remaining 32 isolates showed no sign of gametangia production.

It is clear that gametangia production may depend on the growth medium. For example, isolate IA7008 produced gametangia profusely on CV8A and PA, slightly on CMA and CV8CMOA and not at all on CA. Other isolates (e.g. IB13004, IIC130027) produced gametangia profusely on CA.

When gametangia-producing isolates were retested on their "best" medium, not only were the results frequently different from those in Table 2.10, but the replicate plates themselves were often different. Table 2.11 illustrates this feature. Isolates could produce more, fewer or no gametangia with respect to their performance in the previous test.

**b. Single zoospore cultures**

None of the single zoospores cultures from the 4 isolates were able to produce gametangia. However, gametangia were produced by some tetrad germlings as shown in Table 2.12. While no response was detected in tetrads of isolate IC140010, gametangia were produced in 2 or 3 of the 5 plates from all the other isolates. These gametangia were produced at the contact point of the single zoospore colonies.

**c. Gametangial dimensions**

Dimensions of oogonia, antheridia, oospores and thickness of the

Legend to Tables 2.10 and 2.11.

| Symbol | Media | Description |
|---|---|---|
| - | All | No gametangia production |
| + | CA<br>CV8A<br>CV8CMOA | Few gametangia in a limited region (2-4 $mm^2$) of the plate, the region is densely covered with with the hyphae compared with therest of the plate. Region always close to the agar plug of the original inoculum |
| + | CMA<br>PA | Few gametangia (1-3), but no sectoring visible. Gametangia can be found only after thorough search all over the plate. |
| ++ | CA<br>CV8A<br>CV8CMOA | One sector producing gametangia, larger than (+), close to inoculum plug. |
| ++ | CMA<br>PA | 10 - 15 gametangia, no sectoring. |
| +++ | CA<br>CV8A<br>CV8CMOA | One large sector, 3/4 plate covered, for IIC130027 on CV8CMOA dark brown pigment was noticed in the medium under the sector. |
| ++++ | All media | Gametangia produced all over the plate. |

Figure 2.10. Production of gametangia in 12 days by *Ph.nicotianae*

| Isolate* | CA | CMA | CV8A | PA | CV8CMOA |
|---|---|---|---|---|---|
| IB13004 | ++++ | - | ++ | - | - |
| IC8001 | + | - | ++ | +++ | + |
| IC14001 | - | ++ | - | - | - |
| IIA30012** | + | - | - | - | - |
| IIB7006 | + | - | + | + | - |
| IIE80012** | + | - | - | - | - |
| IA7008 | - | ++ | ++++ | ++++ | ++ |
| IC140010 | ++ | ++++ | + | - | - |
| IIA4001 | ++ | - | + | - | - |
| IIB13001 | ++ | - | ++++ | - | - |
| IID3007 | - | ++++ | + | + | ++ |
| IIC130010 | + | + | - | - | - |
| IIC130027 | ++++ | + | - | - | ++ |
| IID3001 | ++++ | ++ | + | ++++ | - |
| IIE80010 | - | - | ++ | - | - |
| IIB10006 | + | - | - | - | - |
| Total no. of isolates producing gametangia | 12 | 7 | 10 | 5 | 4 |

* All other isolates did not produce gametangia

** Isolates which took 6 weeks to produce gametangia

Table 2.11. Comparison between results of gametangia production shown in Table 2.10. and retesting the same isolates in duplicate on the "best" medium for gametangia production.

| Isolate* | Medium | Results from Table 2.10 | Replicates 1 | 2 |
|---|---|---|---|---|
| IB13004 | CA | ++++ | - | - |
| IC8001 | CV8A | ++ | +++ | +++ |
| IC14001 | CMA | ++ | ++++ | - |
| IIA30012 | CA | + | ++ | + |
| IIB7006 | CV8A | + | - | - |
| IIE80012 | CA | + | ++ | + |
| IA7008 | CV8A | ++++ | + | + |
| IC140010 | CMA | ++++ | ++++ | ++ |
| IIA4001 | CMA | + | - | - |
| IIB13001 | CV8A | ++++ | ++++ | +++ |
| IID3007 | CMA | ++++ | ++++ | +++ |
| IIC130010 | CA | + | - | - |
| IIC130027 | CA | ++++ | ++++ | + |
| IID3001 | CA | ++++ | +++ | +++ |
| IIE80010 | CV8A | ++ | ++ | ++ |
| IIB10006 | CA | + | - | - |

**Table 2.12.** Gametangia production by tetrad of single zoospore isolates

| Isolate | Medium | Plates* |
|---|---|---|
| IC140010 | CMA | 0 |
| IID3007 | CMA | 3 |
| IB13001 | CV8A | 2 |
| IIC130027 | CA | 3 |

* Number of plates (maximum 5) producing gametangia between two or more zoospore isolates.

**Table 2.13** **Gametangial dimensions of 5 *Ph.nicotianae* isolates taken from 50 gametangia of each isolate**

| Isolate | Oogonia diameter µm | Oospore diameter µm | Oospore wall thickness µm | Antheridia | |
|---|---|---|---|---|---|
| | | | | Length µm | Breadth µm |
| IC14001 | (24.74 ± 1.6) | (18.42 ± 1.8) | (3.04 ± 0.94) | (12.18 ± 0.92) | (9.93 ± 0.98) |
| | 23.22 - 28.38 | 15.48 - 21.93 | 1.29 - 5.16 | 10.32 - 12.9 | 7.74 - 10.32 |
| IC8001 | (23.37 ± 2.77) | (20.49 ± 3.12) | (2.95 ± 0.73) | (12.48 ± 0.65) | (9.8 ± 0.75 |
| | 19.35 - 28.38 | 12.9 - 23.22 | 1.29 - 5.58 | 11.61 - 12.9 | 9.03 - 10.32 |
| IB13004 | (26.32 ± 2.25) | (20.17 ± 2.9) | (2.89 ± 0.85) | (11.93 ± 1.87) | (9.8 ± 1.12) |
| | 21.93 - 29.67 | 14.19 - 23.22 | 1.29 - 5.16 | 10.32 - 12.9 | 7.74 - 10.32 |
| IID3007 | (25.59 ± 1.52) | (16.82 ± 2.84) | (3.52 ± 0.67) | (12.5 ± 2.1) | (9.65 ± 1.24) |
| | 21.93 - 28.38 | 12.9 - 23.22 | 1.29 - 6.45 | 9.68 - 14.19 | 7.74 - 11.61 |
| IIB13001 | (24.62 ± 1.81) | (19.68 ± 2.32) | (3.04 ± 0.94) | (11.8 ± 1.7) | (9.71 ± 0.81) |
| | 20.45 - 28.53 | 16.21 - 23.12 | 1.29 - 4.52 | 10.12 - 13.11 | 7.74 - 10.42 |
| Average | (24.93 ± 0.99) | (19.12 ± 1.51) | (3.11 ± 0.21) | (12.18 ± 0.28) | (9.78 ± 0.1) |
| | 19.35 - 29.67 | 12.9 - 23.22 | 1.29 - 6.45 | 9.68 - 14.19 | 7.74 - 11.61 |

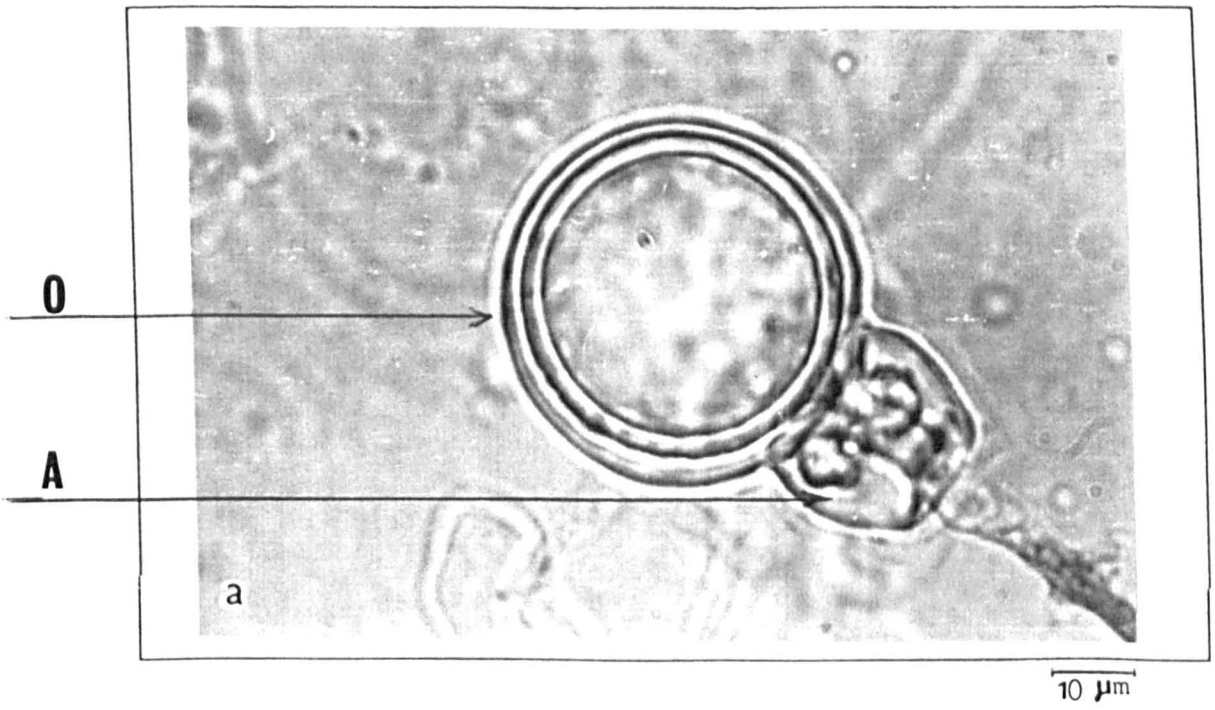
**Plate 5 .** Gametangia of *Ph.nicotianae*

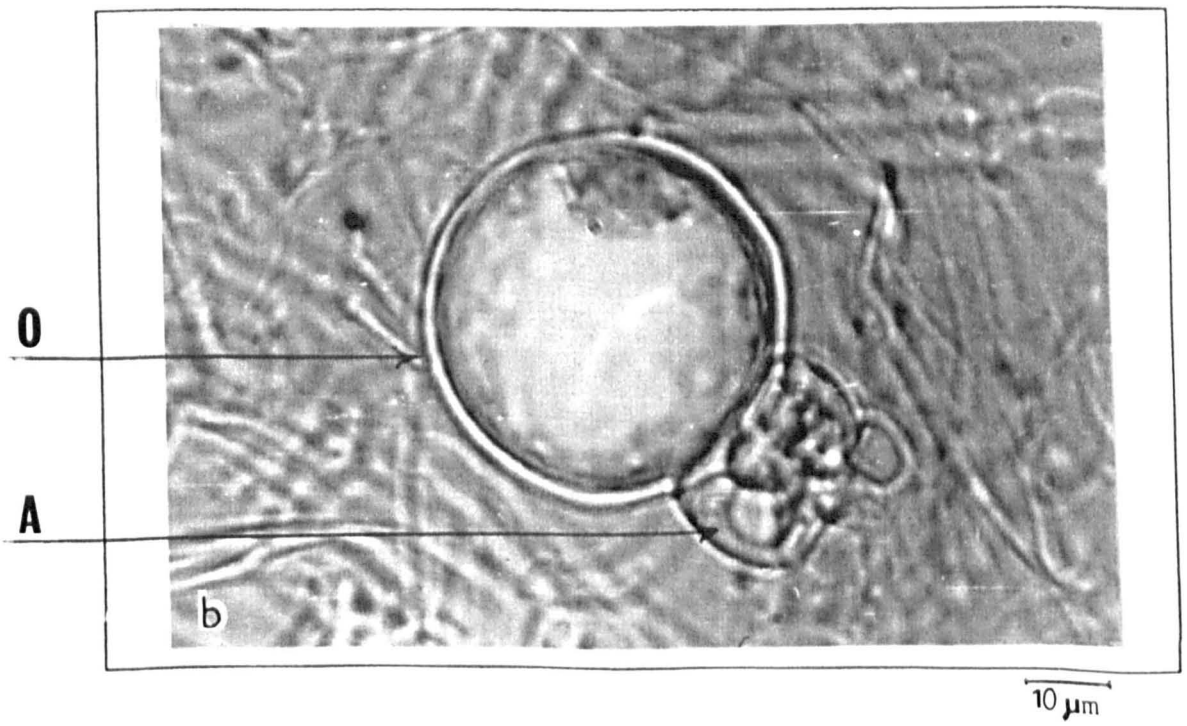
**a.** Gametangium showing amphigynous antheridium, oospore wall 2.7 μm thick.

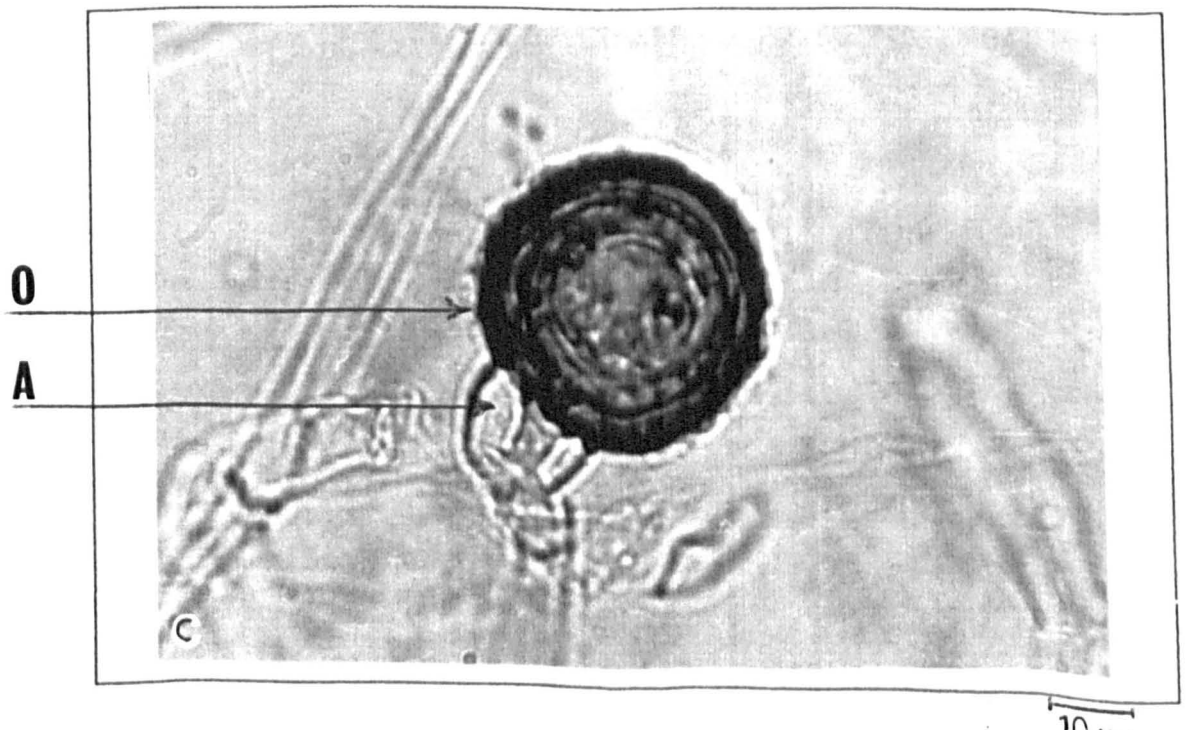
**b.** Oval antheridium, with thin oospore wall ( 1.3 μm)

**c.** Partially amphigynous antheridium, with thick oospore wall (6.45 μm)

A. Antheridium O. Oospore


O
A
a
10 µm


O
A
b
10 µm


O
A
c
10 µm

oospore wall for 5 isolates are shown in Table 2.13.

The diameter of the oogonia varied between 19.4 µm and 29.7 µm (mean 24.0 ± 1.0). Antheridia were amphigynous (Plate 5.a), oval (Plate 5.b), or partially amphigynous (Plate 5.c) with a length of 9.7 to 14.2 µm (mean 12.2 ± 0.3) and a breadth of 7.7 to 11.6 µm (mean 9.8 ± 0.1). The oospores were spherical, aplerotic, 12.9 to 23.2 µm (mean 19.1 ± 1.5) in diameter, with a wall varying in thickness from 1.3 µm (Plate 5.b) to 6.45 µm (Plate 5.c) with an average thickness of 3.1 ± 0.2 µm, varying in colour from colourless to yellow-brown.

### 2.2.6 Independent species assignment

Species identification was supported by a report from Dr. Geoffrey Hall, CAB International Mycological Institute, Kew on 12 isolates, six tentatively assigned to each species.

He agreed with the identity of 11 of the 12, as *Ph.citrophthora* (Smirh & Smith ) Leonian and *Ph.nicotianae* van Breda de Haan . The twelfth isolate did not produce sporangia. Details are shown in Appendix 3.

## 2.4 Discussion

It can be difficult to identify isolates of *Phytophthora* to species level (Waterhouse *et al* ., 1983; Gallegly, 1983). This identification has been based on morphology rather than other criteria. Keys have been proposed to tabulate these characters in order to identify *Phytophthora* species on this basis (Waterhouse, 1963; 1970; Newhook *et al* ., 1978; Ho, 1981; 1982). The main characters for identification include cultural characteristics, temperature requirements, production and dimensions of sexual and asexual organs of reproduction.

All reports from the area east of the Mediterranean on *Phytophthora* isolates from *Citrus* whether from Palestine (Reichert, 1932; Reichert & Littauer, 1931; Perlberger, 1936), Israel (Afek and Sztejnberg, 1988; Sneh & Katz, 1988), Lebanon (Hartmann, 1969; Hartmann & Nienhaus, 1974a, b), Iran (Mansoori & Fassihiani, 1985), Iraq (Hassan *et al* ., 1989) or Turkey (Cinar& Tuzcu, 1976; Tuzcu *et al* ., 1984) have revealed either the first or both of *Ph. citrophthora* and *Ph. parasitica* . There is a single report of a third species *Ph. hibernalis* Carne, being found on *Citrus* in the region (Schiffman, 1951). *Ph. hibernalis* falls in group IV of Waterhouse (1963; 1970), Newhook *et al* .,(1978) and Waterhouse *et al* ., (1983) being semi-papillate and homothallic with an optimum growth temperature of 20°C, 4-8°C less than *Ph.citrophthora* and 5-10°C less than *Ph.nicotianae.*

All of the isolates recovered during the survey were able to grow at 24°C, produced papillate sporangia and fell into one of the two cultural morphologies of the colonies. So, the presence of any isolates of *Ph.hibernalis* within the isolates is unlikely.

The isolates were divided into two groups in the preliminary work based on colony morphology and then identified as *Ph.citrophthora* and *Ph.nicotianae* based on their colonial morphology, temperature tolerance, chlamydospores and gametangia production.

All the isolates recovered from the *Citrus* orchards, not just the few tested in detail, had one of the two very obviously different morphologies. Each morphology correlated with a different group of temperature requirement and chlamydospore or gametangial production abilities. The features of putative *Ph.citrophthora* and *Ph.nicotianae* isolates matched closely the description of the species in the literature (Waterhouse and Waterson, 1964, a,b, c; Waterhouse, 1970; Newhook *et al* ., 1978; Rebiero, 1978).

Sporangial dimensions of all isolates overlap and there was no clear

species distinction in their size or shape (Table 2.7 and 2.8) which are the same as those reported in the literature (Tables 2.2 and 2.3) which are not of valuable differential value.

It has been well documented in the literature that *Ph.citrophthora* and *Ph.nicotianae* are heterothallic (e.g. Savage *et al* ., 1968; Ho, 1981). None of the *Ph.citrophthora* isolates were able to produce gametangia alone or in dual cultures with other *Ph.citrophthora* or *Ph.nicotianae* isolates in the present study. There is a disagreement in the literature about the heterothallism of *Ph.nicotianae* ( Tucker, 1931; Waterhouse, 1963; 1970; Newhook *et al* ., 1976; Ho, 1981).

A third of the *Ph. nicotianae* isolates (37%) were able to produce gametangia in single cultures. The ability was not consistent, not exhibited on all media or with the same intensity. Since *Ph. nicotianae* is a heterothallic species this result was unexpected but would agree with some earlier reports (Waterhouse, 1963; 1970; Tsao *et al* ., 1980; Ho & Jing, 1989). This inconsistency was reported to be lost after a few subcultures (Tsao *et al* ., 1980; Ho & Jing, 1989). The ability to produce gametangia was investigated by producing authentic single zoospore cultures (i.e. single nucleus) some of which produced gametangia on pairing in groups but failed to produce them in single cultures.

Chlamydospores were not produced by *Ph.citrophthora* , where reports in the literature state a variability, abundance or absence of these organs (Waterhouse, 1963; Ribiero, 1978; Newhook *et al* ., 1978; Ho, 1981). These asexual bodies were formed abundantly by *Ph. nicotianae* isolates, which matches with reports in literature regarding size and abundance (Waterhouse & Waterson, 1964 b, c; Newhook *et al* ., 1978), and was of a significant value in differentiating this species from *Ph. citrophthora* which did not produce them at all.

The present study has demonstrated that:

1. The isolatable *Phytophthora* from *Citrus* orchards in the West Bank can be assigned to *Ph.citrophthora* (Smith & Smith) Leonian and *Ph.nicotianae* Van Breda de Haan.

2. The two species can be identified and separated on the basis of morphological and physiological differences.

3. *Ph. hibernalis* which was isolated once from the area (Schiffman, 1951) does not fit into either of the morphological or physiological forms of isolates in this study. It may be either absent or it was not isolated due to its limited activity except in winters of low temperatures (Sneh & Katz, 1988).

# CHAPTER THREE

## Distribution and abundance of *Phytophthora* populations in *Citrus* orchards

## 3.1. Introduction

The disease potential of a plant pathogen is commonly related to the inoculum potential, which is the the ability of the pathogen to infect its host resulting in disease which manifests itself as host damage, e.g. root rot, bark canker stem or leaf lesions.

This chapter reports a survey conducted with the aim of examining the extent and distribution of *Phytophthora* species in *Citrus* orchards on the West Bank of the Jordan River.

Populations of *Phytophthora* species are multi-state being composed of mycelia, sporangia, chlamydospores, zoospores, zoospore cysts and, in many species oospores. Overall, the biomass of the propagule community is related to host infection, with the general relationship that the higher the abundance of propagules that persist in a habitat, the more probable is host infection (Weste, 1983).

### 3.1.1. The distribution of Phytophthora

#### I. Survey of natural forests

Tree decline is one of the major problems of forestry (Zentmyer,1976). In an attempt to relate tree decline to *Phytophthora* populations, forests

from the Georgia Piedmont district in America were surveyed by Otrosina and Marx (1975). They related differences in abundance of *Ph.cinnamomi* to the host trees inhabiting the soil. Soil sampled from under short leaf pine revealed more isolates than soil sampled from under lobolly pine, as the former is more susceptible to infection by *Ph.cinnamomi* than the latter (Zak, 1961). But they could not explain clearly the low numbers of isolates recovered during the 12 months survey, relating it to the unusual meteorological conditions of a mild winter and a cooler summer during the survey period. However, they reported the absence of any relationship between disease severity and the numbers of *Ph.cinnamomi* (the feeder root) pathogen recovered from areas where trees with signs of infection compared with those from areas with no apparent signs of infection. The same species was isolated by Marks *et al* .,(1975), indicating that the causative agent of the severe die back disease in flat, poorly drained Australian coastal forests was also *Ph.cinnamomi* . Weste and Ruppine, (1975, 1977) confined their survey sites to discrete areas of diseased vegetation at different stages of the disease. They found differences in *Ph.cinnamomi* population densities between decline and non-decline areas according to the host availability. Kliejunas *et al* . ; (1977) isolated *Ph.cinnamomi* as the only *Phytophthora* species causing decline in Hawaiian *Eucalyptus* forests. Weste and Vithange (1977, 1978) stressed the effect of climatic variation on the population fluctuations of *Ph.cinnamomi* . This indicates that in spite of the difference in geographic location (Hawaii or Australia or Georgia, USA) and the difference in host plant species , they all shared the fact of being infected by *Ph.cinnamomi* . However it was noticed that the plant species did not suffer the same degree of decline, which might have resulted from differences in population densities, host resistance or *Ph.cinnamomi* virulence.

## II. Orchards

Orchard tree plantations differ from natural forests in their tendency for genetic uniformity of species, the imposition of cultivation practices, economic importance and often nutritional status. Even though an orchard may be planted with a single plant species, it may reveal more than one *Phytophthora* species. *Ph.megasperma* and *Ph.cactorum* were isolated from 10 apple orchards (*Malus pumila* Miller) during two consecutive years by Jeffers *et al* . ,(1982), even though the latter species had never been isolated from New York apple orchards before. They emphasised the sublethal invasion of apple trees as a possible cause of damage, which Mulder (1969) stated regarding the possible role of chronic latent root disease of apples caused by *Pythium* where no clear symptoms accompany the infection. This fact indicates the insufficiency of choosing only tree decline sites for surveys of orchards. Pointing out a problem of agricultural practices in California, Mircetich and Mathron (1983) related the direct contact between free water and the trunk for long periods of time as the major cause of crown rot of walnut trees. Six identified species of *Phytophthora* were isolated namely *Ph.cactorum*, *Ph.citricola*, *Ph.cinnamomi*, *Ph.citrophthora*, *Ph.megasperma* and four different unidentified *Phytophthora* species. They concluded that careful water management could reduce losses in the orchard sites infected with *Ph.citricola* but not *Ph.cinnamomi* . All species could be isolated regardless of walnut host or irrigation system, thus negating the argument about the role of moisture in spreading or delimiting the spread of the infective agent in different sites of the host range, in spite of the fact that *Phytophthora* species require water for their survival and infection process. Two years later Matheron and Mircetich (1985) isolated two

more species i.e; *Ph.drechsleri* and *Ph.parasitica* in addition to the former six, which supports the need for definite species identification as it is unlikely that the latter two species were not in the orchards during the first survey but might have been either passed over or were from the 4 unidentified species. Doster and Bostock ( 1988 ), surveying three almond orchards under different irrigation techniques attributed pruning wound cankers to *Ph.syringae* . Their finding suggests that pruning of almond trees ( and possibly other trees ) could increase the possibility of *Phytophthora* cankers.

Studies on *Citrus* orchards have been carried out in different parts of the world (Uppal & Kamat, 1936; Doepel, 1966; Ann, 1984; Timmer *et al.*, 1988). After sampling 16 citrus plantations in Eastern Corsica for three years, Vallavieille and Erselius, (1984) were unable to relate the varying results to the sampling times of the survey, concluding that the dominant factor could have been differences in climatic conditions between 1977 and the following two years. However, their survey revealed three species with different densities; *Ph.nicotianae* , *Ph.citrophthora* and *Ph.citricola* . The first two were more prominant than the last, but they only isolated *Ph.nicotianae* in their first samples and not on three successive occasions sampled over two years. Surveying *Phytophthora* in Taiwan, Ann (1984) isolated seven species ( *Ph.parasitica* , *Ph.palmivora* , *Ph.citrophthora* , *Ph.cinnamomi* , *Ph.citricola* , *Ph.hevae* and *Ph.insolita* ) from 108 orchards out of 229 orchards surveyed. The results may indicate that the other 121 orchards were free from any *Phytophthora* species but did not disclose whether the *Citrus* trees were free from any signs of disease or that *Phytophthora* species could not be isolated from the soil of the orchards only. Three mature *Citrus* orchards were chosen by Timmer *et al* . , (1988) to survey the soil for *Ph.parasitica* . The

orchards were similar in soil types, but different in moisture and drainage conditions and host range. Moisture and temperature had a variable effect on the efficiency of isolation of *Ph.parasitica* .

**III. Effect of edaphic factors**

Although fluctuations in propagule numbers do occur, there is substantial evidence that edaphic factors such as organic matter content, pH, water content, and temperature play a vital role in survival and recovery of *Phytophthora* from soil. Water content and temperature are the most important parameters in the isolation, survival and variation in the number of isolates. Schiffmann (1951) found that the relationship between rain fall and temperature is responsible for fluctuations in the number of *Ph.citrophthora* isolated from *Citrus* orchards in Israel. The survey which took place between May and November for four successive years, showed an increase in isolate recovery during summer and decrease in winter. *Ph.parasitica* was never isolated at occasions when *Ph.hibernalis* was isolated during the coolest winter months. However climatic details of the site and the geographical area of Israel where the survey was conducted were omitted, and as temperature fluctuations are inevitable among sites, the conclusion may be limited. In contrast, Perlberger (1936) isolated *Ph.citrophthora* and *Ph.nicotianae* var. *parasitica* only from infected *Citrus* seedlings in Israel, while Sneh and Katz (1988) isolated the same two species from *Citrus* roots and fruits (which were showing rot symptoms) at other sites in the same country but failed to isolate *Ph.hibernalis* , due to the comparatively warm weather prevailing in the area during the winter of that year. They stated in their report that this species is less common in Israel and can be isolated from the field

only in winters of especially low temperatures. Similarly, Hartman (1969) failed to isolate any *Phytophthora* species from lesions of *Citrus* trees showing signs of gummosis in Lebanon between May and September (dry season), but was able to isolate *Ph.citrophthora* and *Ph.parasitica* between February and April, and during October (wet weather). The explanation of this was formation of gum on the bark preventing exposure of the external part of the bark to *Phytophthora* during the dry season. Once the rain had washed the gum away, the bark was rendered more susceptible to infection, with more *Phytophthora* isolates being recoverable.

### 3.1.2. Survey and sampling methods

In any plant pathology survey, the choice of specific sites plays a vital role in determining the scope of inference concerning pathogen populations. Populations in different sites will reflect variability which is both phenotypic and genotypic. Species characteristics in the plant will determine the area over which the species is dispersed and limit the numbers of that species, its distribution and abundance. The design of a survey should reflect its objective(s) , the information required and the capacity of the survey to deliver this information. This leads to decisions on the scale and the duration of the survey so as to achieve its goal within the available timescale. Sampling methods play a crucial role in the estimation of abundance of species from a given site. The sample must adequately reflect population characteristics and may require increases in sample size to lead to improved precision in estimation.

## Soil Samples

Common survey methods for isolation of plant pathogens deal mainly with soil sampling in which volumes of soil can be collected from specific level(s) of sites and transferred to the laboratory to be diluted and cultured on selective media, but baiting techniques can also be used by placing germinated seeds, whole fruits or leaf discs on water flooded soil and assessing the infection of these baits.

### a. Depth of sampling in soil

A tendency towards decrease in population level was found by Otrosina and Marx (1975) when sampling in pine forests for *Ph.cinnamomi* at depths of 10, 30 and 45 cm. These results were not considered by Kliejunas *et al* ., (1977) when they took their soil samples from a depth of 30 cm under Hawaiian forest trees to survey for *Ph.cinnamomi* . But noting that it was not essential to sample at a particular depth, Kliejunas and Nagata (1979) surveyed Hawaiian forests to evaluate the effect of temperature and organic content on the isolation of *Ph.cinnamomi* , by collecting soil samples from a depth of 8 - 10 cm. Weste and Vithange (1977,1978) collected their samples from a depth of 6 cm and were able to detect seasonal variations in total population densities. Ann (1984) sampled *Citrus* orchards from Taiwan taking soil and roots from a depth of 0 - 15 cm when surveying for the distribution of *Phytophthora* where he was able to recover 628 isolates from 68 soil samples. Timmer *et al* . ; (1988) when collecting *Citrus* orchards soil from 0-23, 23-46, 46-69 and 69-92 cm deep isolated the least number of *Ph.parasitica* from the deepest soil and *vice versa*.

This leads to the fact that satisfactory isolation of *Phytophthora* can be acquired without any requirement to collect the samples from deep soil levels, and 0 - 10 cm deep samples would fulfill the requirements for *Citrus* orchards.

**b. Sampling positions**

Whether it is better to collect soil from under trees or from area between one tree and another is of importance in a survey, as the former might represent the rhizosphere while the latter might not. A plot centre was established by Kleijunas *et al* ., (1977) to sample Ohio forests and samples were taken at 15.3 m from the centre point of each cardinal direction from the root zone. However, the reason for sampling around this centre using this exact distance was not made clear. In a later survey Kleijunas and Nagata (1979) collected their soil samples from within 1 $m^2$ area at each of 5 sites . In order to have a satisfactory representative specimen of the sampling site in general, it seems better to collect a number of subsamples from sections within the site, bulking them together so as to make one sample (Weste and Vithange, 1977; Weste and Ruppine, 1977; Ann, 1984). Timmer *et al* ., (1988) compared samples collected from between rows of *Citrus* trees and near trunks or within the drip line of the trees. The least numbers of *Phytophthora nicotianae* were isolated from inbetween rows . This fact was explained by assuming that the *Phytophthora* propagules mainly stick to the roots where they stay as pathogens under better conditions than the soil itself (Weste & Ruppin, 1975).

c. Size of sample

The volume of soil collected at a sampling site is very important as it contributes towards the chances of the recovery of isolates. There has been a vast difference between researchers in the sample volume of soil collected.Weste and Ruppin (1975) collected 500 g as each sample, since 50g gave very variable results. On one occasion no isolates could be recovered, on another 256. However, Otrosina and Marx (1975) used only 30 g of soil, diluted with 0.3 % medium of water agar then plated on selective media to recover 2.3 - 45 isolates per 30 g of soil. This is a clear indication that the volume of soil is not necessarily associated with a success in isolating *Phytophthora* species.

d. Transfer and storage of soil samples

Transfer of soil samples to the laboratory may determine the number of isolates available for recovery in the collected soil especially in hot weather. Using an ice box has been highly recommended in order to prevent any fluctuations in the number of soil propagules , while keeping the sample for days in a refrigerator could lead to losses in the isolates (Otrosina & Marx, 1975). Timmer *et al* . , (1988) studied the effect of storage conditions on *Ph.parasitica* isolates within *Citrus* orchard soil, where the number decreased with prolonged storage. The number was 25% less than the number recovered by direct isolation of fresh samples, whether using 4°C or 21-24°C storage under moist conditions for the previously dried samples.

### 3.1.3. Isolation methods

#### a.Choice of selective media

Isolation of *Phytophthora* species from soil and infected material has been a problem due to interference from other fungi and bacteria within the soil and plant material. Addition of antibiotics to render the medium selective has been tried by many plant pathologists for the isolation of different *Phytophthora* species. There has been a requirement for the addition of antibacterial and antifungal antibiotics. Some of these antibiotics are toxic to either some or all of the *Phytophthora* species, either at high concentrations or in combination with others. Massago *et al* ., (1977) report that hymexazole reduces *Pythium* species interference with *Phytophthora* , and propose its addition as a selective agent. No significant differences were found between the use of 25 or 50 ppm for the inhibition of *Pythium* , the germination of zoospores or linear growth of several *Phytophthora* species. Papavizas *et al* .,(1981) found that the use of rose bengal in combination with hymexazole was toxic for *Ph.capsici* when isolated from soil and plant parts. Addition of 50 ppm hymexazole reduced linear growth and prevented the production of sporangia. Solel & Pinkas (1984 ) reached the same conclusion when testing different antibiotics including hymexazole at 50ppm which reduced zoospore germination of *Ph.citrophthora* by 40 %, and *Ph.nicotianae* by 30 %, while addition of 25ppm had no adverse effect.

In this study, addition of toxic antibiotics reported previously was avoided and concentrations of other antibiotics were reduced to the extent tolerated by *Phytophthora* species. A modification of P5VPPBH medium used by Papavizas *et al* ., (1981) and of VP3 medium (Ali-Shtayeh *et al*.,1986) were followed initially. Hymexazole was added to the media

at 25ppm with other antibiotics non-toxic for *Phytophthora* . Table 3.1 summarises the results of several studies on the use of antibiotics in *Phytophthora* recovery.

**b.Soil dilution**

Although soil may contain a large number of *Phytophthora* propagules, it is usually diluted before plating onto growth media. This dilution is necessary to reduce the number of *Phytophthora* propagules and to avoid overgrowth by other microorganisms within the soil. Use of water agar for dilution is more convenient than distilled water, since it prevents sedimentation of soil particles and simplifies spreading the diluted soil over the selective medium. Flowers and Hendrix (1969 ) inoculated chilled plates of selective media with 1 $cm^3$ of 1:50 soil suspension diluted with 0.5% water agar for the isolation of *Ph.nicotianae* var *parasitica* from soil. The colonies were examined microscopically to identify *Phytophthora* and counts converted to number per gram of dried soil. The high concentration of water agar used would produce a semi solid medium which might prevent the dispersion of the soil particles. Weste and Vithange (1978 ) sieved soil, mixed it with water and incubated the plates at 22°C for 6 days. The plates were washed and counts recorded. However, since the *Phytophthora* colonies were grown over 6 days, individual spore colonies may have coalesced, or been overgrown by *Pythium* species. . Ali-Shtayeh *et al* ., (1986) used 0.08% water agar to dilute soil samples for the isolation of *Pythium* from soil, incubated the soil for 36 h at 24°C and were able to get isolated colonies. This concentration of agar used for the soil dilution seems satisfactory, as using a higher concentration of agar might produce a semi solid medium.

Table 3.1. Selective media used world wide for isolation of *Phytophthora* species

| Reference | Antibiotics added | Base Medium | Species isolated & notes |
|---|---|---|---|
| Eckert & Tsao 1960, 1962 | Pimaricin 100ppm<br>Penicillin 50 ppm<br>Polymyxin50ppm | CMA | *Ph.citrophthora*, *Ph.parasitica* & *Ph.cryptogea* from plant tissue. |
| Hass 1964 | Pimaricin 2ppm<br>Penicillin 80unit/$cm^3$<br>Polymyxin 370unit/ $cm^3$<br>PCNB 10ppm | CMA | *Ph.megasperma* from soil |
| Ocana &Tsao 1966<br>Tsao & Ocana 1969 | Pimaricin 10 ppm<br>Vancomycin200ppm<br>PCNB 100ppm | CMA | *Ph.citrophthora* , *Ph.parasitica*, *Ph.cinnamomi* ,*Ph.palmivora* from soil & plant tissue |
| Tsao & Manyonga 1966 | Pimaricin 100ppm<br>Vancomycin200ppm | CMA | *Phytophthora* species |
| McCain *et al* ., 1967 | Nystatin 50 ppm<br>Vancomycin100 ppm<br>PCNB 100 ppm | V8A | *Ph.cinnamomi* |
| Flowers & Hendrex 1969 | Nystatin 100 units/ $cm^3$<br>PCNB 25 ppm<br>Penicillin 80 units/m<br>Rose bengal 0.5ppm<br>Gallic acid 425ppm | SA +<br>YE +<br>Thiamine | *Ph.parasitica* var *nicotianae* |
| Green & Pratt 1970 | Pimaricin 100 ppm<br>Penicillin 50 ppm<br>Neomycin 35 ppm | PDA | *Ph.citricola* from walnut root & bait |
| Follin 1971 | Benomyl 25 ppm<br>Penicillin 50ppm<br>Plymyxin 50ppm | LBA | *Ph.parasitica* |
| Wheeler & Boyle 1971 | Pimaricin 100ppm<br>Chloramphenicol 125ppm | WA | *Ph.parasitica* |
| Ponchet *et al* 1972 | Benomyl 15 ppm<br>PCNB 100ppm<br>Penicillin250ppm<br>Polymyxin 250ppm | | *Ph.parasitica* from carnation tissue |
| Mircetich & Matharon 1976 | Pimaricin 5ppm<br>Vancomycin300ppm<br>PCNB 100ppm | CMA | *Ph.megasperma* , *Ph.cambivora* & *Ph.drechsleri* |

| | | | | |
|---|---|---|---|---|
| Newhook & Jackson | 1977 | Pimaricin 10ppm<br>Penicillin 50ppm<br>PCNB | CMA | *Ph.palmivora*. from plant tissue |
| Massago *etal* | 1977 | Benomyl 10ppm<br>Nystatin 25ppm<br>PCNB 25ppm<br>Rifampicin 10ppm<br>Ampicillin 500ppm<br>Hymexazole 50 ppm | PDA | *Phytophthora* spp from soil |
| Papavizas *et al* | 1981 | Pimaricin 5ppm<br>Vancomycin200ppm<br>Penicillin 100ppm<br>PCNB 100ppm<br>Benomyl 2.5ppm<br>Hymexazole 20ppm | CMA | *Ph.capsici* from plant tissue & soil |
| Solel & Pinkas | 1984 | Pimaricin 10 ppm<br>Ampicillin 125ppm<br>PCNB 100ppm<br>Hymexazole 50ppm<br>Rifampicin 10ppm<br>Iprodione 400ppm | CMA | *Ph.cinnamomi* from avocado |
| Ann | 1984 | Hymexazole 25ppm<br>Bavestin 10ppm<br>Mycostatin 25ppm<br>Rifampicin 10ppm<br>Ampicillin 200ppm | V8A | *Ph.palmivora* , *Ph.citricola* , *Ph.citrophthora* , *Ph.hevae* , *Ph.cinnamomi* , *Ph.insolita* from *Citrus* soil & roots |
| Ann | 1984 | Mycostatin 50ppm<br>Ampicillin 10ppm<br>PCNB 2ppm | V8A | *Citrus* stem,leaves & fruits |
| Pittis & Colhoun | 1984 | Pimaricin 5ppm<br>Rifampicin10ppm<br>Ampicillin 500ppm<br>Penicillin 100ppm<br>PCNB 100ppm<br>Benomyl 2.5 ppm<br>Hymexazole 20ppm | CMA | *Phytophthora* spp from soil |
| Ali-Shtayeh *et al* | 1986 | Pimaricin 5ppm<br>Vancomycin75ppm<br>Penicillin 50ppm<br>PCNB 100ppm<br>Rose bengal 2.5ppm | CMA | *Pythium* from soil |

Note:

| | | | | | |
|---|---|---|---|---|---|
| CMA | : | Corn meal agar | PDA | : | Potato dextrose agar |
| V8A | : | V 8 juice agar | YE | : | Yeast extract |
| LBA | : | Lima bean agar | PCNB | | :Pentachloronitrobenzene. |
| WA | : | Water agar | SA | : | Synthetic agar |

c. Baiting Technique

Before the development of soil dilution techniques, plant material was generally used to isolate *Phytophthora* from soil. Tsao (1960) used fruits of *C.limon* as baits for the isolation of *Ph.citrophthora* and *Ph.parasitica* quantitatively from artificially infested soil samples. Other fruits have been used as baits. For example Mercetich and Matheron (1983) used ripe, green, unblemished fruits of *Pyrus communis* var *bartlett* as bait for isolation of other *Phytophthora* species.

Lupins produce radicles in 3 days, so are a very suitable source of rapidly germinating material. These were used as baits by Chee and Newhook(1965), Marks *et al* ., (1972), and Dance *et al* ., (1975). Weste (1983) compared the use of lupin radicles with germinated *Eucalyptus* seeds in surveying forest soil for *Ph.cinnamomi* and pointed out that in spite of the lower number of isolates recorded using the lupins compared with *Eucalyptus* , the former was more convenient due to rapid germination. Grimm and Alexander (1973) were the first to use young *Citrus* leaf discs to isolate *Phytophthora* species from *Citrus* soil. Ann and Ko (1980), and Ann (1984) used *Citrus* leaf discs to survey *Citrus* orchards in Taiwan for *Phytophthora* .

Considering the procedures mentioned above, it was decided that the use of *Citrus* fruits was more difficult than using leaf discs. Fruits cost more and were not available all the time . Leaf discs were easily collected from the orchards while collecting the specimen, infection with *Phytophthora* is indicated by change of green to olive-green and they belong to the same species as the *Citrus* orchard. Lupins were cheap and available at all times. Both of these baits require less space than fruits and therefore they were the baits of choice for the survey .

## 3.2. Materials and Methods

### 3.2.1.Sampling sites

Two main sites were surveyed extensively over a period of 8 months and twelve additional sites were assessed once during the survey period. Temperature, pH, organic matter content and moisture content of each soil sample were measured and the soil samples or plant parts were prepared before plating them on selective media.

*Citrus* orchards in the West Bank cover an area of 254.2 ha (Table 3.2.). Due to the low winter temperature prevalent in the southern districts of Ramallah, Bethlehem and Hebron *Citrus* is grown as an ornamental tree, producing a low grade crop unsuitable for marketing. However, the *Citrus* crop from orchards in the central and northern districts with warmer winter temperatures forms an important source of income for the owners. This survey covers orchards from districts where *Citrus* forms a major crop spreading over substantial areas of the district .

Two main *Citrus* orchards were chosen for this survey, from Tulkarem and Jericho districts , selected because of differences in their geography, topography and climatic conditions. These two orchards are referred to as Permanent sites.

Twelve more orchards , were chosen from the Tulkarem, Jericho, Jenin and Nablus districts. These orchards were called Other sites. ( Figure 3.1. shows a map of the West Bank with Permanent and Other sites).

**Permanent sites**

**Site I**

This site is located in the middle of the Jordan Valley ( Great Rift

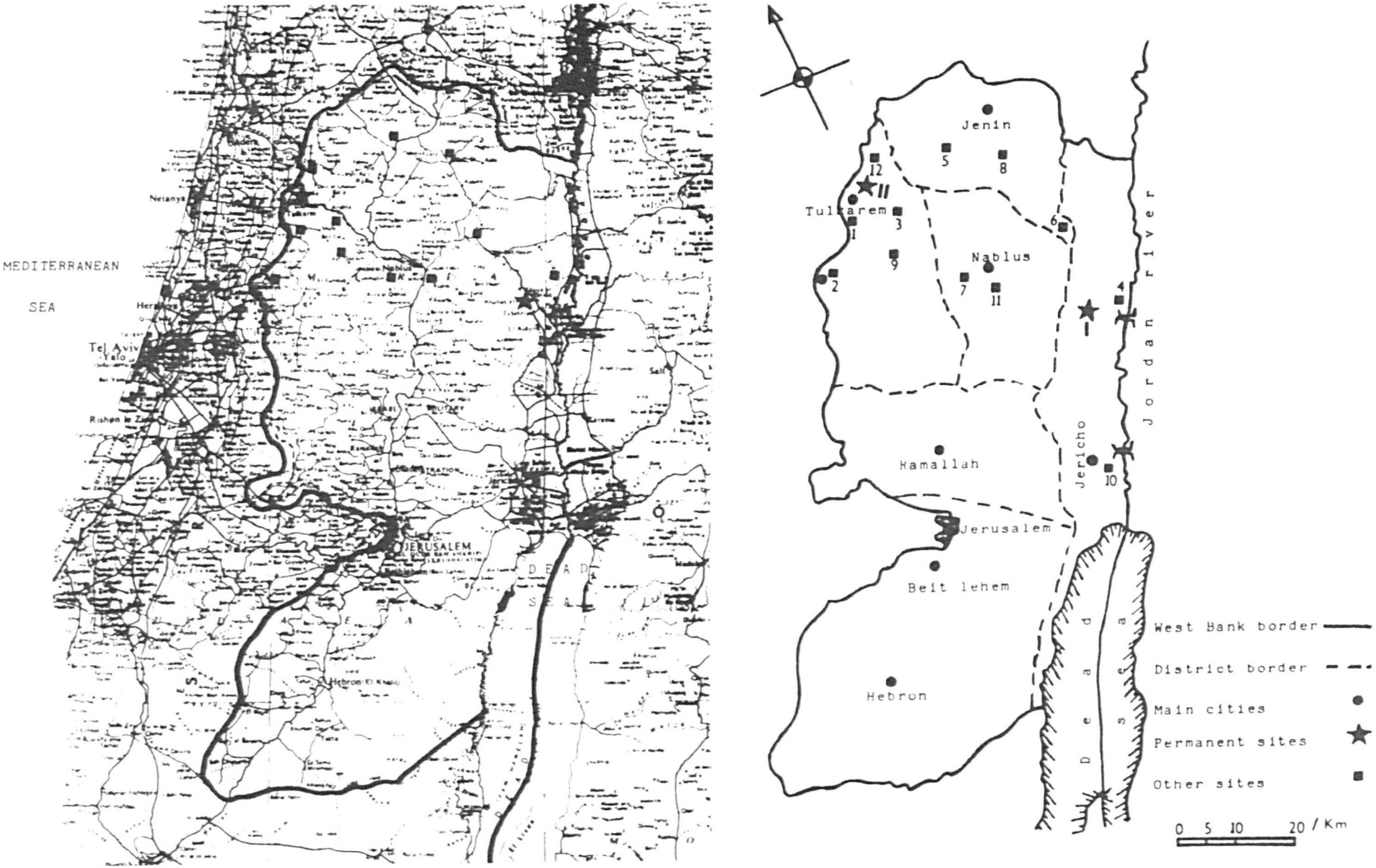



**Figure 3.1.** Map of the West Bank of the River Jordan, showing locations of Permanent Sites and the Other Sites.

**Table 3.2.** Surface area planted with *Citrus* on various districts of the West Bank. ( from Rural Studies Centre Annual report, 1987)

| District | Surface area |
|---|---|
| Tulkarem | 172.23 |
| Jericho | 37.59 |
| Jenin | 27.35 |
| Nablus | 14.25 |
| Ramallah | 1.95 |
| Hebron & Beit lehem | 1.14 |
| Total (ha) | 254.51 |

Valley extension ), 12 km to the west of the river Jordan at 380 m below sea level. It occupies an area which falls within a narrow green valley (500-1200 m wide), in between dry high mountains which rise to an altitude of 800m above sea level. The topographic conditions are the major reason for extreme temperatures in the valley with extreme evaporation potential, as the mountains from the western side form a boundary that prevents most of the annual rain from falling into this area. The annual rain falls in a short period of 40 - 60 consecutive days in the winter season (October to April), which leaves the valley under semi arid conditions for most of the year. (Karmon, 1971; Rosenan, 1976; Borsuk *et al* .,1976). These conditions lead to the need for extra irrigation (every 2 weeks instead of 3) especially during hot summer time when water is in a great demand and not always available.

The orchard was divided to 5 subsites taking into consideration the physical features of the orchard , the neighbouring landscapes and plant fields. Most of the site is planted with monthly fruiting *C.limon* , except subsite A which is planted with *C.clementina* . During visits to the orchards, it was obvious that about a third of the trees showed signs of gummosis and bark canker. Some more severely affected ones showed signs of decline and a few trees were partially dried up. Figure 3.1. shows the geographic location of the site in relation with the West Bank. Figure 3.2. shows a plan of the orchard, and Table 3.3 shows graft stocks, rootstocks, age, and fruiting season for *Citrus* trees in this site.

**Site II**

This site is 70 km to the west of Site I and lies in a green hilly area of land, with a Mediterranean climate. Rain falls over a longer period of

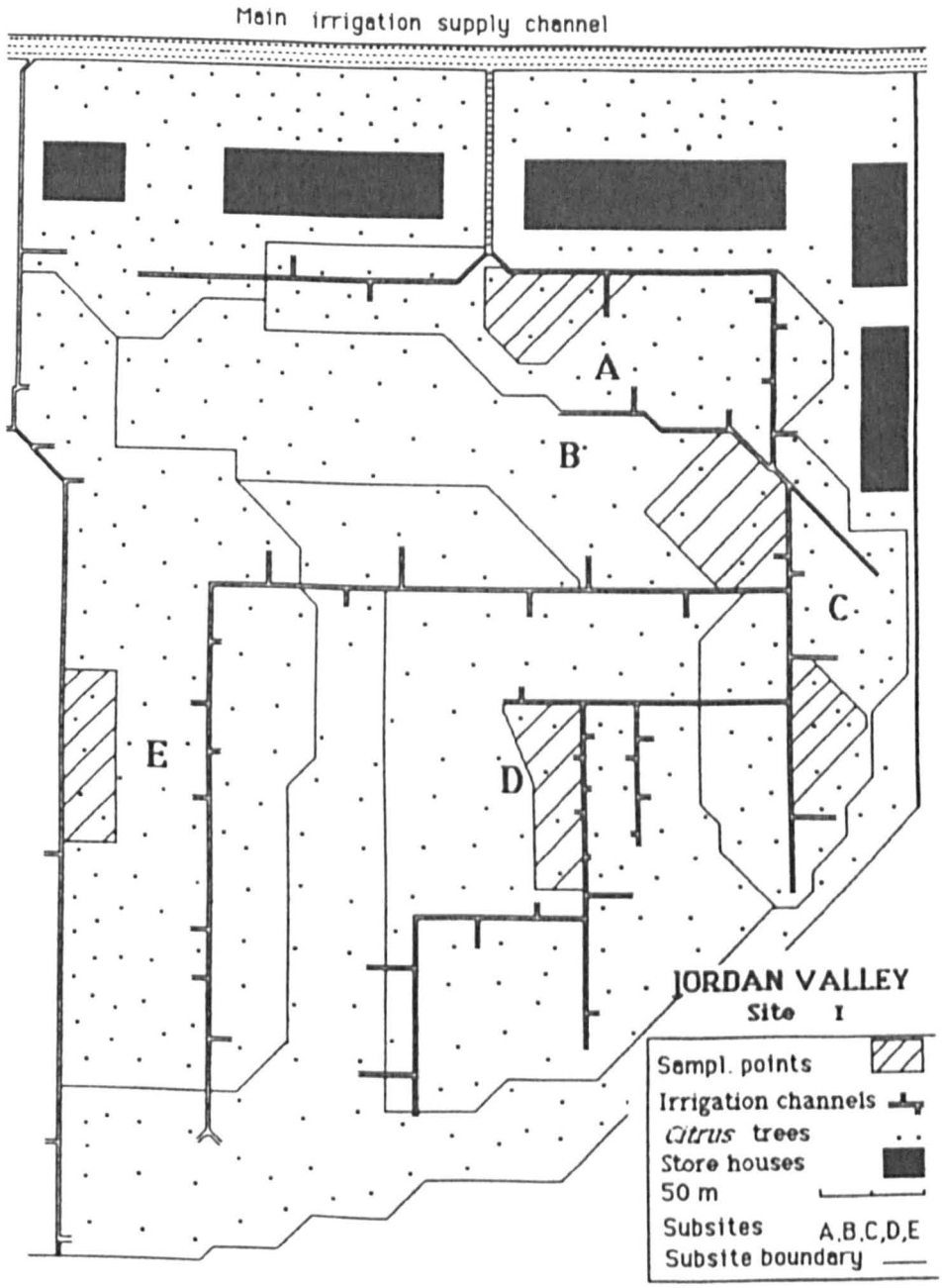



**Figure 3.2.** Plan of SiteI ( Jordan Valley)

Table 3.3 *Citrus* cultivars in Site I

| Sub site | Graft stock | Root Stock | Trees number | Fruiting time | Age (years) |
|---|---|---|---|---|---|
| A | *C.clementina* | *C.aurantium* | 42 | Dec-Jan | 20 |
| B | *C.limon* | *C.limmitioides* | 45 | All year | 30 |
| C | *C.limon* | *C.limmetioides* | 40 | All year | 30 |
| D | *C.limon* | *C.limmetioides* | 60+6* | All year | 30 |
| E | *C.limon* | *C.limmetioides* | 100 | All year | 20 |

Note:

*The 6 trees of other graft stocks in this subsite are:
2.*C.oblonga* x *C.sinensis* 2.*C.mandarine* 2.*C.sinensis*.

Table 3.4 *Citrus* cultivars in Site II

| Sub site | Graft stock | Root stock | Trees number | Fruiting time | Age (Years) |
|---|---|---|---|---|---|
| A | *C.oblonga* x *C.sinensis* | *C.aurantium* | 35 | Mar-Apr. | 30 |
| B | *C.clementina* | *C.aurantium* | 75 | Jan-Feb. | 20 |
| C | *C.oblonga* | *C.limmetioides* | 50 | Apr-May | 30 |
| D | *C.oblonga* x *C.sinensis* | *C.aurantium* | 48 | Mar-Apr. | 30 |
| E | *C.sinensis* | *C.aurantium* | 70 | Jan-Feb | 22 |

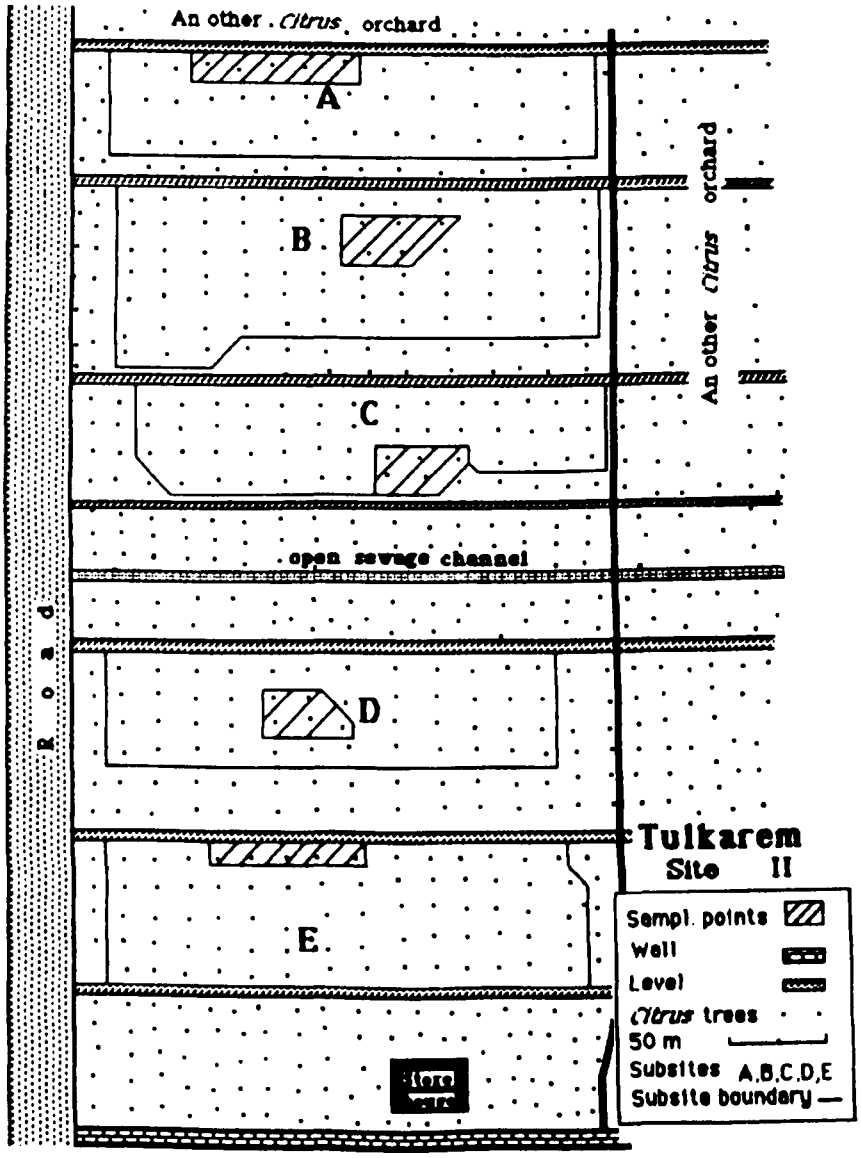



Figure 3.3. Plan of Site II ( Tulkarem)

in this area (Rosenan,1976; Borsuk *et al* .,1976), and the evaporation rate is much less than that in Site I. Despite that irrigation is required every three weeks, yet there is no need to irrigate fortnightly even during summer.

This site was surveyed using the stratified random sampling method as the orchard was not on the same level. Each level was planted with a different *Citrus* species forming a distinct part, and information about each subsite was thus required separately. A part of the orchard towards the centre was avoided in this survey due to the presence of an open shallow sewage channel passing through the site. Signs of *Phytophthora* disease were less obvious at this site. About a fifth of the trees showed gummosis and bark canker and none exhibited decline symptoms. Figure 3.1.shows the geographic location of the orchard in relation to the West Bank, Figure 3.3 shows a plan of the orchard, and Table 3.4. shows graft stocks, age, and fruiting season for *Citrus* trees in site II.

A comparison between the Permanent sites is shown in Table 3.5.

## The Other sites

The twelve Other orchards were distributed throughout the West Bank. (See Figure 3.1 for the locations of these sites). The sites were sampled once during the survey. Table 3.5. shows the names, districts and surface areas of these orchards.

**Table 3.5.** Other sites included in the survey

| Site number | District | Orchard | Surface area (ha) |
|---|---|---|---|
| 1. | Tulkarem | Abdullah Atair | 2.3 |
| 2. | " | Ahmad Sabri | 3.8 |
| 3. | " | Dair Al-Ghsoon | 2.6 |
| 4. | Jericho | Damyah Bridge | 2.7 |
| 5. | Jenin | Ersan | 2.7 |
| 6. | " | Far'ah | 3.2 |
| 7. | Nablus | Fawwar | 1.7 |
| 8. | Jenin | Habash | 1.6 |
| 9. | Tulkarem | Nathanya Street | 2.3 |
| 10. | Jericho | Na'oorah Park | 1.8 |
| 11. | Nablus | Wadat | 1.5 |
| 12. | Tulkarem | Zaita Atteel | 2.1 |
| | | Surface area of all orchards (ha) | 28.1 |

### 3.2.2.Cultivation practices

Information on cultivation practices followed in the West Bank was collected from the orchard owners and farmers . These methods had been practiced regularly for the last 30 - 50 years with slight improvements due to new fertilizers or anti infective agents.

**Site I**

Trees have been planted at about 10 m intervals and the soil is kept clear of weeds manually, with no plants growing underneath or amongst the *Citrus* trees. Any grass or plants growing under the trees, or fallen leaves (which form a layer of 5 cm or more covering the whole orchard in autumn ) are collected and buried or burnt at one corner of the orchard . A 25 - 30 cm high earth wall was erected around the base of each tree for spot irrigation. Maintainance of these depressions is carried out whenever necessary but mainly at the end of winter or autumn. The orchard was irrigated every three weeks ( about 150 litre per tree ) except during high temperature periods in summer (air temperature < 40 $^{o}$C) when the orchard is irrigated fortnightly provided enough water is available.

Water is drawn into the orchard through a cement channel (2 m wide) supplied from Far'a, Badan and Ain-Shibly water springs at 37, 30, and 15 km to the west respectively. Water enters the orchard from the north and runs through a 30 cm wide cement channel straight to the south of the orchard to irrigate the farthest trees before flowing back north until the whole orchard is irrigated.

Fertilizers are added to the depressions around the trees as 0.5 - 1 kg/tree twice a year, in spring (April) and Autumn (September). It is

usually composed of N P K 20:20:20 and Mn 1.45%, Zn 0.42%, Fe 1.0% and Cu 0.11%. Trees are pruned after harvest in autumn ( December ) for *C.clementine* or continuously for the monthly *C.limon.* All dry or broken branches are removed as well as the new small branches from the centre of the tree. If the tree is drying up due to bark canker ( sometimes split into halves ) then the whole trunk is cut off and some 4 - 6 new young branches inserted between the bark of the stock and the wood of the tree. If the bark canker has reached the stock, then the whole tree is uprooted and replaced by a new one.

The harvest was collected using shears or clippers to cut the stalk of the fruit. The crop is either collected in plastic buckets (e.g. *C.clementine* ) or (sometimes) thrown directly on to the soil below the tree ( e.g. *C.limon* ), then eventually sorted out and packed in wooden boxes for marketing.

## Site II

All cultivation practices mentioned above apply to this site too, except for the irrigation system. The orchard is irrigated from one artesian well 100 m to the north located within another citrus orchard. Irrigation water passes along soil channels within the orchards, which narrowed gradually to irrigate each tree alone . Site E is irrigated from the same artesian well through a different route from a third orchard to the north. A comparison between the Permanent sites is shown in Table 3.6.

Table 3.6. A comparison between the Permanent sites

| Site | I | II |
|---|---|---|
| District | Jericho | Tulkarem |
| Geographical location | Jordan Valley | Mediterranean plain |
| Surface area ( ha ) | 3.62 | 2.75 |
| Orchard age ( yr ) | 30 | 50 |
| Height from sea level (m) | -380 | +100 |
| Annual Rainfall ( mm ) | 200-300 | 500-600 |
| Environment | semi arid | Mediterranean |
| Soil pH | 7.3 - 7.8 | 7.3 - 7.8 |
| Soil organic matter | 1.6-2.7 | 1.8-3.2 |
| Surrounding area: | | |
| East | dry area | 20mroad+*Citrus* orchard |
| West | 10 yr old *Citrus* orchard | *Citrus* orchard |
| South | 4 ha planted with tomato | 10mroad + cucumber under polythene tunnels |
| North | dry area | *Citrus* orchard |

### 3.2.3. Sampling methods

#### a. Soil

Soil samples were collected from a depth of 0 - 10 cm using a trowel, after sweeping off any fallen or decaying leaves .

Each sample consisted of 4 - 5 aliquots, each of 200 - 250 g from an area of 4 $m^2$ under the same 4 - 5 *Citrus* trees at each sampling occasion. The aliquots were placed in a plastic bag and mixed thoroughly. The bags were labelled and taken to the laboratory in an ice box for processing on the same day. However, if the conditions did not allow immediate processing, samples were kept in closed bags at 4 - 8 °C until the next day.

#### b. Plant parts

As *Citrus* roots grow near the surface they were collected along with the soil using a sterile scalpel if necessary. Other plant parts were collected using a scalpel to cut off small pieces from the large portions of cracked bark. Trunks with signs of gummosis, brown rotten fruits and water soaked leaves were also collected. Each group of plant parts was kept in a separate plastic bag, and dealt with individually.

### 3.2.4.Selective media

VP3HB medium was prepared by dissolving the agar, corn meal agar, and the sucrose in 1L of water first, then adding trace elements which were prepared as stock solutions. After autoclaving for 15 min at 121 °C and 15 lb pressure, the medium was cooled down to 50 °C before addition

Table 3.7. VP3HB selective medium,

(A modification of VP3 , Ali-Shtayeh *et al* . , 1986)

| Material | Weight per litre |
|---|---|
| Agar | 20 g |
| Corn meal agar (Difco) | 17 g |
| Sucrose | 20 g |
| Thiamine HCl | 100 µg |
| Trace elements: | |
| $CaCl_2$ | 10 mg |
| $MgSO_4.7H_2O$ | 10 mg |
| $ZnCl_2$ | 1 mg |
| $CuSO_4.5H_2O$ | 20 µg |
| $FeSO_4.7H_2O$ | 20 µg |
| $MnCl_2$ | 20 µg |
| $MoO_3$ | 20 µg |
| Antibiotics: | |
| Ampicillin | 500 mg |
| Benlate | 2.5 mg |
| Hymexazole | 25 mg |
| Penicillin G | 50 mg |
| PCNB | 100 mg |
| Pimaricin | 5.0 mg |
| Rifampicin | 10 mg |

Table 3.8. P5VPPBH selective medium

(A modification of Papavizas *et al* . ,1981.)

| Material | Weight per litre |
|---|---|
| Corn meal agar | 17 g |
| Ampicillin | 500 mg |
| Benlate | 2.5 mg |
| Hymexazole | 25 mg |
| Penicillin G | 100 mg |
| PCNB | 100 mg |
| Pimaricin | 5.0 mg |
| Rifampicin | 10 mg |

of the antibiotics which were dissolved in 70% ethanol, except PCNB which was dissolved in acetone, then the filtered thiamine was finally added.

Corn decoction was sometimes used as an alternative to corn meal agar. This was prepared by boiling sixty grams of corn meal in one litre of water, then the cooled paste was filtered through cheese cloth to produce a clear liquid. Failure to do so resulted in great difficulty in observing *Phytophthora* colonies through the turbid agar. The volume was completed to one litre with water ,35g of agar added and the above mentioned procedure was followed as shown in Table 3.7.

P5VPPBH was prepared according to Papavizas *et al* .,( 1981) by dissolving 17g CMA ( or the corn decoction with 15g of agar ) in a litre of water, autoclaving then adding the antibiotics in the same concentrations mentioned for VP3HB as shown in Table 3.8.

To assess the differences between the two methods of isolation, 20 replicate plates of each medium were tested for 9 soil samples collected at different occasions from 3rd June to 31st July, including both sites. The samples were dealt with as mentioned in section 3.2.5.1. for soil dilution technique.

The results were tabulated and a chi - square test was performed to detect significant differences between the two media.

### 3.2.5. Isolation methods

#### 3.2.5.1. Soil dilution technique

A soil suspension was made from each soil sample by taking 50 g of wet

soil, and aseptically diluting it to 250 $cm^3$ with 0.08 % water agar. The suspension was stirred using a sterile glass rod, then poured into a sterile 500 $cm^3$ erlenmeyer flask. The aluminium foil stoppered flask was placed in the handle of a wrist shaker ( Burrell model 75), and shaken vigorously for 20 min. The flask was then released from the shaker, hand shaken vigorously once and 10 $cm^3$ of the soil suspension were transferred aseptically to a sterile measuring cylinder and made up to 100 $cm^3$ with 0.08 % agar. The suspension was then stirred using a sterile glass rod, 10 $cm^3$ removed using a sterile pipette and 1 $cm^3$ spread evenly over the VP3BH selective medium surface using a sterile bent glass rod. Twenty replicate plates were used.

Thirty six hours after incubating the plates at 25°C, the soil layer was removed from the agar surface using a gentle stream of running tap water with the help of a wet piece of cotton wool. Care was taken to avoid scratching the agar surface. Visual examination with a slide viewer was made. Colonies of *Phytophthora* or any mycelia producing fungi were marked on the back of the plate with a needle. Then microscopic examination was carried out. If the plates were allowed to grow for longer periods at this temperature, it was very difficult to differentiate the growing colonies which overlap to form a single large colony. All or part of the marked colony within a block of agar was transferred to a second sterile Petri dish using a sterile scalpel and covered with a 1:1 mixture of sterile pond and distilled water. One sterile maize kernel was added, and the plates were then incubated at 25°C under a 40 W fluorescent lamp, to enhance the production of the specific *Phytophthora* papillated sporangia in which the zoospores were differentiated. Forty eight to seventy two hours later, the plates were examined microscopically for the production of *Phytophthora* sporangia, which were characteristic of *Ph.citrophthora*

or *Ph.nicotianae* as non- papillated sporangia were not detected in any of the isolates.

Fungal mycelia which failed to produce sporangia were allowed more incubation time, but eventually discarded after two months of incubation. The isolates were freed from any fungal or bacterial contaminants by plating the contaminated colonies on selective media for a second time. If any bacterial colonies were left, the pancake method (Sleeth, 1945) was followed by inverting a part of the contaminated colony on the selective medium. The extended growth of *Phytophthora* penetrating the medium was scraped off the surface using a scalpel and subcultured in a pure form on PDA and CA where the isolates were separated into two distinct groups depending on the colonial morphology. The isolates were then transferred to 100 $cm^3$ flasks containing 50 $cm^3$ of sterile distilled water and a maize kernel, then stored at room temperature.

Later on they were transferred to slopes of PDA or CMA and sent by air to Liverpool University. These isolates were eventually subcultured on PDA to confirm their survival and freedom from further contamination, then transferred to OMA slopes, allowed to grow into a mycelial mat for two months and stored under a layer of sterile mineral oil.

### 3.2.5.2. Soil baiting techniques

#### a. *Citrus* leaves as baits

Young *Citrus* leaves regardless of the cultivar were cut into small squares (0.5 $cm^2$), surface sterilised using 2% sodium hypochlorite solution for 30 seconds, washed thrice with sterile distilled water and scattered to float on the water surface as 15 per 500 $cm^3$ beaker containing soil up to the 200 $cm^3$ mark. Sterile distilled water was added up to the

300 $cm^3$ mark, and the beaker was then covered with aluminium foil, pierced to allow ventilation.

After 4 days incubation at room temperature (20 - 25 °C) leaves were inspected visually for any signs of infection, and those which changed in colour from green to olive-green with a sticky appearance, were examined microscopically for *Phytophthora* sporangia. However, when the room temperature fell below 15°C signs of infection were few or non existant. The beakers were thus incubated at 25°C in an incubator for the same period of time. If any were detected, the leaf squares were surface sterilised then transferred to the VP3BH medium, placing 3 infected squares on each plate. The plates were incubated at 25°C for 4 days and dealt with as described for soil dilution.

**b. Lupin baiting**

Lupin ( *Lupinus angustifolius* L.) seeds were surface sterilised as for *Citrus* leaves and incubated at 25°C inside a flask covered with sterile cotton gauze topped with a Whatman number 3 filter paper . Daily inspection of seeds was made to remove any rotted ones ( dark in colour and sticky ). Four to five days later, intact radicles were surface sterilised and used as baits. The intact radicles ( about 2 cm long) were mounted in holes in surface sterilised expanded polystyrene discs. The discs were layered on top of 500 $cm^3$ sterile beakers containing soil and flooded with sterile distilled water as described for *Citrus* leaf discs. Five radicles were mounted inside each beaker, covered with pierced foil.

After 4 days incubation at room temperature (20-25°C), the lupin radicles were visually inspected for any infection reflected as a browning rot at the junction between the radicle and water surface. The diseased ones were examined microscopically for the presence of

*Phytophthora* sporangia. The infected areas with sporangia were cut off, surface sterilised, embedded in the selective medium and followed up as described for soil dilution .

### 3.2.6. Plant parts plating method

Plant parts collected in separate plastic bags were cut into small pieces 1 $cm^2$ (or 1 cm long for the roots which showed any rotting), and washed with running tap water to remove any adhering soil or dust. After surface sterilisation with 2% sodium hypochlorite solution for 30 seconds, the parts were washed thrice with sterile distilled water, blot dried using a sterile filter paper. Three plant parts were embedded in each plate of the selective media. Ten replicate plates were prepared from each sample. Growing hyphae from the sides of the plant parts were marked. Growth of one colony or more out of the plant part was considered as a single isolate, and was dealt with as described previously.

### 3.2.7. Edaphic factors

Soil dry weight (Weber, 1977), pH (Schofield & Taylor, 1955 ) and organic content (Weber, 1977) measurements were taken for each soil sample following the procedures in literature as shown below.

#### a. Estimation of soil dry weight ( after Weber, 1977)

Approximately 50 g of moist soil was placed in a preweighed 100 $cm^3$ porcelain crucible. Its exact weight was recorded, then the crucible with sample was placed into a hot-air oven and dried at 105 $^oC$ for 24 hrs. The soil sample was removed from the oven, and cooled in a desiccator

over $CaCl_2$ . The sample was weighed again and the dry weight was recorded. The water content was calculated as follows:

$$\% \text{ Water} = \frac{\text{Weight of wet soil - Weight of dry soil}}{\text{Weight of dry soil}} \times 100$$

**b. Soil pH** (after Schofield & Taylor, 1955 )

**Reagents**

1. Stock $CaCl_2$ solution, 3.6 M.
2. Working $CaCl_2$ solution, 0.1 M. Prepared by diluting 5 $cm^3$ of the stock solution to 1.8 litre with water. The pH of this solution must be between 5 and 6.5.

**Procedure**

Twenty $cm^3$ of 0.1 M $CaCl_2$ solution was added to 10 g of soil in a 50 $cm^3$ beaker and stirred several times during the next 30 min. The suspension was allowed to stand for a further 30 min, then the pH was measured by immersing a glass electrode into the partly settled suspension.

**c. Soil organic matter content** (after Weber, 1977)

**Reagents**

1. $K_2Cr_2O_7$ 1M
2. $H_2SO_4$ 18M

**Procedure**

Soil samples were dried at 105 $^{o}C$ overnight, then ground finely in a disc mill. Soil samples ( 0.2- 2.0 g, depending on the carbon content ) were weighed into a 200 $cm^3$ erlenmeyer flask. Ten $cm^3$ of the 1M $K_2Cr_2O_7$ solution was poured into the flask, followed by 20 $cm^3$ of 18M $H_2SO_4$, added quickly from a measuring cylinder. The flask was swirled vigorously for one min in a fume hood, placed on an asbestos sheet and left to stand for one hr to allow a complete oxidation of the organic matter. One hundred $cm^3$ of water was then added, mixed and allowed to stand overnight. About 3 $cm^3$ of the supernatent liquid was drawn out and the optical density was measured at 625 nm using a digital Bausch and Lomb Spectronic-21 spectrophotometer. A standard curve was prepared using a sucrose standard made by dissolving 11.876 g sucrose in water and made up to 100 ml with water ( equivalent to 50 mg carbon $cm^{-3}$ ). The solution was diluted with water to produce standards from 2.5 - 15 mg carbon $cm^{-3}$. A blank reference was prepared by adding 10 $cm^3$of $K_2Cr_2O_7$ solution to 20 $cm^3$ of $H_2SO_4$.

## 3.3. Results

### 3.3.1. Choice of selective medium

Use of selective media plays an important role in the recovery of *Phytophthora* species from soil and plant parts. Two media were used initially; P5VPPBH (Table 3.8) a modification of Papavizas *et al* . , (1981),

and VP3HB(Table 3.7) a modification of VP3, Ali-Shtayeh *et al* ., (1986). Finally only VP3HB was used for the isolation of *Phytophthora* species from soil and plant parts. Both media contain corn meal agar ( CMA ), but the latter contains 20 g extra of agar, 20 g of sucrose and trace elements. Hymexazole and benlate were added while rose bengal was removed from the recipe as it is reported to be toxic to *Phytophthora* when used in combination with benlate or hymexazole (Papavizas *et al* .,1981). Vancomycin was substituted with rifampicin and ampicillin (Pettis and Colhoun, 1984).

The results ( Table 3.9 ) obtained indicated that both media were able to support selectively the isolation of a similar number of isolates at separate sampling occasions . However, VP3BH was favoured over P5VPPBH due to the more solid surface which made it easier to handle. P5VPPBH tended to split in use, making soil removal by washing difficult. This rendered the *Phytophthora* colonies difficult to count. Also the appearence of colonies on the former was more clear cut. The results indicated significant differences in responses between the two methods ($\chi^2$ =12.24 , $P < 0.05$), but there were slight differences between the isolates recovered at each of the sampling times.

### 3.3.2.Isolation techniques

From the data in Table 3.10. it can be seen that soil dilution and *Citrus* bait techniques yielded the largest number of isolates. However, the soil dilution technique is more suitable for quantitative measures than the *Citrus* bait and other techniques used. Because the weight of soil is known, it is possible to determine the colony forming units per gram of soil and relate it to the moisture content or any other edaphic factor, or

**Table 3.9.** The number of *Phytophthora* isolates recovered by soil dilution method using two selective media.

| Sampling date | Sampling site | P5VPPBH | VP3HB |
|---|---|---|---|
| 03.6.87 | I | 9 | 19 |
| 13.6.87 | I | 20 | 20 |
| 21.6.87 | II | 0 | 3 |
| 29.6.87 | I | 12 | 12 |
| 06.7.87 | II | 12 | 18 |
| 12.7.87 | I | 49 | 46 |
| 21.7.87 | II | 50 | 65 |
| 26.7.87 | I | 0 | 3 |
| 31.7.87 | II | 20 | 38 |
| Total | (9 samples) | 172 | 224 |

infection of the trees in the orchard. While with the baiting technique, the zoospores infect the bait after being released from the sporangia and swimming towards the bait. The baiting technique is also affected by the incubation temperature of the container where the soil is being baited. When the room temperature fell to 15°C the *Citrus* baits did not show any manifestations of infection, as no change in colour took place and no sporangia were detectable on microscopic inspection . However, when the temperature of incubation for baited soil was increased to 24 ± 2°C during the cooler months of the survey period (from September, 1987) it was possible to recover some isolates, after positive observation of the sporangia on the olive green *Citrus* baits.

The low number of isolates obtained using the intact lupin radicles indicates that the isolated species do not infect lupin radicles as readily as *Citrus* baits. This can be easily explained as the isolated species are well known as *Citrus* pathogens and more numbers would be isolated from lupin radicles if the soil was planted with of a different plant species.

Direct isolation from plant parts other than the roots was unsuccessful, and even the number of isolates from roots was low in comparison with soil.

The results in Table 3.11. suggest that the soil dilution technique was more efficient than *Citrus* leaf discs in recovering *Ph.nicotianae*. However, both seem equal in recovering both *Ph.citrophthora* and the unidentified species of *Phytophthora* .

As the numbers obtained by using lupin baits and the direct isolates from roots were low, a statistical analysis was carried out to compare the efficiency of only the soil dilution and the *Citrus* bait techniques in recovering *Phytophthora* from the soil. Chi square testing of the pooled number of isolates from both orchards indicated a significant difference between the two methods ( $\chi^2 = 12.77$, $p < .002$ ), confirming initial impressions.

**Table 3.10.** Isolates recovery using the four isolation techniques

| Method | Total isolates recovered |
|---|---|
| Soil dilution | 377 |
| *Citrus* leaf bait | 359 |
| Lupin radicle bait | 15 |
| Direct isolation from roots | 40 |
| Total number of isolates | 791 |

**Table 3.11.** A comparison between soil dilution and *Citrus* leaf bait techniques in regard to the isolated species.

| Isolated species | Soil dilution | *Citrus* leaf bait |
|---|---|---|
| *Ph.citrophthora* | 160 | 171 |
| *Ph.nicotianae* | 38 | 13 |
| *Phytophthora* (unidentified) | 179 | 175 |
| Total | 377 | 359 |

### 3.3.3. Sampling sites

A summary of information about the two sites is shown in Table3.6, which shows the district, geographical loci, surface areas, age of orchards, height from sea level, annual rainfall, general environmental condition, soil pH, soil organic matter content and areas surrounding orchards from the four cardinal directions.

### 3.3.4.Recovery of *Phytophthora* species

#### Site I (Jordan Valley)

A total of 335 isolates were recovered from the site using four isolation methods. Of these isolates 180 ( 54% ) were *Ph.citrophthora* , 20 ( 6% ) *Ph.nicotianae* and 135 ( 40% ) remained unidentified.

The distribution of *Phytophthora* isolates, both across subsites, and in relation to isolation methods was analysed statistically by chi-square and contingency analysis. In addition, isolation methods were compared to investigate differential recovery within subsites. It was already known (Table 3.11) that *Ph.nicotianae* was not recovered as successfully using *Citrus* baits as by using the soil dilution method.

To examine the relationship between isolation method and subsite, the results for soil dilution and *Citrus* bait method were analysed using logarithmic linear chi-square. Only these two isolation methods were examined because the number of isolates recovered using the other two methods were very low. Only 15 isolates were recovered during the whole survey period using lupin bait method and 40 using direct isolation from roots, compared to 377 and 359 isolates using soil dilution and *Citrus* bait methods respectively.

Testing the total isolates of all species recovered from subsites A, B, C, D, and E in Site I (Table 3.12), revealed that there was a significant difference in the numbers of isolates among the subsites ( $\chi^2 = 37.12$, $P < 0.001$). Analysis for the ability of the *Citrus* bait and soil dilution to recover *Phytophthora* from each of the subsites (Table 3.13) again indicated a significant difference among them ($\chi^2 = 15.44$, $P < 0.05$) as shown in Table 3.14.

Comparison of the three groups of *Phytophthora* species isolated using both isolation methods (Table 3.12 Totals) indicated no significant difference between the subsites ($\chi^2 = 13.8$ , $P < 0.1$) in the distribution of the three groups. However, when the numbers recovered in each group using only the soil dilution technique was compared, there was a significant difference between the subsites ($\chi^2 = 20.06$, $p < 0.01$) . The number of isolates was between 14 isolates from subsite D, to 62 from subsite A.

**Site II ( Tulkarem )**

A total of 457 isolates were recovered from this site. Of these isolates, 179 (39%) were *Ph.citrophthora* , 42(9%) were *Ph.nicotianae* , and 236 (52%) remained unidentified *Phytophthora* species.

Logarithmic linear chi-square analysis of the total number of isolates recovered from subsites A, B, C, D, and E of this site(Table 3.15), indicated no significant differences in the numbers of isolates between the subsites ($\chi^2 = 8.6$, $P < 0.15$), when *Citrus* bait and soil dilution techniques were pooled together.

Testing for variation in total isolate numbers recovered from each subsite using *Citrus* bait and soil dilution techniques (Table 3.16), there was a significant difference among the subsites ($\chi^2 = 15.48$,

Table 3.12. Distribution of *Phytophthora* among subsites of Site I using soil dilution and *Citrus* bait techniques only.

| Subsite | *Ph.citrophthora* | *Ph.nicotianae* | *Ph.*unidentified | Total |
|---|---|---|---|---|
| A | 44 | 9 | 44 | 97 |
| B | 23 | 1 | 19 | 43 |
| C | 44 | 2 | 27 | 73 |
| D | 25 | 7 | 22 | 64 |
| E | 16 | 0 | 18 | 34 |
| Total | 162 | 19 | 130 | 311 |

Table 3.13. Recovery of *Phytophthora* isolates from the five subsites of Site I using two recovery methods.

| Subsite | *Citrus* bait | Soil dilution | Total |
|---|---|---|---|
| A | 35 | 62 | 97 |
| B | 17 | 26 | 43 |
| C | 16 | 57 | 73 |
| D | 50 | 14 | 64 |
| E | 8 | 26 | 34 |
| Total | 126 | 185 | 311 |

**Table 3.14.** Analysis of variance table for logarithmic linear chi-square of two methods of isolation ( *Citrus* bait and soil dilution methods) interaction with the five subsites in Site I.

| Source | DF | Chi-square | Probability |
|---|---|---|---|
| Intercept | 2 | 41.58 | 0.0001 |
| Subsites | 8 | 11.06 | 0.1986 |
| Method | 2 | 0.37 | 0.8320 |
| Subsite X method | 8 | 15.44 | 0.0511 |

P < 0.051) in the recovery of *Phytophthora* using either methods as shown in Table 3.17.

When the three groups of *Phytophthora* species were compared(Table 3.15), using the two isolation methods of recovery from the five subsites, there was no significant difference ($\chi^2$ = 4.053 , P= 0.90) in the efficiency of the method used to recover groups from the subsites . However, testing differences in the three groups of the *Phytophthora* species between the subsites using only the soil dilution technique, contingency chi-square analysis suggested a statistically significant difference ($\chi^2$ = 15.37, P = 0.052).

## The Other Sites

These 12 orchards were sampled once only to cover the distribution of *Phytophthora* species and find out if any species other than the two isolated from the Permanent sites were present throughout the West Bank.

The total of 45 isolates were recovered from 9 of the 12 sites, of which 33 (73%) were *Ph.citrophthora* and two *Ph.nicotianae* while 10 (22%) were not identified up to species level. No *Phytophthora* isolates could be recovered from the other three orchards (Dair al-Ghsoon, Wadat and Zaita Atteel orchards).

Applying soil dilution technique it was possible to recover 24 isolates, while *Citrus* bait recovered 21 and none were recovered applying lupin bait technique. However, two isolates were recovered from roots. These results are summarised in Table 3.18.

**Table 3.15.** Recovery of *Phytophthora* isolates from the five subsites of Site II, using two recovery methods.

| Subsite | *Citrus* bait | Soil dilution | Total |
|---|---|---|---|
| A | 12 | 41 | 53 |
| B | 35 | 49 | 84 |
| C | 49 | 55 | 104 |
| D | 52 | 61 | 113 |
| E | 35 | 36 | 71 |
| Total | 183 | 242 | 425 |

**Table 3.16.** Distribution of *Phytophthora* among subsites of Site II using soil dilution and *Citrus* bait techniques only.

| Subsite | *Ph.citrophthora* | *Ph.nicotianae* | *Ph.* unidentified | Total |
|---|---|---|---|---|
| A | 33 | 10 | 47 | 90 |
| B | 32 | 5 | 36 | 73 |
| C | 40 | 8 | 53 | 101 |
| D | 30 | 3 | 38 | 71 |
| E | 34 | 6 | 50 | 90 |
| Total | 169 | 32 | 224 | 425 |

**Table 3.17.** Analysis of variance table for logarithmic linear chi-square of two methods of isolation ( *Citrus* bait and soil dilution methods) interaction with the five subsites in Site II.

| Source | DF | Chi-square | Probability |
|---|---|---|---|
| Intercept | 2 | 61.47 | 0.0001 |
| Subsite | 8 | 19.47 | 0.0125 |
| Method | 2 | 3.02 | 0.2208 |
| Subsite X method | 8 | 15.48 | 0.0506 |

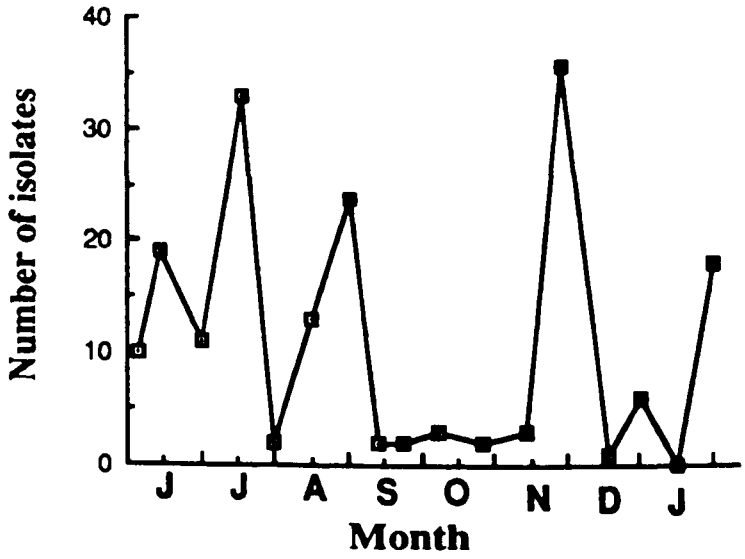



Figure 3.4. Total number of Isolates from site I recovered using soil dilution technique.

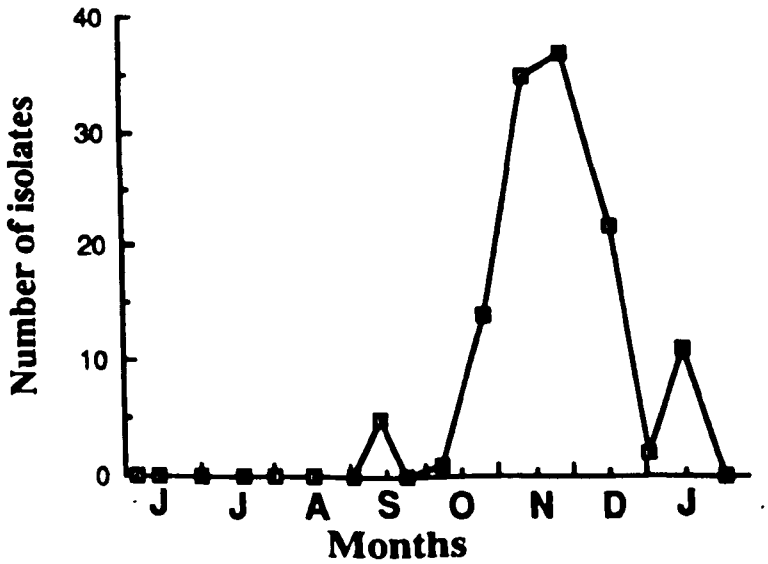



Figure 3.5. Total number of Isolates from site I recovered using *Citrus* baiting technique.

### 3.3.5.Seasonal variation

#### Site I

Table 3.19 shows that the number of isolates recovered from this site varied from 3 (29th July) or 4 (4th October) to 70 on 21st November.

Total recoveries were usually between 10 and 40 isolates. Few (<3) isolates were recovered by soil dilution on 8 of the 17 sampling occasions ( Figure 3.4). While none were recovered on 7th January, the highest numbers were isolated on 16th July (33 isolates), 29th August (24 isolates) and 21st November when the maximum number of isolates (36) were recovered.

*Citrus* baiting produced few isolates until the 18th of October. The highest numbers of isolates were recovered during November when 35 and 37 isolates were recovered in two successive samples. Numbers then declined to two isolates at the end of December, but later, on the 7th of January 1988, 11 isolates were recovered. However, no isolates were recovered on the 22nd of the same month( Figure 3.5).

No relation was evident between moisture content and the number of propagules at various sampling times, (Figure 3.6) or between the temperature and the isolates (Figure 3.7).

#### Site II

Table 3.20 summarises isolate recovery from this site. Numbers varied from one only in the first sample ( 1st June, 1987) to 85 on 11th December. Recoveries were highest in June and then in November/ December, with distinctly low numbers between. This declined to 5 during August, remaining constant through September and most of October.

Table 3.18. *Phytophthora* isolates of the three groups from the Other Sites using soil dilution and *Citrus* bait methods

| Site No. | Orchard | S.D. | C.B. | Total | P.c | P.n. | P.ud |
|---|---|---|---|---|---|---|---|
| 1 | Abdullah Atair | 5 | 7 | 12 | 10 | 0 | 2 |
| 2 | Ahmad Sabri | 0 | 6 | 6 | 6 | 0 | 0 |
| 3 | Dair Al-Ghsoon | 0 | 0 | 0 | 0 | 0 | 0 |
| 4 | Damyeh Bridge | 0 | 6 | 6 | 3 | 0 | 3 |
| 5 | Ersan | 3 | 0 | 3 | 3 | 0 | 0 |
| 6 | Far'ah | 0 | 3 | 3 | 3 | 0 | 0 |
| 7 | Fawwar | 4 | 0 | 4 | 2 | 0 | 2 |
| 8 | Habash | 3 | 0 | 3 | 3 | 0 | 0 |
| 9 | Nathania Street | 0 | 2 | 2 | 0 | 2 | 0 |
| 10 | Na'oorah Park | 6 | 0 | 6 | 3 | 0 | 3 |
| 11 | Wadat | 0 | 0 | 0 | 0 | 0 | 0 |
| 12 | Zaita Atteel | 0 | 0 | 0 | 0 | 0 | 0 |
| | Total | 21 | 24 | 45 | 33 | 2 | 10 |

Note: C.B. = *Citrus* bait

S. D. = Soil dilution

P.c. = *Phytophthora citrophthora*

P.n. = *Phytophthora nicotianae*

P.ud= Unidentified *Phytophthora* species.

Table 3.19. *Phytophthora* species recovered from Site I ( Jordan Valley orchard) at each sampling date using all isolation techniques.

| Date | *Ph.citrophthora* | *Ph.nicotianae* | *Ph.* unidentified | Total |
|---|---|---|---|---|
| 4.6.1987 | 5 | 0 | 4 | 9 |
| 13.6 | 11 | 0 | 8 | 19 |
| 29.6 | 6 | 1 | 4 | 11 |
| 16.7 | 26 | 1 | 10 | 37 |
| 29.7 | 1 | 0 | 2 | 3 |
| 13.8 | 6 | 0 | 7 | 13 |
| 29.8 | 14 | 11 | 4 | 29 |
| 10.9 | 4 | 1 | 6 | 11 |
| 20.9 | 4 | 0 | 2 | 6 |
| 04.10 | 4 | 0 | 0 | 4 |
| 18.10 | 14 | 3 | 8 | 25 |
| 05.11 | 14 | 1 | 23 | 38 |
| 21.11 | 42 | 2 | 26 | 70 |
| 09.12 | 13 | 1 | 9 | 23 |
| 23.12 | 7 | 0 | 1 | 8 |
| 07.1.88 | 9 | 0 | 2 | 11 |
| 22.1 | 0 | 0 | 18 | 18 |
| Total | 180 | 21 | 134 | 335 |

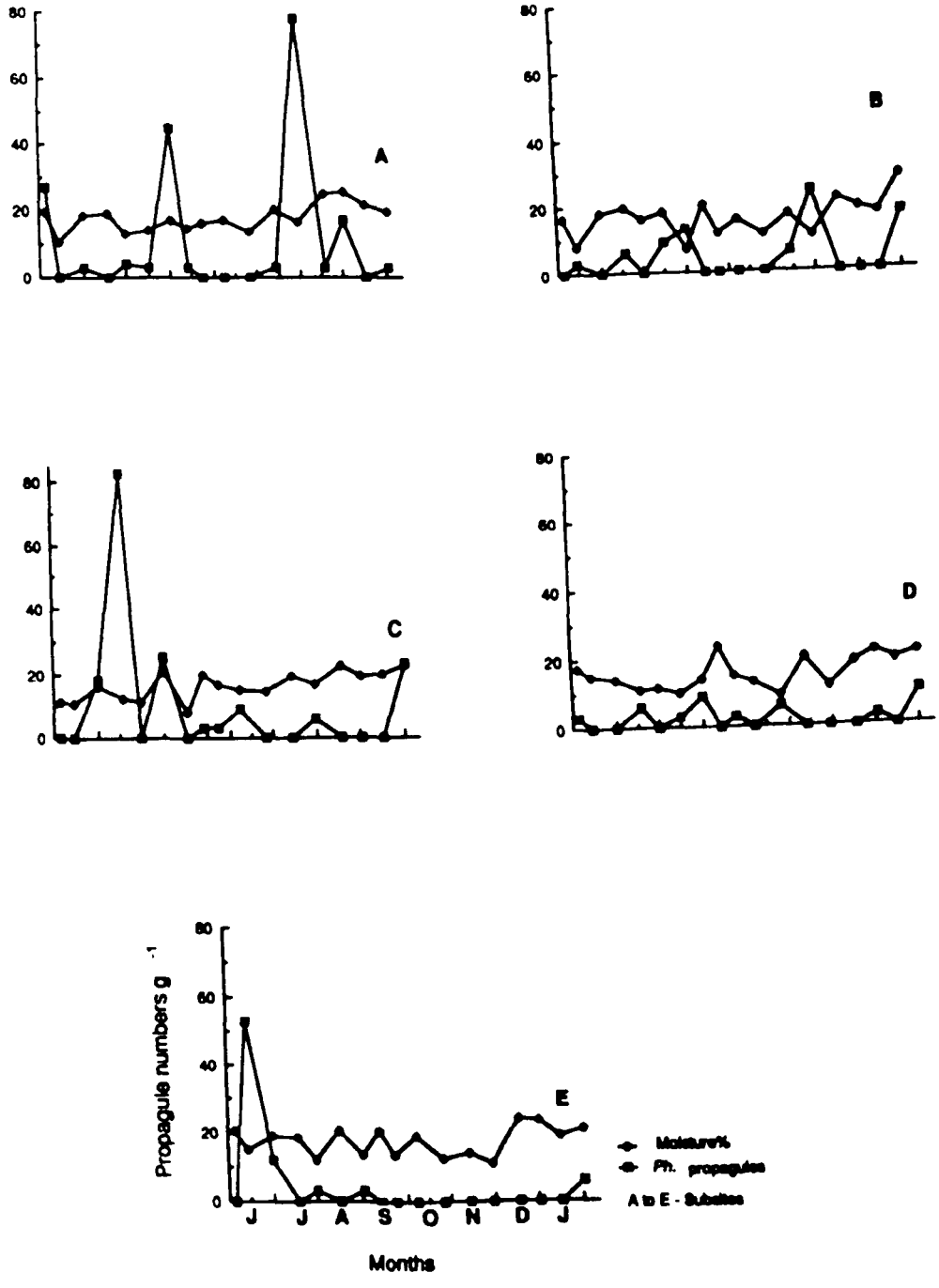



**Figure 3.6.** Relationship between moisture content of soil and *Phytophthora* propagules in various subsites of Site I.

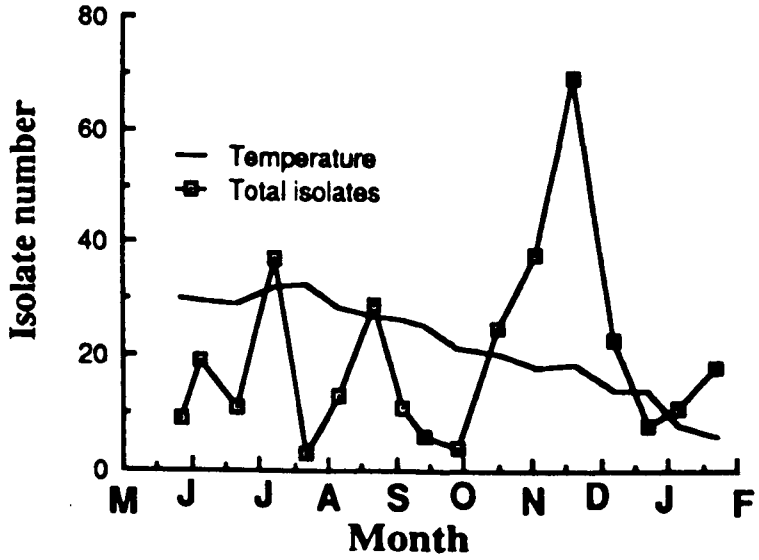



**Figure 3.7.** Relationship between total number of *Phytophthora* isolates recovered from Site I and soil temperature.

If the soil dilution technique was considered alone ( Figure 3.8), the numbers of isolates declined slowly, from a peak in late June so that no isolates were recovered on two successive sampling times in October. The numbers went up again during November and early December to decline again in mid January.

*Citrus* bait produced few isolates until 4th September when the recovery started to increase and at the time when no isolates could be isolated from soil on two successive occasions (26th September and 9th October) using soil dilution, it was possible to isolate 19 and 14 isolates respectively using *Citrus* bait. Never the less, this number went down to 8 on 13th November but it later increased on three successive occasions reaching a peak on 27th November. These numbers then declined in January to reach 35 isolates (Figure 3.9).

Considering the relationship between the sampling occasion and temperature, there seemed to be an absence of any clear effect of soil temperature on isolation as shown in Figure 3.10. No correlation between the isolates and moisture content of the soil and the number of propagules was evident (Figure 3.11)

## 3.4. Discussion

### 3.4.1.Species composition

The presence of *Ph.citrophthora* and *Ph.parasitica* in *Citrus* orchards of the Mediterranean basin has been reported earlier (Reichert & Littauer, 1931; Reichert, 1932; Perlberger, 1936; Schiffman, 1951; Hartman, 1966, 1969; Favaloro & Sommarco, 1970; Vanderweyen, 1983; Sneh & Katz,

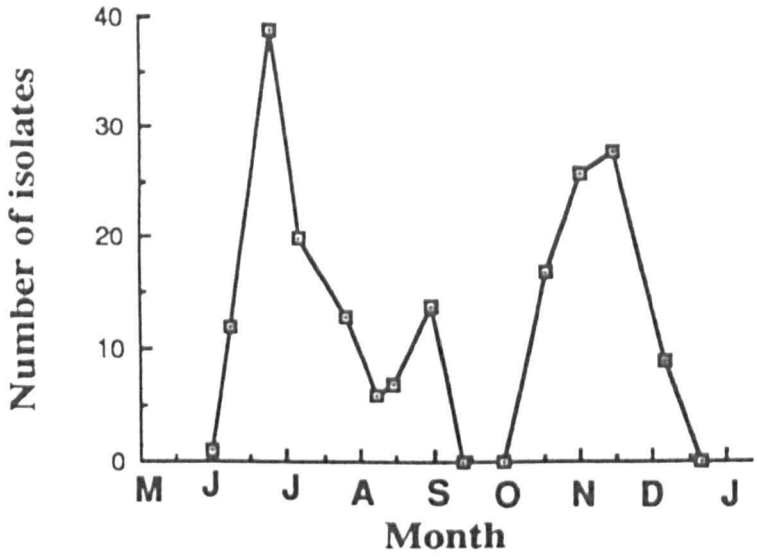



Figure 3.8. Total number of Isolates from site II recovered using soil dilution technique.

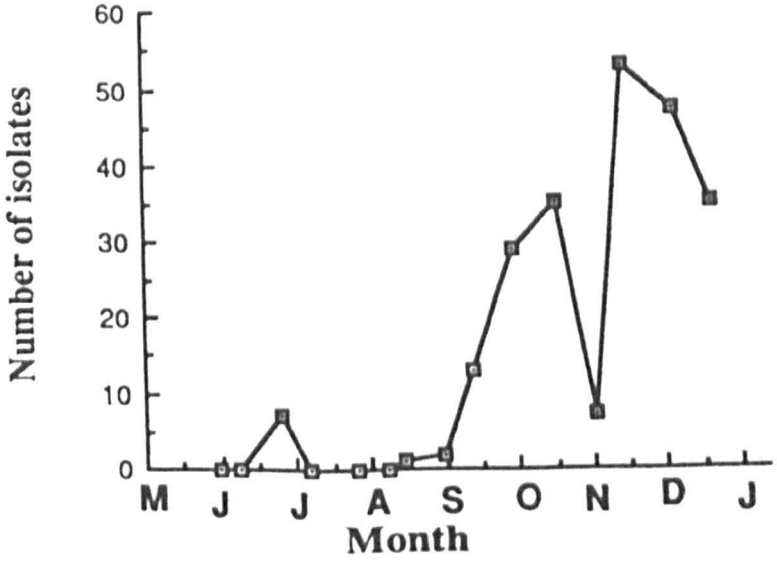



**Figure 3.9.** Total number of Isolates from site II recovered using *Citrus* baiting technique.

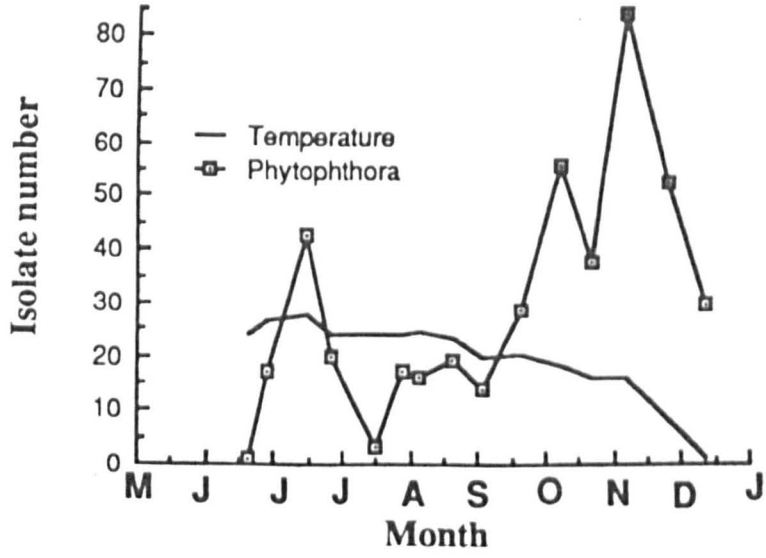



**Figure 3.10.** Relationship between total number of *Phytophthora* isolates recovered from Site II and soil temperature.

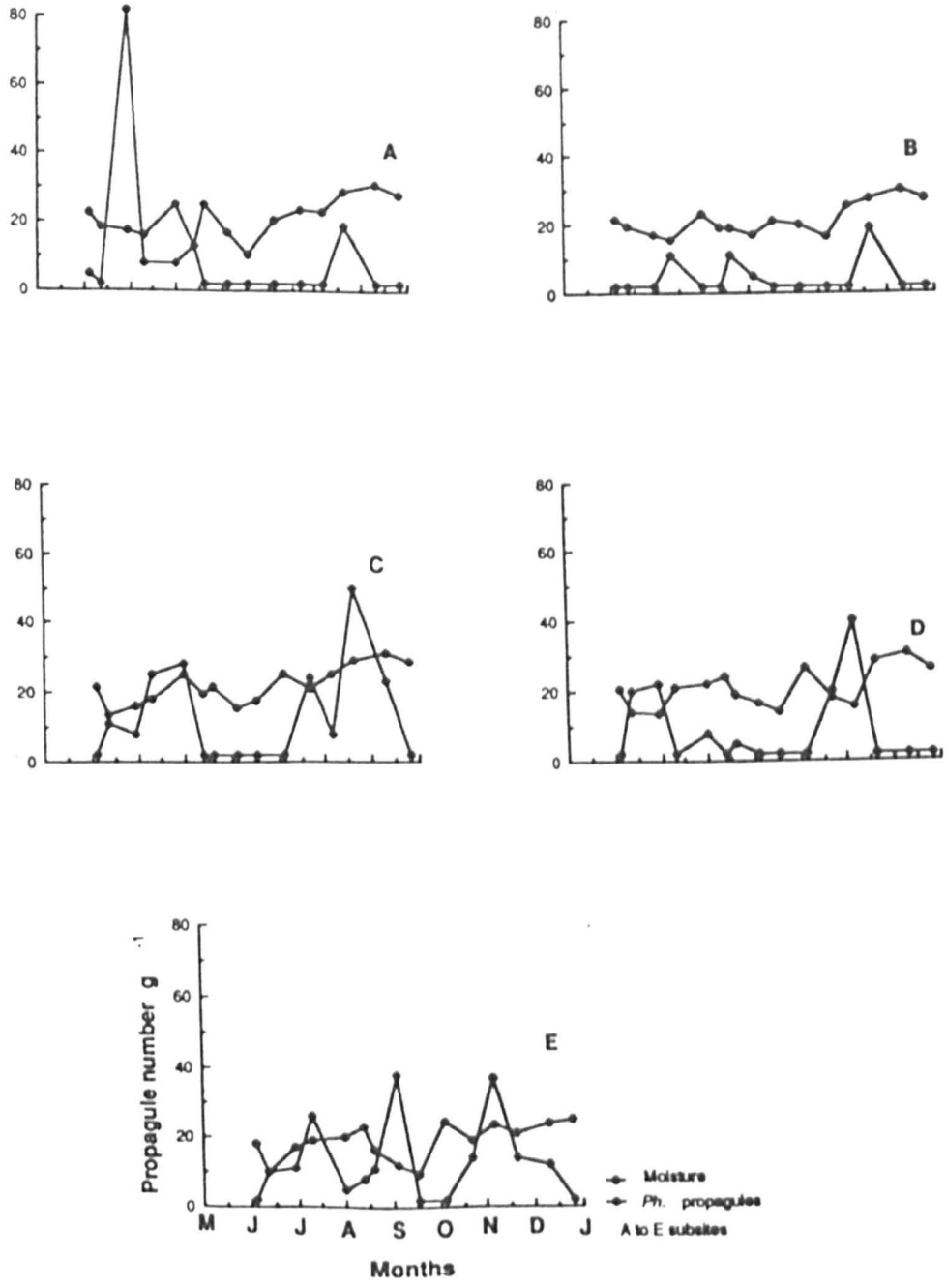



**Figure 3.11.** Relationship between moisture content of soil and *Phytophthora* propagules in various subsites of Site II.

Table 3.20. *Phytophthora* species recovered from Site II (Tulkarem orchard ) at each sampling date using all isolation techniques

| Date | *Ph.citrophthora* | *Ph.nicotianae* | *Ph.* unidentified | Total |
|---|---|---|---|---|
| 22.6.87 | 0 | 0 | 1 | 1 |
| 06.7 | 5 | 1 | 11 | 17 |
| 23.7 | 10 | 5 | 32 | 47 |
| 03.8 | 2 | 6 | 10 | 18 |
| 23.8 | 2 | 0 | 3 | 5 |
| 04.9 | 3 | 0 | 14 | 17 |
| 11.9 | 6 | 3 | 8 | 17 |
| 26.9 | 13 | 4 | 2 | 19 |
| 09.10 | 5 | 2 | 7 | 14 |
| 26.10 | 16 | 6 | 7 | 29 |
| 13.11 | 24 | 1 | 43 | 68 |
| 27.11 | 6 | 5 | 16 | 27 |
| 11.12 | 56 | 8 | 21 | 85 |
| 31.12 | 31 | 0 | 27 | 58 |
| 15.1.88 | 0 | 0 | 35 | 35 |
| Total | 179 | 42 | 236 | 457 |

1988). This study has emphasized that *Ph.citrophthora* and *Ph.nicotianae* are prevalent within *Citrus* orchards of the West Bank of the Jordan, which coincides with the reports mentioned above. However, Schiffman(1951) isolated a third species, *Ph.hibernalis* in small numbers, but the exact number of isolates and geographic location of the site was not mentioned. No previous workers have reported the isolation of this species, nor it was isolated later by others, whether from orchards of Palestine (Reichert & Littauer, 1931; Reichert, 1932; Perlberger, 1936), Israel (Sneh and Katz, 1988), Lebanon (Hartman, 1969), Iran (Benihashemi,1983; Mansoori & Fassihiani, 1985) or Iraq (Hassan *et al* ., 1989).

### 3.4.2. Isolation techniques

#### a. Soil dilution

A soil dilution technique using 0.08 % WA (Ali-Shtayeh *et al* ., 1986) was followed in order to reduce the numbers of both *Phytophthora* species and other accompanying microorganisms. This WA concentration produced a more homogenous soil suspension than a higher concentration of 0.5 % (Flowers & Hendrix, 1969) or water alone ( Weste & Vithange, 1978). In the concentration of 0.5% it is harder to distribute the soil within the semi solid WA, while alone water would allow some soil components to float and the others to settle to the bottom of the container. In addition, using agar allows a smooth and even distribution of the diluted soil over the selective medium.

#### b. Baiting

Use of baiting techniques allows larger volumes of soil to be assayed

and can be highly specific if the correct bait is used. Lupin baits were not as satisfactory as the *Citrus* leaf disc baits, which came from the same host as the *Citrus* orchards under investigation. Lupin baits needed support, 3 - 4 days to grow, and less number can be used in the same beakers, while the *Citrus* leaf discs floated, could be used immediately and more numbers could be used in the same space. However, no *Phytophthora* was isolated from any discs that sank to touch the soil in the beakers. The site of infection of the lupin baits was at the water level. The part of the radicle which touched the soil or was below the water meniscus and showed discolouration did not reveal any *Phytophthora* . The lupin bait technique was not highly successful in the recovery of isolates, with a total of 15 during the whole survey which may be due to the difference between the original host plant and this bait (Weste, 1983).

### c. Selective media

The two selective media used initially differed in the increased concentration of agar, addition of sucrose, thiamine and trace elements to the modified VP3HB. This difference did not have any effect on the recovery of *Phytophthora* from soil at each sampling time. The VP3HB was adopted in order to avoid the technical problems with P5VPPBH when washing the soil off the surface of the selective medium and the ability to to get a clear cut shape of the *Phytophthora* colonies on the VP3HB.

Use of hymexazole (Massago *et al* ., 1977) at a concentration of 25 ppm (Ann, 1984) was followed after reports about toxicity at double this concentration (Papavizas *et al* ., 1981). In spite of that Massago *et al* ., (1977) did not find any problems in their evaluation of this antibiotic, reporting no significant differences between the use of 25 and 50 ppm hymexazole. It was reported later that addition of 50 ppm hymexazole

would reduce the RER of the *Phytophthora* colonies, prevent sporangial production (Papavizas et al ., 1981; Middleton, 1982), and prevent zoospore germination (Sneh & Katz, 1988).

A problem arose in the present study from the interference of *Pythium* species which were not entirely inhibited at 25 ppm concentration of hymexazole. While it was possible to purify many of the mixed isolates that contained *Phytophthora* and *Pythium* together, most of the *Phytophthora* colonies which were in the centre of the *Pythium* colonies could not be taken into pure culture for final identification to the species level. However, at this stage the identity of the isolates at the genus level was confirmed from the morphology of sporangia produced in pond water and the mode of release of the zoospores from these sporangia. Repeated attempts at purification ended with failure most of the time. This problem was reported by Middleton (1982) in the isolation of *Phytophthora* from irrigation water.

### 3.4.3. Species recovery

Both soil dilution and *Citrus* baiting techniques proved to be equally suitable for isolation of *Ph.citrophthora* and the unidentified group, but soil dilution was more efficient in recovering *Ph.nicotianae* . The increase in number of isolates obtained using *Citrus* baits in both sites, starting in September 1987, is mainly attributed to change in the incubation conditions, from room temperature (12-15 °C) to the 24 ± 2 °C incubator (Figure 3.5, 3.9). No similar improvement in numbers is seen for isolates recovered by soil dilution where incubation was at 24 ± 2 °C throughout the sampling period with variations in numbers at different times. These findings draw attention to fact that incubation temperature may be important in increasing chances for recovering more isolates. However,

only 13 isolates of the 51 *Ph.nicotianae* were recovered by *Citrus* bait technique which indicates that it is not as dependable as the soil dilution, which recovered three times this number. *Citrus* baits were equally good for the isolation of *Ph.citrophthora* , which was isolated in similar numbers by soil dilution (1:1.05).

These numbers of recovered isolates might represent the quantitative population of *Phytophthora* in the West bank which do vary in between one sampling occasion and the other. However, it can be illustrated that even using 4 recovery methods over such a period of extensive sampling time shows the difficulty in making any definite statements about the numbers at all.

### 3.4.4. *Phytophthora* distribution within orchards

The *Citrus* orchards surveyed as Permanent sites in this study represent the two main environments suitable for this economically important crop in the West Bank. These environments contrast in their geological, topographical, and climatic features (Zohary, 1942; Karmon, 1971). The other 12 sites represent the major *Citrus* plantations in the area ( Rural Studies Research Centre, 1987).

The sampling methods adopted for this survey were the most suitable for the topography of the orchards. The random sampling method was used for Site I, being at the same level with predominantly *C.limon* host only (except subsite A of the *C.clementina* host). The stratified method was used for Site II due to the variety of the *Citrus* hosts and site terracing.

Comparing the recovered numbers of *Phytophthora* isolates from Site I with those from Site II , the following points can be concluded:

1. Fewer isolates were recovered from Site I (335) than from Site II

(425).

2. A larger number of isolates could be identified from Site I up to the species level ( 58% ) than those from Site II ( 48% ).

3. Isolates of *Ph.nicotianae* recovered from site II ( 42 ) were double the number recovered from Site I ( 20 ), but those of *Ph.citrophthora* were the same (162:164) from both sites.

These differences might be due to one or more of the following factors:

1. In the spot irrigated orchard ( SiteI ), water comes directly in a cement channel from the water springs, the channel narrowing to irrigate each individual tree. While in Site II the irrigation water supplied from an artesian well has to pass through the soil of other orchards and thus might transport more colony forming units from that soil or soil of the same orchard directly to individual trees.

2. Age of the orchards, Site I is located in a more recent orchard area, while Site II falls in an old established orchard area.

### 3.4.5. Seasonal variation

Although fluctuations in the numbers of recovered isolates can be seen in both orchards, there was no obvious relationship between the numbers of these isolates and temperature or moisture content (Figures 3.7 and 3.10). These findings contradict the positive relationship between increase in temperature during the summer and *Phytophthora* isolates reported by West and Ruppin(1977), Weste (1983), and Tsao, (1983), but agree with Timmer *et al* .; (1989) who report no seasonal variation of *Ph.nicotianae* in Florida *Citrus* orchards. However, the increase in numbers of recovered isolates might not be related to the moisture content (Weste, 1983) but to the water matric potential (Gisi, 1977; Duniway,1979; Weste, 1982), which was not measured in this study.

# CHAPTER FOUR

## Genotype-environment interactions

## 4.1 Introduction

Many organisms including fungi can exist in a variety of environments. *A priori* , environmental factors affect the relative performance (growth and development) of a species but also the performance of individuals within a species (Apple, 1957; Christie, 1958; Cameron & Milbrath, 1965; Cameron, 1966; Zentmyer *et al.*, 1974; Hussain, 1980; Jennings, 1987). Many investigators have reported this phenomenon in a variety of higher organisms and for terrestrial plants it has led to the development of methods termed the analysis of genotype-environment interactions. This technique was first employed to examine the stability of a group of barley varieties in a range of environmental conditions (Finlay & Wilkinson, 1963). In their analysis, the individual mean yields of each barley variety in each environment were plotted against the overall mean yield recorded for each of the environments, so that the mean of yields of all the varieties grown in one environment provided an estimate, or rank, of the status of that environment which was comparable with other environments.

Figure 4.1 shows the range of linear responses that may be expected to occur when examining individual mean yields in relation to environmental rank. These responses may be quantified by linear regression analysis using the regression coefficient as an index of stability. An average stability is indicated if the coefficient approximates to a value of 1.0, since the individual variety yields are plotted against each

environmental mean yield (Case A, Figure 4.1). Case B illustrates a good overall adaptability to the environment which results in a slope of unity associated with a higher than average yield. If the adaptation is poor, as in Case C, there is a lower than average yield. If regression coefficients are significantly different from unity, this implies that the varieties are sensitive to environmental changes. Case D indicates this with a below average stability, Case E with above average stability.

This type of analysis has been used in studying a range of organisms especially plants (Hill, 1975) and fungi (Fripp & Caten, 1971, 1973; Fripp, 1972; Hussain, 1980). Many studies have indicated that species and isolates within species vary in their response to environments which provides a valuable aid in taxonomy (Bumbieris, 1974; Kanniyan *et al.*, 1980; Kellam & Zentmyer, 1986; Faris *et al* ., 1989). Genotype-environment interaction has been shown to be a significant source of variation in phenotypic responses (Mather & Jinks, 1971). The environment will affect the genotypic composition of a population in the long term through natural selection. In addition, genotype and environment will interact in the short term thus causing differences in character expression among individual isolates.

Application of this analysis for *Ph.citrophthora* and *Ph.nicotianae* is considered in section 4.2.1 of this chapter, using the term isolate in place of 'genotype' as genotypes could not be identified. The environments are various natural media.

The extent of variation within a group of isolates from both species and between the two species measured as the ability to utilize a particular carbon source is presented in section 4.2.2.

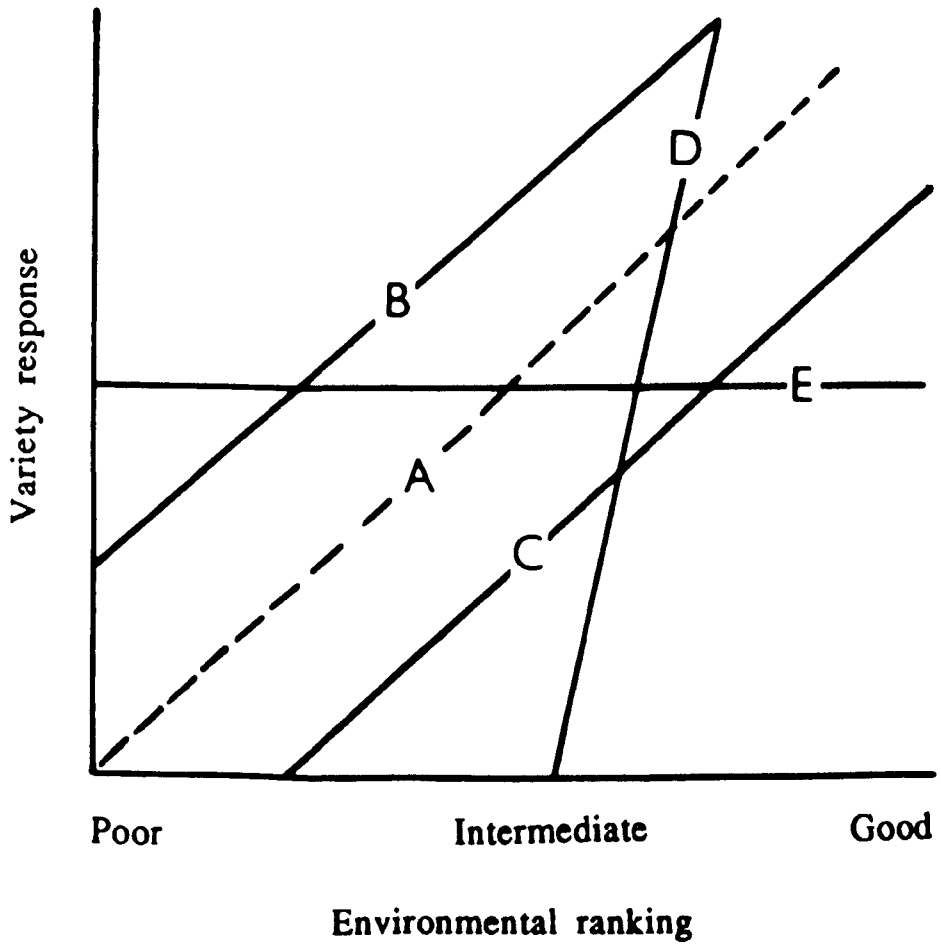



**Figure 4.1.** Hypothetical regression lines showing the relationship of individual variety responses and the population mean responses over a range of environments ( after Finlay & Wilkinson, 1963).

## 4.2 Materials and Methods

### 4.2.1 Isolate-environment interactions

Twelve isolates of *Ph.citrophthora* and 12 isolates of *Ph.nicotianae* were chosen randomly (source shown in Table 4.1) for this assessment. After growing the cultures on PDA for 3 days, 5 mm agar plugs were subcultured from the actively growing margin of the colonies on to the centre of 9 cm plastic petridishes containing PDA, CA, CV8A, PA or CMA. Three replicates of each isolate on each medium were incubated in the dark at 25°C for three days. Three measurements were taken of colony diameter at right angles to each other on each colony.

#### Statistical analysis

Linear regression analysis was performed for each isolate, regressing individual isolate performance upon average environment score. Values of calculated regression coefficients were compared against unity using a t-test.

### 4.2.2 Effect of carbon source and temperature on variability

The experiment investigating the effect of temperature on *Ph.citrophthora* and *Ph. nicotianae* was extended to include a number of carbon sources. Sucrose, galactose, fructose, lactose or sorbitol (all at 25 mM) were substituted for glucose in the original Ribiero's Synthetic medium (see Section 2.3). The plates were inoculated in duplicates using the same 8 isolates (4 of each species) as in experiment 2.2.1, incubated at 25, 30, 32, 35, 37 and 45°C for 5 days and radial extension rates (mm $d^{-1}$)

**Table 4.1** Source of isolates assessed in section4.2.1 collected from Site I (Jordan Valley) and Site II (Tulkalerm) orchards.

| No. | Isolate | Site | Species | Origin |
|---|---|---|---|---|
| 1 | IB7005 | I | *Ph. citrophthora* | Soil dilution |
| 2 | IB13001 | I | " " | " " |
| 3 | IE2006 | I | " " | " " |
| 4 | IB8002 | I | " " | Citrus root |
| 5 | IB120012 | I | " " | " bait |
| 6 | IE5002 | I | " " | " root |
| 7 | IIA13003 | II | " " | Soil dilution |
| 8 | IIE5001 | II | " " | " " |
| 9 | IIE12001 | II | " " | " " |
| 10 | IIA2005 | II | " " | Citrus bait |
| 11 | IIE7004 | II | " " | " root |
| 12 | IIE80014 | II | " " | " root |
| 1 | IA7003 | I | *Ph. nicotianae* | Soil dilution |
| 2 | IB13004 | I | " " | " " |
| 3 | ID7001 | I | " " | " " |
| 4 | IC8001 | I | " " | Citrus bait |
| 5 | IC14001 | I | " " | " root |
| 6 | ID11003 | I | " " | " bait |
| 7 | IIA30012 | II | " " | Soil dilution |
| 8 | IID3006 | II | " " | " " |
| 9 | IIE80012 | II | " " | " " |
| 10 | IIA10004 | II | " " | Citrus root |
| 11 | IIB7006 | II | " " | " root |
| 12 | IIC130026 | II | " " | " bait |

were recorded.

The RERs on the six carbon sources at 25°C and 30°C were analysed by analysis of variance according to a mixed model design with the four isolates of each species grown independently on the 6 carbon sources.

The radial extension rates were indistinguishable amongst isolates (see Table 4.7) but the carbon sources had a very noticeable effect on overall colony morphology. On three of them (galactose, lactose and sorbitol) hyphae were sparse with few aerial branches and the agar was easily visible beneath each colony. On the other three carbon sources (glucose, sucrose and fructose) the isolates displayed dense aerial hyphae and completely covered the agar surface.

Data for the latter three carbon sources (glucose, sucrose, and fructose) was analysed alone by analysis of variance as described above.

## 4.3 Results

### 4.3.1 Isolate environment interactions

The average isolate performance on each medium was plotted against the averages of all scores for each medium (Figure 4.2 and 4.3) for the twelve isolates of *Ph.citrophthora* and *Ph.nicotianae* . Mean radial extension rates for the isolates are shown in Tables 4.2 and 4.3.

Ten of the *Ph.citrophthora* behaved similarly and displayed average stability to changes in environment (slopes were not significantly different from 1, Table 4.4). Isolate IB7005 however, was responsive to changing environment whilst isolate IIA13003 displayed lower than average growth rates (Table 4.2) and was insensitive to changes in environment.

The regression lines were plotted by plotting the average RER of each individual isolate on the five media aganist the overall mean of RER of all isolates on each medium ( shown on the x axis of the Figures) forming one point. The other points were plotted using the following equation :

$y = mx + c$

where y is the mean isolate RER

x is the over all mean of RER of all isolates on all media

m is the slope (Bi) from Table 4.4 for *Ph.citrophthora*

and Table 4.5 for *Ph.nicotianae* .

Thus the factor c can be calculated.

Now, plotting 3 more hypothetical points on the x axis, and using the above equation ( where m and c are known) , it would be possible to calculate the value of y.

After plotting the three other points on the graph, the regression lines can be drawn as seen in Figure 4.2. and 4.3.

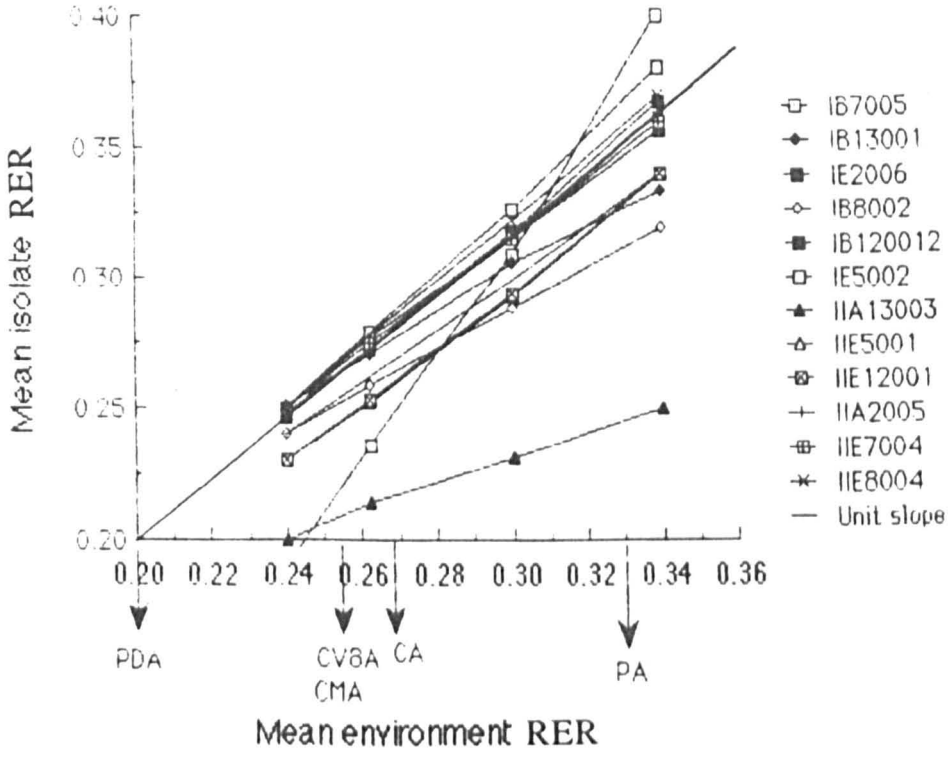



**Figure 4.2.** Isolate-environment interactions of 12 *Ph.citrophthora* isolates plotted as the average RER of each isolate on five media against the over all mean of RER of all isolates on these media, using the slope in Table 4.4. for each individual isolate.

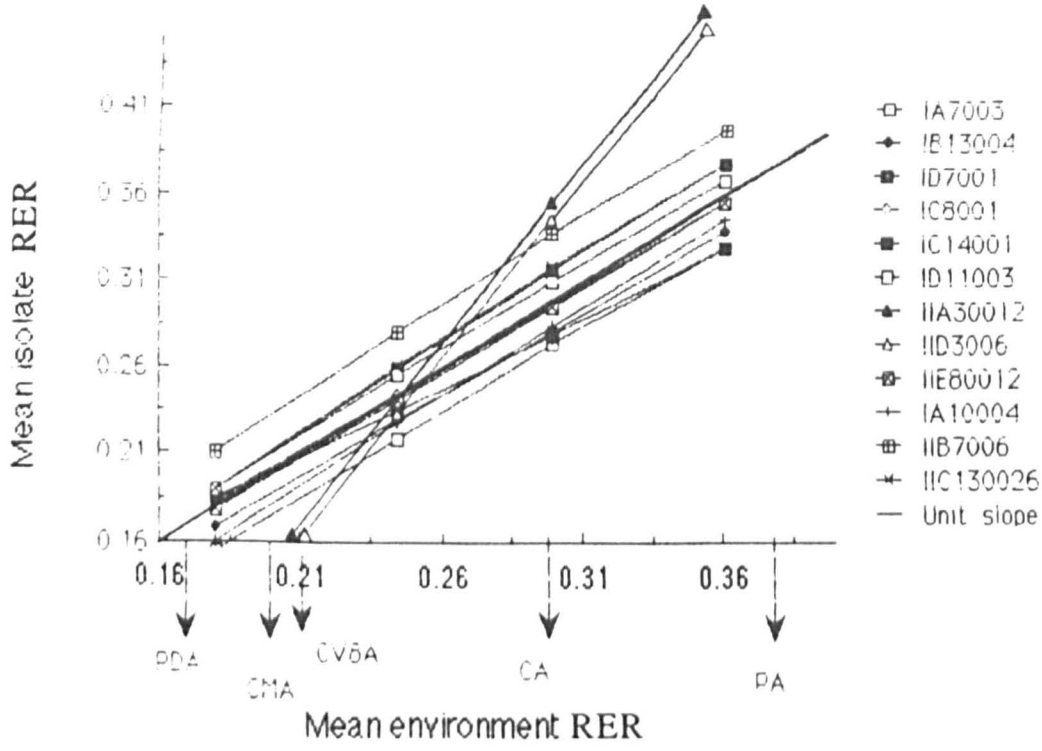



**Figure 4.3.** Isolate-environment interactions of 12 *Ph. nicotianae* isolates plotted as the average RER of each isolate on five media against the over all mean of RER of all isolates on these media, using the slope in Table 4.5. for each individual isolate.

Nine of the twelve isolates of *Ph.nicotianae* behaved similarly showing average stability. Isolate IC8001 showed a general adaptability (being of average RER and unresponsive to environmental change) whilst IIA30012 and IID3006 had a below average stability (Table 4.3) being conspicuously sensitive to the environment (Table 4.5).

### 4.3.2 Effect of carbon source on RER

The growth rates on the six carbon sources at each temperature (25, 30, 32, 35 and 37°C) are tabulated in Appendix 4. Maximum radial extension rates were observed at 25°C for all *Ph.citrophthora* isolates utilizing each of the six carbon sources (Figure 4.4).The optimum RER *Ph.nicotianae* was found to be at 30°C (Figure 4.5). Mean RER values for both species are shown in Table 4.6.

Analysis of variance (Table 4.7) indicated that there was no significant difference between the RER of the two species at 25°C ( F ratio = 1.84, $P_{(Ho)} > 0.05$). However, there were significant differences in the reaction of individual isolates towards different carbon sources and significant variation within species (amongst isolates).

At 30°C (Table 4.8) there were statistically significant differences between the species whilst intraspecific variation was no longer significant. Differences between individual carbon sources were still retained.

The growth of all isolates on galactose, lactose or sorbitol was sparse,whilst that on media containing sucrose, glucose or fructose was dense being of a characteristic colonial morphology (see section 4.2.2 for description). Analysis of variance for growth at 25°C on media containing

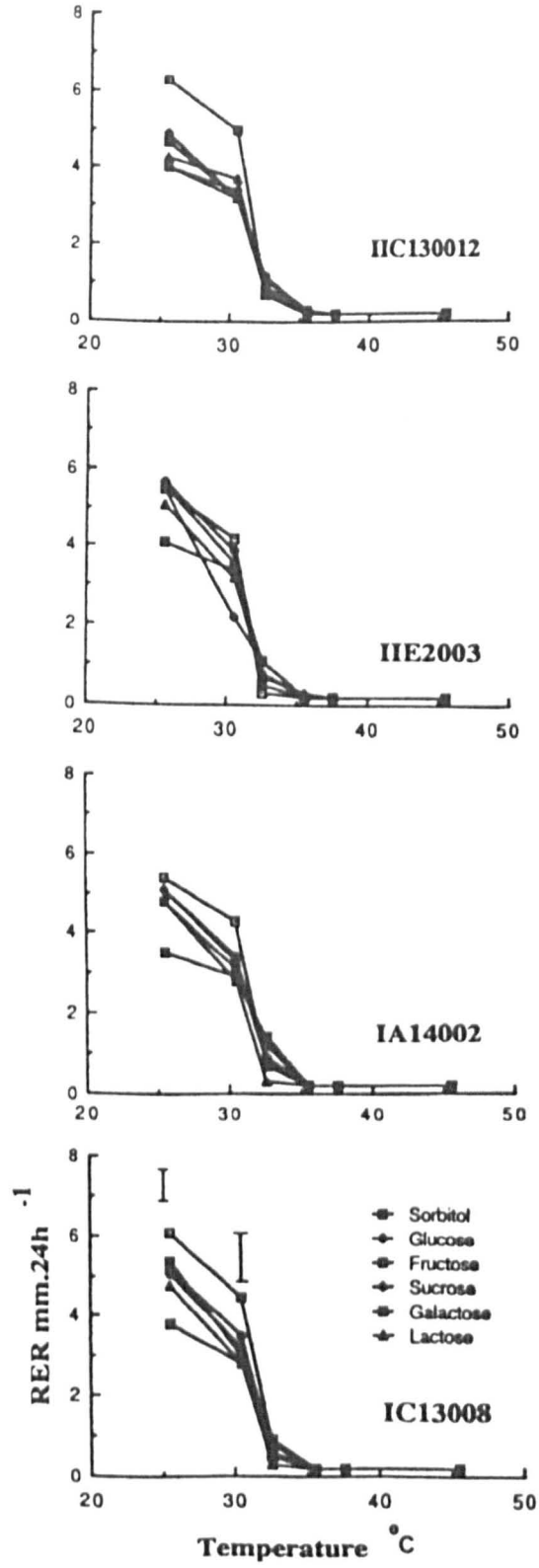



**Figure 4.4.** Effect of carbon source on RER of *Ph.citrophthora* at different temperatures. Bars indicate LSD.

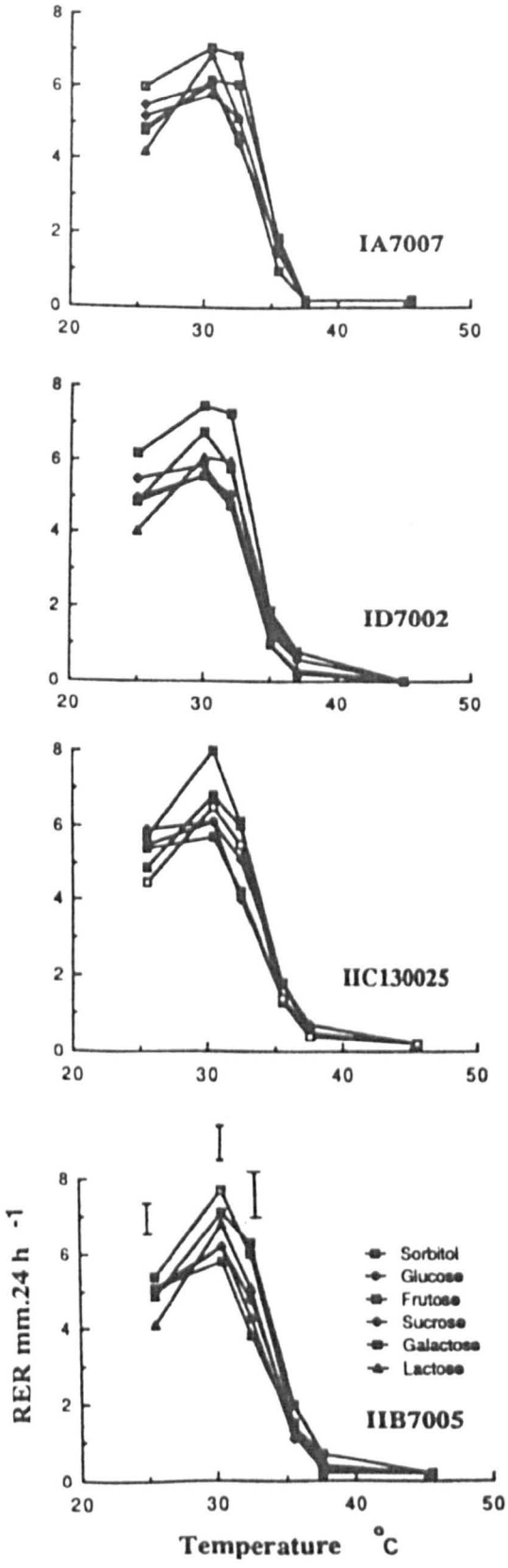



Figure 4.5. Effect of carbon source on RER of *Ph.nicotianae* at different temperatures. Bars indicate LSD.

Table 4.2 *Ph.citrophthora* RER on various media $mm.h^{-1}$

| | Isolate | PDA | CA | CV8A | PA | CMA | Average isolate performance | S.E. |
|---|---|---|---|---|---|---|---|---|
| 1 | IB7005 | 0.182 | 0.256 | 0.209 | 0.300 | 0.232 | 0.236 ± | 0.02 |
| 2 | IB13001 | 0.220 | 0.285 | 0.252 | 0.332 | 0.232 | 0.271 ± | 0.017 |
| 3 | IE2006 | 0.216 | 0.282 | 0.277 | 0.345 | 0.265 | 0.277 ± | 0.018 |
| 4 | IB8002 | 0.198 | 0.273 | 0.276 | 0.299 | 0.248 | 0.259 ± | 0.015 |
| 5 | IB120012 | 0.204 | 0.273 | 0.266 | 0.357 | 0.265 | 0.273 ± | 0.022 |
| 6 | IE5002 | 0.216 | 0.279 | 0.274 | 0.370 | 0.256 | 0.279 ± | 0.023 |
| 7 | IIA13003 | 0.196 | 0.190 | 0.214 | 0.254 | 0.214 | 0.214 ± | 0.01 |
| 8 | IIE5001 | 0.184 | 0.268 | 0.236 | 0.322 | 0.251 | 0.252 ± | 0.02 |
| 9 | IIE12001 | 0.198 | 0.257 | 0.226 | 0.333 | 0.252 | 0.253 ± | 0.02 |
| 10 | IIA2005 | 0.215 | 0.282 | 0.251 | 0.355 | 0.261 | 0.273 ± | 0.021 |
| 11 | IIE7004 | 0.214 | 0.289 | 0.272 | 0.347 | 0.252 | 0.275 ± | 0.02 |
| 12 | IIE80014 | 0.220 | 0.292 | 0.264 | 0.361 | 0.254 | 0.278 ± | 0.021 |
| | Mean | .205 | 0.269 | 0.251 | 0.331 | 0.252 | 0.262 ± | 0.010 |
| | S.E. | ±0.004 | ±0.028 | ±0.007 | ±0.010 | ±0.005 | | |

**Table 4.3** *Ph.nicotianae* RER on various media $mm.h^{-1}$.

| | Isolate | PDA | CA | CV8A | PA | CMA | Average isolate performance | S.E. |
|---|---|---|---|---|---|---|---|---|
| 1 | IA7003 | 0.114 | 0.282 | 0.186 | 0.318 | 0.195 | 0.219 ± | 0.036 |
| 2 | IB13004 | 0.147 | 0.276 | 0.205 | 0.337 | 0.187 | 0.230 ± | 0.034 |
| 3 | ID7001 | 0.148 | 0.323 | 0.212 | 0.383 | 0.234 | 0.260 ± | 0.042 |
| 4 | IC8001 | 0.176 | 0.266 | 0.209 | 0.368 | 0.205 | 0.245 ± | 0.03 |
| 5 | IC14001 | 0.175 | 0.228 | 0.203 | 0.353 | 0.220 | 0.236 ± | 0.027 |
| 6 | ID11003 | 0.173 | 0.313 | 0.218 | 0.365 | 0.212 | 0.256 ± | 0.032 |
| 7 | IIA30012 | 0.144 | 0.299 | 0.212 | 0.363 | 0.191 | 0.242 ± | 0.035 |
| 8 | IID3006 | 0.156 | 0.285 | 0.180 | 0.358 | 0.188 | 0.233 ± | 0.034 |
| 9 | IE80012 | 0.176 | 0.283 | 0.205 | 0.361 | 0.186 | 0.242 ± | 0.031 |
| 10 | IIA10004 | 0.157 | 0.275 | 0.192 | 0.355 | 0.198 | 0.229 ± | 0.034 |
| 11 | IIB7006 | 0.187 | 0.316 | 0.282 | 0.407 | 0.213 | 0.281 ± | 0.035 |
| 12 | IIC130026 | 0.192 | 0.290 | 0.214 | 0.402 | 0.202 | 0.261 ± | 0.035 |
| | Mean | 0.163 | 0.286 | 0.210 | 0.364 | 0.203 | 0.245 ± | 0.008 |
| | S.E. | ± 0.006 | ±0.007 | ±0.007 | ±0.007 | ±0.004 | | |

**Table 4.4** The sensitivity of *Ph.citrophthora* isolates. Analysis of regression coefficients, testing the hypothesis that the slope (Bi)=1. P is the probability of the null hypothesis. N.S. indicates P>0.05.

| | Isolate | Bi | t-value | $P_{(Ho)}$ |
|---|---|---|---|---|
| 1 | IB7005 | 0.972 | 0.22 | N.S. |
| 2 | IB13001 | 0.917 | 0.64 | N.S. |
| 3 | IE2006 | 1.027 | -0.212 | N.S. |
| 4 | IB8002 | 0.782 | 1.681 | N.S. |
| 5 | IB120012 | 1.215 | -1.661 | N.S. |
| 6 | IE5002 | 1.251 | -1.939 | N.S. |
| 7 | IIA13003 | 0.442 | 4.298 | <0.05 |
| 8 | IIE5001 | 1.103 | -0.796 | N.S. |
| 9 | IIE12001 | 1.095 | -0.731 | N.S. |
| 10 | IIA2005 | 1.149 | -1.147 | N.S. |
| 11 | IIE7004 | 1.088 | -0.68 | N.S. |
| 12 | IIE80014 | 1.1712 | -1.3236 | N.S. |

**Table 4.5** The sensitivity of *Ph.nicotianae* isolates. Analysis of regression coefficients, testing the hypothesis that the slope (Bi)=1. P is the probability of the null hypothesis. N.S. indicates P>0.05.

| | Isolate | Bi | t-value | $P_{(Ho)}$ |
|---|---|---|---|---|
| 1 | IA7003 | 0.985 | 0.141 | N.S. |
| 2 | IB13004 | 0.946 | 0.507 | N.S. |
| 3 | ID7001 | 1.055 | 0.564 | N.S. |
| 4 | IC8001 | 0.947 | 0.493 | N.S. |
| 5 | IC14001 | 0.805 | 1.820 | N.S. |
| 6 | ID11003 | 0.991 | 0.080 | N.S. |
| 7 | IIA30012 | 1.097 | 0.902 | N.S. |
| 8 | IID3006 | 1.062 | 0.576 | N.S. |
| 9 | IIE80012 | 0.977 | 0.211 | N.S. |
| 10 | IIA10004 | 0.996 | 0.036 | N.S. |
| 11 | IIB7006 | 1.059 | 0.547 | N.S. |
| 12 | IIC130026 | 1.059 | 0.547 | N.S. |

**Table 4.6.** Mean radial extension rate ( ± S.E) of 4 isolates from each of *Ph.citrophthora* and *Ph.nicotianae* at 25°C on Ribiero's synthetic agar utilizing 6 carbon sources as mm.24h $^{-1}$.

| Species | Carbon sources | | | | | | Mean growth rate |
|---|---|---|---|---|---|---|---|
| | Sorbitol | Glucose | Fructose | Sucrose | Galactose | Lactose | |
| *Ph.citrophthora* | 5.6±0.2 | 4.7±0.3 | 4.8±o.37 | 5.0±0.15 | 3.6±0.1 | 4.5±0.15 | 4.7±0.31 |
| *Ph.nicotianae* | 5.7±0.2 | 5.4±0.15 | 4.9±0.1 | 4.9±0.15 | 4.7±0.05 | 4.1±0.1 | 5.0±0.27 |

glucose, fructose and sucrose alone (resulting in dense mycelial growth) is shown in Table 4.9. This indicates that there were now no significant differences due to the carbon sources. It was thus again not possible to differentiate the two species at this temperature.

At 30°C (Table 4.10), significant differences among the three carbon sources and between isolates of each species were also undetectable. However, the two species could be differentiated at this temperature and there was a significant difference in utilization of the three carbon sources by the isolates.

From this assessment it appears that both *Ph.citrophthora* and *Ph.nicotianae* can grow on synthetic media and can be distinguished from one another at 30°C. Moreover, at incubation temperatures of 25°C (for *Ph.citrophthora* ) and 30°C (for *Ph.nicotianae* ) intra-isolate variation within species was evident and for *Ph.nicotianae* different to the ranking observed at 25°C. In spite of similar extension rates of both species at 25°C, there were significant differences amongst the isolates within each of the two species. At 30°C this difference could not be detected but the species still could be differentiated. At higher temperatures this effect was clearer in detecting variation in utilization of various carbon sources by isolates of *Ph.citrophthora* only. At 32°C sorbitol initiated faster linear extension growth than glucose for *Ph.citrophthora* isolate no IC13008 (Appendix 4.c), but it was the opposite for isolate IIE12003. The same phenomenon could not be observed for *Ph.nicotianae* at this temperature. However, the results of incubations at 37°C indicated variation in temperature sensitivity. Isolate IA7007 was unable to grow at this temperature while the other three isolates were able to utilize all carbon sources (except isolate IB7005 which could not utilize sorbitol). Thus, differences in the over all

**Table 4.7** Analysis of variance of RER at 25°C on glucose, fructose, galactose, sorbitol, lactose and sucrose of 4 isolates from each of *Ph.citrophthora* and *Ph.nicotianae*.

| Source | DF | SS | MS | F ratio | $P_{(HO)}$ |
|---|---|---|---|---|---|
| Carbon source | 5 | 604.1 | 120.8 | 13.4 | <0.05 |
| Species | 1 | 38.5 | 38.5 | 1.84 | >0.05 |
| Isolates within spp. | 6 | 125.69 | 20.95 | 24.36 | <0.05 |
| Carbon source x isolate within spp. | 35 | 314.96 | 9.00 | 10.47 | <0.05 |
| Error | 48 | 41.12 | 0.86 | | |

**Table 4.8** Analysis of variance of RER at 30 °C on glucose, fructose, galactose, sorbitol, lactose and sucrose of 4 isolates from each of *Ph.citrophthora* and *Ph.nicotianae*.

| Source | DF | SS | MS | F ratio | $P_{(HO)}$ |
|---|---|---|---|---|---|
| Carbon source | 5 | 585.9 | 117.18 | 14.6 | <0.05 |
| Species | 1 | 5526.7 | 5526.7 | 1073.14 | <0.05 |
| Isolates with spp. | 6 | 30.87 | 5.15 | 4.18 | >0.05 |
| Carbon source x isolate with spp. | 35 | 280.8 | 8.02 | 6.52 | <0.05 |
| Error | 48 | 59 | 1.23 | | |

**Table 4.9** Analysis of variance of RER at 25°C on glucose, fructose, and sucrose for 4 isolates from each of *Ph.citrophthora* and *Ph.nicotianae*.

| Source | DF | SS | MS | F ratio | $P_{(HO)}$ |
|---|---|---|---|---|---|
| Carbon source | 2 | 8.166 | 4.083 | 0.29 | >0.05 |
| Species | 1 | 15.7 | 15.7 | 2.62 | >0.05 |
| Isolates with spp. | 6 | 167.6 | 27.93 | 25.64 | <0.05 |
| Carbon source x isolate with spp. | 14 | 59.6 | 4.26 | 3.91 | <0.05 |
| Error | 24 | 26.125 | 1.089 | | |

**Table 4.10** Analysis of variance of RER at 30°C on glucose, fructose, and sucrose for 4 isolates from each of *Ph.citrophthora* and *Ph.nicotianae*.

| Source | DF | SS | MS | F ratio | $P_{(HO)}$ |
|---|---|---|---|---|---|
| Carbon source | 2 | 11.7 | 5.85 | 0.87 | >0.05 |
| Species | 1 | 2185.65 | 2185.65 | 3973.9 | <0.05 |
| Isolates with spp. | 6 | 3.318 | 0.55 | 0.608 | >0.05 |
| Carbon source x isolate with spp. | 14 | 94.136 | 6.72 | 7.43 | <0.05 |
| Error | 24 | 21.705 | .9044 | | |

growth response at different temperatures may vary between *Ph.citrophthora* and *Ph.nicotianae*, irrespective of the growth medium.

## 4.4 Discussion

Isolate-environment interactions may play an important role in the variation displayed by fungal species (Perkins & Jinks, 1968). The analysis of these interactions in this study did not show substantial variation in response in either *Ph.citrophthora* or *Ph.nicotianae*. Most of the isolates displayed an average stability in their reaction to environmental changes. However, some isolates did show departures from the common pattern. Three out of 12 isolates in *Ph.nicotianae* and two out of 12 in *Ph.citrophthora* were statistically significantly different from the average response, i.e. 25% and 16% of the samples respectively. Whilst these are based on very low sample sizes, they suggest that populations may contain variable isolates. Such a tentative conclusion can only be confirmed by large sample sizes in further assessment.

Isolate environment interactions detected here by linear regression analysis are limited since they preclude the possibility of detecting non-linear responses to environmental change. Extending the range and type of environments used in assessments, whether carbon source (Christie & Milbrath, 1965), pH (Cameron & Milbrath, 1965) or temperature (Faris *et al.*, 1989) might be expected to reveal further differences. Implicitly however, mean performance of individual isolates and sensitivity to environment will always be dependent on the choice of environments under study.

Isolate-environment differences were detected when comparing, RER s at 25°C and 30°C in *Ph.nicotianae* and RER s at 30°C and 32°C for

*Ph.citrophthora* (Figure 4). The RER of *Ph. nicotianae* which increased between 25 and 30°C dropped slightly at 32°C with a drastic drop at 35°C.

That *Ph.citrophthora* decreased above 30°C, there was a drop to just fractions of a millimeter per day at 32°C (see Appendix 4). If this sensitivity to small temperature changes is maintained in the field, there are implications that the growth of *Ph.citrophthora* rather than *Ph.nicotianae* is inhibited by temperature rise in summer. This is a clear indication of the temperature role on the growth of both species which may take place *in vitro* or *in vivo*.

Temperature mediated differences in the isolate-environment response (e.g. isolate IA7007 and IIC130025) may also constitute a source of intra-population variation.

The noted similarity amongst the isolates in isolate-environment response may have resulted from subsampling the same isolate in the field which might have been distributed over a large area. However, as Table 4.1 shows the isolates used in the genotype environment study came from a range of subsites, and both orchards. Their similarity implies a very widespread homogeneity amongst both presumably asexual *Ph.citrophthora* populations and also those of *Ph.nicotianae*, where sexual recombination is better documented.

The differences in growth between species detected in terms of morphology or RER on a range of carbon sources, or temperature effects must reflect underlying biochemical differences. Differences in enzyme complement and the temperature optima of individual enzymes will give rise to these gross effects. Later work in this study, on enzyme and protein profiles clearly indicates that these differences do exist (see Chapter 6).

## CHAPTER FIVE

### Pathogenicity and virulence characteristics

## 5.1 Introduction

The consideration of host-pathogen interactions is central to description of variation in a plant pathogen. Pathogenic behaviour may be dealt with in two ways, namely virulence and host range. While pathogenicity can be defined as the overall ability of a particular species to attack a particular host genotype (Day, 1960; Hawksworth *et al.*, 1983), virulence is the measure or degree of the attack intensity by various isolates on the hosts (Flentje, 1970).

In this study, the aspect of virulence has been investigated for *Ph.citrophthora* , the more abundant species in the recovered isolates. An attempt had been made to obtain quantifiable differences between *Ph.citrophthora* isolates in terms of their ability to infect *Citrus*. The *Citrus* cultivars were chosen to represent the graft and rootstocks present in Sites I and II, but grown from open pollinated seed obtained on the West Bank, rather than the study orchards themselves. They thus represent possible local host genotypes, rather than ones from the study orchards.

### Disease symptoms

The numerous symptoms of *Citrus* disease caused by *Phytophthora* are well described in the scientific literature, these include brown rot, collar rot, and seedling blight, as well as leaf and fruit decay. Gummosis, exudation of excessive amounts of resins from the trunk,

is an additional destructive symptom (Fawcette, 1923; Perlberger, 1936; Fraser, 1942; Schiffmann, 1951; Mansoori & Fassihiani, 1984; Sneh & Katz, 1988). The infection usually starts at the base of the trunk or crown roots, spreading both laterally through the roots and upwards towards the crown of the tree. Typical symptoms include loss of patches of bark, gummosis, and yellowing and loss of leaves. In the early stages, however the foliage and fruit yield may remain substantially unaffected (Fraser, 1942). Later, or when young seedlings are affected, symptoms of a stem or tip blight are evident, with blackening of the shoot tip and leaf loss (Perlberger, 1936). *Phytophthora* can also cause damping off of seedlings, when the emerging root is attacked, and also the stem as it emerges at the soil surface. The base of the stem becomes watery, collapses and dies (Timmer, 1972).

**Resistance and susceptibility in *Citrus* cultivars:**

There is a considerable interest in developing *Citrus* cultivars which are resistant to *Phytophthora* . The genus *Citrus* is divided into two subgenera, *Eucitrus* which includes the commonly cultivated species, and *Papeola* which bears non-edible fruit. The genus is endemic in South East Asia, but species hybridise easily, leading to considerable taxonomic controversy (Vardi, 1983). Susceptibility to infection by *Phytophthora* varies considerably both between and within species and varieties. When plants have to be propagated from seed, rather than cuttings, outcrossing helps to perpetuate susceptibility traits (Fawcette & Bitacourt, 1940; Cinar *et al* ., 1976; Longe *et al* ., 1977; Sawant *et al* ., 1984).

Difficulties in comparing the susceptibility of individual varieties are not helped by the inadequate descriptions of plant material and assessment methods given by many workers. The *Citrus* material is frequently poorly identified. For example, Sawant and co-workers describe

a study of seven varieties of *C. jambhiri* where two were rated as resistant, four as tolerant and one as highly susceptible (Sawant *et al* ., 1984). In a study of fourteen *Citrus* cultivars citrange was both the most resistant (Carrizo citrange) and most sensitive (Savage citrange) (Cinar & Tuzcu, 1976).

All infectious diseases are governed by host-pathogen interactions (Vanderplank, 1982). While lack of aggressiveness within pathogens is equivalent to horizontal resistance in the host, this resistance may ascribe the host action against the pathogen.

Resistant genes in the host can be classified according to the behaviour of corresponding virulence genes in the pathogen and virulence or avirulence towards the host may lead to formation of pathogen races. The presence of a gene in the host means the presence of at least two races in the pathogen, one virulent and another avirulent. This forms the basis for the gene to gene hypothesis meaning that for each resistant gene in the host there is a matching or corresponding gene for pathogenicity in the fungus. The range of pathogenicity within a race of pathogens may be associated with pathogenic factors for each resistant factor possessed by the host. (Flentje, 1970).

The research for resistant cultivars in *Citrus* has led to massive breeding programmes and the great genetic diversity and abundant heterozygosity of cultivars has to be considered. The many interspecific and intergeneric crosses have resulted in a large number of cultivars (Purseglove, 1968).

There is no standard method for objectively measuring susceptibility of *Citrus* to *Phytophthora*. Many methods of experimental infection, plant site or part and *Phytophthora* inoculum have been used. Nevertheless, some results have been established clearly. *Ph.citrophthora* has a wide host range (Tucker, 1931) and it is capable of producing symptoms

associated with *Phytophthora* disease in *Citrus* alone. Inocula of *Ph.citrophthora* as diverse as local Palestinean soil (Perlberger, 1936), infected alfalfa seeds (Stolzy *et al* ., 1965) and mycelium homogenates (Marks & Kassaby, 1974; Zimmer & Urie, 1967; Fatemi, 1980) have produced disease symptoms in *Citrus* seedlings.

Root rot can be detected in seedlings after keeping their roots in tanks of aerated water containing zoospores alone (Klotz *et al* ., 1958; Klotz & De Wolfe, 1960; Wong & Varghese, 1966) or a mixture of hyphae,sporangia and zoospores (Grimm & Whidden, 1962). Simply dipping roots in zoospore suspensions before repotting has been used to initiate an infection (Ann, 1984). Mycelial plugs, applied to wounded roots (Grimm & Hutchinson, 1977; Sawant *et al*., 1984) or trunks (Klotz *et al* ., 1958; Rosettie, 1947; Klotz *et al* ., 1969; Tuzcu *et al*., 1984; Oxenhm & Stone, 1969; Afeq & Szenberg, 1988) will also produce infection symptoms. Similar methods have been used to inoculate non-*Citrus* host plants (Tucker, 1931; Smillie *et al* ., 1989). Inoculating leaves, despite its widespread use in assessing potato cultivars for resistance to *Ph.infestans* (e.g. Tooley *et al* ., 1985; Cuypers & Hahlbrock, 1987; Caten, 1970) has not been used extensively with *Citrus*. Ann (1984) has recorded using zoospores to inoculate *Citrus* petioles. Detached fruit and intact (Schiffmann & Cohen,1969; Ridings *et al*., 1977; Ann, 1984) or wounded (Oxenham & Stone, 1969) has been inoculated with zoospores to produce disease symptoms.

Difference in the susceptibility of *Citrus* cultivars to infection has been detected, but the mode of experimental infection can influence the results. For example, Tucker (1931) reports that *C.sinensis* and *C. paradisi* seedlings are resistant to *Ph.citrophthora* attack, but the stems of *C.paradisi* become susceptible once wounded. Oxenham and Stone (1969) reported a 75% infection of uninjured fruits inoculated with

*Ph.citrophthora* zoospores compared with a 100% infection on injured fruits by reporting sites of inoculated zoospores which developed infection

Ranking of cultivars resistance varies between workers as shown in Table 5.1. Nevertheless, cultivars such as *Pinocirus trifoliata* and *C.sinensis* are consistently recorded as more resistant to *Phytophthora* than others (e.g. mandarine oranges and grapefruit) by most workers. The ranking of *Citrus* species was reported in terms of lesion size of inoculated fruits (Klotz *et al* ., 1968) or stem (Grimm & Hutchison, 1973; Dimitman & Klotz, 1960). Others reported the ranking in relation to the amount of root rot (Klotz *et al* ., 1958; Grimm & Hutchison, 1973). However, Matheron and Matejka (1986) related the ranking with the size of cankers on excised stem sections.

Diversity within the pathogen population has received much less attention. Single zoospore cultures of *Ph.infestans* derived from a single isolate have been shown to differ markedly among themselves in virulence (Caten, 1970).

To differentiate between *Ph.citrophthora* isolates an objective method of assessing the degree of virulence of standard amounts of each isolate against a set of *Citrus* hosts is required. A method involving inoculating sets of leaf discs with zoospore suspensions has been developed in an attempt to achieve this. Leaf discs were chosen as the test material, despite possible complications from, for example, phytolexin production after wounding because it represented the only source of sufficient *Citrus* material for this study.

Table 5.1. Ranking of resistance in *Citrus* cultivars to *Phytophthora* as reported in the literature (from resistant to susceptible).

| Reference | Ranking of *Citrus* cultivars |
|---|---|
| Fawcette & Bitacourt, 1940 | Tangerine, valencia, grape fruit |
| Fraser, 1942 | Trifoliata, sour oranges, rough lemon |
| Rosettie, 1947 | *C.sinensis* , sweet lime, citrange |
| Furr & Carpenter, 1961 | Citranges(carrizo & yomma), swingle trifoliate |
| Rebour, 1966 | Sour oranges, trifoliate |
| Wonge & Vorghese,1966 | Sour oranges, lemon, mandarine oranges |
| Klotz *et al* ., 1969 | Trifoliate, citrange, mandarine |
| Mendel, 1971 | Poorman, sour orange, sweet lime, rough lemon |
| Grimm & Hutchison, 1973 | Macrophylla, sour oranges, grapefruit |
| Vanderweyen, 1983 | Morton & troyer citranges, sour orange, macrophyla, trifoliate |
| Whiteside, 1974 | Trifoliate, troyer & carrizo citranges |
| Cinar *et al* ., 1976 | Citromelo CRC 1452, trifoliate, sour oranges |
| Cinar & Tuzcu, 1976 | Carrizo citrange, citromelo CRC 1452, sour oranges, trifoliate, macrophylla |
| Tuzcu *et al* ., 1984 | *C.aurantium* , *Pinocirus trifoliata* , *C.aurantium* , *C.micraathe* |
| Sawant *et al* ., 1984 | Cleopetra mandarine, rough lemon, trifoliate |

## 5.2 Materials and methods

### 5.2.1 Host plant cultivation

Seeds of six *Citrus* species (*C.aurantium*, *C.clementine*, *C.josephine*, *C.limmitioidis*, *C.mandarine*, and *C.sinensis* ) were collected from fresh fruits in the West Bank, washed with warm water and dried at room temperature (20-22°C). The seeds were then packed in protective plastic bags and sent by air to Liverpool.

Small plastic pots of 100 $cm^3$ capacity were filled with John Innes seed compost and the seeds were planted, watered and transferred to a heated glasshouse 24 $\pm$ 2°C to germinate. Sixty seeds were chosen randomly from the collection as representative of each *Citrus* species (except for *C.limmitioidis* where all 32 available seeds were planted). Table 5.2 shows germination success.

Seedlings were transferred to 10 cm diameter plastic pots when they were 5 cm tall, using John Innes potting compost supplied with John Innes base fertilizer as 100g per bushel (250 x 250 x 500 cm) measuring composts by volume.

Germination and growth of *C.josephine* was poor and it was excluded from the trials. The seedlings were watered daily, Standard Vitax foliarfeed N,P,K; 20:20:20) being added as 184 g/100 litre of water once a week.

An insecticide (Diflueren zuron) was sprayed prophylectically onto the seedlings to control sciarid fly. Dicofel smokes were used in the glasshouse to control *Tetranychus urticae* mite. *Cryptolaemus motrouzieri* brown beetle was used as a biological control of the *Planococcus citri* mealy bugs.

**Table 5.2.** Germination capacity for the six *Citrus* cultivers

| *Citrus* cultivar | Seeds planted | Seeds germinated | % germination |
|---|---|---|---|
| *C.aurantium* | 60 | 48 | 80 % |
| *C.clementina* | 60 | 38 | 72 % |
| *C.josephine* | 60 | 3 | 5 % |
| *C.limmitioidis* | 32 | 20 | 63 % |
| *C.mandarine* | 60 | 10 | 17 % |
| *C.sinensis* | 60 | 20 | 42 % |
| Total | 332 | 139 | 42 % |

The temperature of the glasshouse varied between 22°C and 35°C all through the cultivation period, relative humidity between 60 and 75% with 12h light of 350-650 µmol $m^{-2}$ $s^{-1}$ intensity and 12 h darkness.

### 5.2.2 Assessment of virulence test method:

#### 5.2.2.1 Determination of leaf surface sterilization conditions:

*Citrus aurantium* leaves were surface sterilized by soaking in 10% Domestos for 5, 10, 20, 30, 40 or 60 min, then washed three times in SDW. NA was cooled to 40°C after autoclaving, leaves were then placed singly inside petridishes and NA was poured over them , the plates rotated to spread the agar evenly and were left to solidify. The plates were inspected after 48 h. The control leaves were washed thrice with SDW only.

#### 5.2.2.2 The effect of zoospore concentration on virulence

*Ph.citrophthora* isolates number IIE3003 and IIE80011 were used in this assessment and *C.clementina* and *C.limmitioidis* leaf discs.

Zoospore concentrations from $10^{6}$-$10^{-1}$ $ml^{-1}$ were prepared by diluting an initial counted suspension appropriately. A volume of 50 µl of each concentration was applied aseptically to the centre of each leaf disc. Eight discs of each *Citrus* cultivar were used for each zoospore concentration. Then the discs were incubated and asessed as described in section 5.3.

### 5.2.2.3 Evaluation of mycelial plugs v Zoospores as inoculum

Both zoospores and mycelial plugs have been used by previous workers to inoculate *Citrus* seedlings or plant parts (see section 5.1). Their use to inoculate leaf discs was compared with respect to differential infection arising from alternate pathogen sources and ease of use.

Six *Ph.citrophthora* isolates were used (IB7002, IB8001, IB8002, IB12002, IB12004) and agar plugs (5mm diameter) cut from the actively growing margin of 3 day old cultures on PDA were placed on the abaxial surface of leaf discs. A total of 15 leaf discs were inoculated for each fungal isolate, 3 discs from each of the *Citrus* cultivars. Incubation and evaluation methods were otherwise as described in section 5.3. Table 5.6 shows the results for the six isolates tested in the same manner, compared with the same six evaluated by zoospore inoculation (as described in section 5.33) at a separate occasion.

### 5.2.2.4 Comparison of *Ph.citrophthora* virulence towards sterile and non-sterile leaf discs

Two sets of leaf discs cut from middle aged leaves of *C.clementina*, *C.limmitioidis* , *C.sinensis* , *C. aurantium* and *C.mandarine* were prepared. Each set contained 38 discs, eight to represent each *Citrus* species (only 6 for *C.mandarine* ). One set was surface sterilized in10% Domestos for 30 min and the other simply washed three times in SDW. The zoospore inoculation and incubation procedures were as described in section 5.3.

### 5.2.2.5 Assessment of disease symptoms by direct inspection or by sterile soil extract assessment

Two methods of disease assessment were compared. To do this, sets of leaves from each of the 5 *Citrus* species were inoculated with zoospores of 16 isolates chosen randomy for the *Ph.citrophthora* collection and incubated as described in section 5.3.

After 8 days, the appearance of the leaves was recorded. Any disease symptoms, namely a change to an olive colour or water-soaked areas were noted. The leaves were then surface sterilized and flooded with SSE.

After 24 h incubation in the light, the plates were assessed. Sporangium production by the SSE flooded discs was recorded as infected.

### 5.2.3 The effect of leaf age on susceptibility to *Ph.citrophthora*

Two isolates of *Ph.citrophthora* were chosen randomly from each of Sites I (IB13002 & IB4002) and II (IIE8001 & IIE12001) and one from the Other Sites (JRD004). These isolates were tested on *C.limmitioidis* and *C.aurantium* leaf discs. Two 12 month old seedlings of each *Citrus* species were chosen randomly and divided into 3 sectors upper (young leaves), middle and lower (old leaves). The leaves were collected from each of the sections and kept in separate plastic bags. The young leaves were bright green, pliable and fully expanded, the middle aged leaves were dark green and leathery, while the old leaves were also dark green and leathery. The four basal leaves (nearest to the soil surface) were not used.

Four leaf discs were used for each leaf age class, from each seedling. The leaf discs were inoculated, incubated and the results recorded as described in section 5.3.3.

### 5.2.4 Standard virulence test method

#### Leaf disc preparation

Leaf discs (26 mm diameter) were cut using a sharp cork borer No.13, from fully expanded, dark green, leathery (middle aged: see section 5.2.3) leaves, avoiding the main vein. They were surface sterilized in 10% Domestos for 30 min washed in 3 changes of sterile distilled water, and then placed 2-3 per petri dish, abaxial surface uppermost. A piece of continually wetted cotton wool in the centre of the petridish maintained a humid atmosphere. The leaf discs were usually cut the day before they were inoculated and stored at 4°C until required.

#### Preparation of zoospores

Isolates of *Ph.citrophthora* were transferred from under oil on OMA slopes to the centres of standard PDA dishes, incubated in the light at 25°C for 3 days. Five mm discs were then cut from the growing edges of the colonies and transferred to CA plates and incubated under fluorescent light at 25°C until sporangia formed (4-6 days), which was assessed by microscopic examination through the bottom of the plates. After ensuring that the mycelia were bearing sporangia they were flooded with SSE:SDW (1:1) and reincubated in the light at 25°C for a further 3 days.

The zoospores were then released after washing the mycelium three times with SDW after which the agar surface was covered with 10 $cm^3$ of SDW. The plates were chilled at 2-8°C for 10-15 min then returned to room temperature (23 $\pm$ 1°C). The sporangia started to release their

zoospores after 10 min and the release was completed within 30-40 min.

The swimming zoospores were counted in a haemocytometer. Concentrations of around 30 x $10^6$ $ml^{-1}$ were routinely obtained. This was diluted to $10^5$ $ml^{-1}$ for inoculation onto leaf discs.

## Leaf disc inoculation

A zoospore suspension of 50 µl (5000 zoospores per disc) and sterile distilled water (50 µl) were placed aseptically in the centre of each leaf disc. The sterile water was added to increase the leaf area exposed to the inoculum. The plates were then wrapped in cling film and incubated in tins for 8 days (dark, 25°C).

## Disease assessment

After 8 days the appearance of the leaf discs was recorded. Discs which had changed colour to olive green, and/or showed water-soaking of part or all the leaf, were recorded as diseased.

All discs were then resterilized (10% Domestos, 30min, washed with 3 changes sterile distilled water) and left in petridishes flooded with sterile soil extract. These plates were incubated for 24 h (35°C, under fluorescent light).

The discs were then inspected microscopically for sporangia, and discs carrying hyphae which produced them were recorded as diseased, by this second criterion.

### 5.2.5 Assessment of virulence of *Ph.citrophthora* isolates

The standard virulence test method (5.2.4) was used. Leaf discs were obtained from *C.clementina*, *C. aurantium*, *C.limmitioides*, *C.sinensis*, and *C.mandarine* . Eight seedlings were used of the first four species, but only 6 of the *C.mandarine* because fewer large plants were available. Sets of 8 (6 of *C.mandarine* ) leaf discs for each species, each disc originating from one seedling were used to test each isolate, a total of 38 discs in all. This strategy was chosen since the *Citrus* seedlings were genetically diverse and none were individually large enough to yield enough material for the whole experiment. Thus by using sets of discs from the same plants, consistency over the experiment was maintained as well as allowing for diversity in resistance to *Ph.citrophthora* within the *Citrus* stocks.

Three sets of *Ph.citrophthora* isolates were assessed for their virulence towards *Citrus* . Two sets represented all isolates obtained from a subsite in each orchard (Subsites IB and IIE), while the third set consisted of all *Ph.citrophthora* isolates obtained during the survey of the Other Sites. Subsites IB and IIE were selected to represent the two intensively surveyed orchards. Subsite IB contained *C.limon* and subsite IIE *C.sinensis* . These two thus contrast in host crop as well as physical location. Isolates from the Other Sites would allow evaluation of diversity across a wider range of geographic and host plant variables. The sets of isolates might also provide information on whether virulence changes with seasons.

### 5.2.6 Statistical analysis

The virulence of isolates was analysed by linear chi-square analysis and by direct chi-square. Log linear analysis was employed where data were collected in a structure and design. Since expected frequencies were less than 5 in many analyses, likelihood estimators were employed. PROC

CATMOD was used for all computations (SAS, 1982). To obtain sufficiently large sample sizes for vigorous statistical testing, data were pooled as required and chi-square calculated directly.

## 5.3 Results

### 5.3.1. Assissment of virulence test method

#### 5.3.1.1 Leaf surface sterilization conditions

The time required to render the leaves sterile as indicated by absence of any contaminants in the NA plates is shown in Table 5.3. The numbers of contaminants dropped drastically at the end of 5 min. A time between 20 and 30 min was the critical time to achieve complete sterilization. The relation between time and the logarithm of contaminated counts is shown in Figure 5.1.

The analysis of variance indicated exponential reduction in the number of contaminants with exposure to Domestos, conforming to conventional death kinetics (Table 5.4).

#### 5.3.1.2 Zoospore concentration and virulence

Table 5.5 indicates that the minimum number of zoospores required from either isolate to cause infection was 500 (A single disc of *C.clementina* was infected at 50 zoospores). At 5000 per disc 75% of *C.clementina* and 37.5-50% of *C.limmitioidis* discs were infected.

This concentration (5000) was chosen as the one for use in the routine test method. This number results in extensive infection of the *Citrus* leaf discs, but also allows some differentiation between cultivars.

Table 5.3. Number of contaminant colonies on exposure of leaves to 10% Domestos.

| Time of exposure | Plates 1 | 2 | 3 | Mean |
|---|---|---|---|---|
| 0 min | 220 | 210 | 190 | 207 ± 8.66 |
| 5 | 40 | 42 | 45 | 42 ± 1.73 |
| 10 | 20 | 30 | 27 | 26 ± 2.88 |
| 20 | 17 | 18 | 15 | 17 ± 1.15 |
| 30 | 0 | 0 | 0 | 0 |
| 40 | 0 | 0 | 0 | 0 |
| 60 | 0 | 0 | 0 | 0 |

Table 5.4. Analysis of variance for contaminant counts along time (min) of treatment with Domestos

| Source | Sum of squares | Degrees of freedom | Mean square | F-ratio | $P_{(HO)}$ |
|---|---|---|---|---|---|
| Regression | 1.619 | 1 | 1.619 | 34.735 | 0.000 |
| Residual | 0.466 | 10 | 0.047 | | |

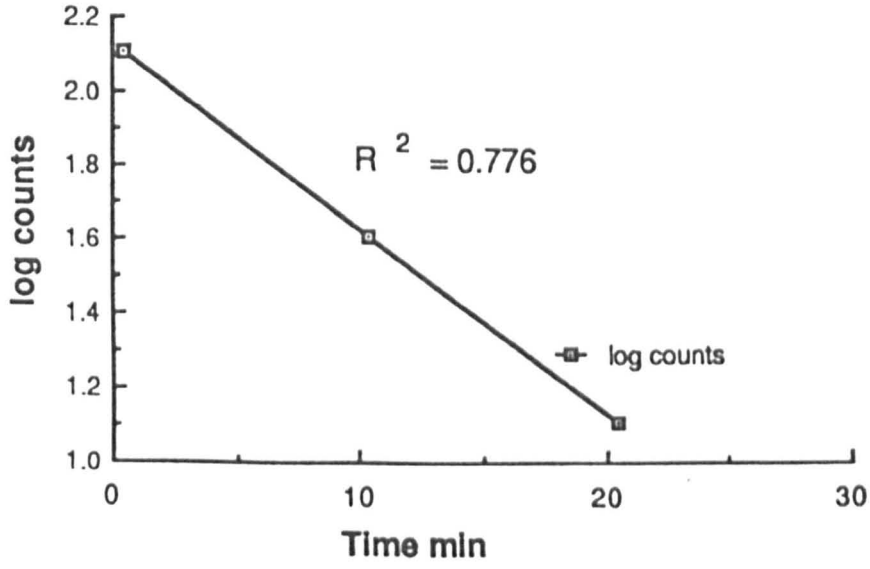



Figure 5.1. Relationship between time and logarithm of number of contaminant counts

**Table 5.5. Effect of zoospore concentration on virulence towards leaf discs of two *Citrus* cultivars.**

| *Citrus* sp. | *C.clementine* | | | | | | | | | | | | *C.limmitioidis* | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Zoospores* | 50,000 | | 5,000 | | 500 | | 50 | | 5 | | 0.5 | | 50,000 | | 5,000 | | 500 | | 50 | | 5 | | 0.5 | 00 |
| Method** | D | S | D | S | D | S | D | S | D | S | D | S | D | S | D | S | D | S | D | S | D | S | D | S | D | S |
| IIE3003 | 8 | 8 | 6 | 6 | 3 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 6 | 7 | 3 | 3 | 2 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| IIE60011 | 8 | 8 | 6 | 6 | 5 | 5 | 1 | 1 | 0 | 0 | 0 | 0 | 6 | 6 | 4 | 4 | 2 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Total | 16 | 16 | 12 | 12 | 8 | 9 | 1 | 1 | 0 | 0 | 0 | 0 | 12 | 13 | 7 | 7 | 4 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

* Actual number of zoospores applied to each disc

**D - Direct inspection S - Sterile soil extract.

### 5.3.1.3 Mycelial plugs and zoospores as inoculum

Table 5.6 shows the results for the six isolates tested in the same manner, compared with the same six evaluated by zoospore inoculum (as described in section 5.2.4) on a separate occasion.

No significant differences were found between mycelial plugs or zoospores as inoculum on leaf discs ($X^2$=1.18, $P_{NH}$=0.3).

This indicates that both methods provide statistically similar results. Zoospores were chosen as the standard inoculum, since concentrations were easily adjusted and they were required for another aspect of the project (growing material for extraction for protein electrophoresis: Chapter 6). Zoospores were thus more convenient.

### 5.3.1.4 Virulence towards sterile and non-sterile leaf discs

The results shown in Table 5.7 indicate that the isolates do not differ in this response towards sterile or non-sterile leaf discs. However, sterilization does significantly lower the number of infected discs as determined by direct inspection ( $X^2$ for direct inspection = 6.8: $X^2_{crit}$ = 3.84). There was no comparable difference when leaf discs were assessed by the sterile soil extract method.

### 5.3.1.5 Assessment of disease symptoms

A comparison between direct inspection and sterile soil extracts is shown in Table 5.8. Neither SSE or DI methods of disease assessment differentiated amongst isolates overal ( $X^2$ = 8.118, P = 0.919). Similarly,

**Table 5.6.** Comparison between the use of zoospores and mycelial plugs as inocula for virulence on leaf discs.

| Isolates | Zoospores | | | | | | | | | | | | Mycelial plugs | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Direct inspection | | | | | | S S E | | | | | | Direct inspection | | | | | | S S E | | | | | |
| | A | B | C | D | E | T | A | B | C | D | E | T | A | B | C | D | E | T | A | B | C | D | E | T |
| IB7002 | 1 | 0 | 1 | 0 | 0 | 2 | 1 | 0 | 1 | 0 | 0 | 2 | 1 | 0 | 0 | 0 | 1 | 2 | 1 | 1 | 1 | 1 | 1 | 5 |
| IB8001 | 2 | 0 | 1 | 1 | 1 | 5 | 2 | 0 | 1 | 1 | 1 | 5 | 2 | 0 | 0 | 0 | 1 | 3 | 2 | 0 | 0 | 1 | 2 | 5 |
| IB8002 | 0 | 0 | 0 | 1 | 1 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 1 | 0 | 1 | 1 | 5 | 1 | 1 | 0 | 1 | 1 | 4 |
| IB11004 | 1 | 0 | 0 | 0 | 2 | 3 | 1 | 0 | 1 | 1 | 2 | 5 | 0 | 1 | 1 | 1 | 2 | 5 | 1 | 1 | 0 | 0 | 1 | 3 |
| IB12002 | 2 | 0 | 1 | 0 | 1 | 4 | 2 | 0 | 1 | 1 | 2 | 6 | 1 | 0 | 1 | 2 | 1 | 5 | 1 | 0 | 1 | 1 | 1 | 5 |
| IB12004 | 1 | 0 | 1 | 1 | 0 | 3 | 0 | 0 | 1 | 0 | 0 | 1 | 2 | 1 | 0 | 1 | 1 | 5 | 2 | 1 | 0 | 1 | 2 | 6 |
| Total | 7 | 0 | 4 | 3 | 5 | 18 | 6 | 0 | 5 | 3 | 5 | 19 | 8 | 3 | 2 | 5 | 7 | 25 | 8 | 4 | 2 | 6 | 8 | 28 |

Note: A.*C.clementina* B.*C.limmitioides* C.*C.sinensis*
D.*C.aurantium* E. *C.mandarine* . T. Total

**Table 5.7. Comparison of *Ph.citrophthora* virulence towards sterile and non sterile leaf discs.**

| Isolate | Sterile leaf discs | | | | | | | | | | | | Non-sterile leaf discs | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Direct inspection | | | | | | sporangia in SSE | | | | | | Direct inspection | | | | | | Sporangia in SSE | | | | | |
| | A | B | C | D | E | T | A | B | C | D | E | T | A | B | C | D | E | T | A | B | C | D | E | T |
| IB8002 | 6 | 2 | 2 | 5 | 4 | 19 | 4 | 2 | 2 | 4 | 3 | 15 | 5 | 4 | 2 | 7 | 5 | 23 | 5 | 3 | 2 | 6 | 4 | 20 |
| IB11004 | 3 | 1 | 3 | 6 | 2 | 15 | 3 | 1 | 2 | 5 | 2 | 13 | 8 | 4 | 4 | 7 | 5 | 28 | 6 | 3 | 4 | 6 | 5 | 24 |
| IIE3003 | 4 | 1 | 5 | 4 | 4 | 18 | 5 | 1 | 5 | 4 | 4 | 19 | 4 | 2 | 6 | 6 | 3 | 21 | 4 | 2 | 5 | 4 | 3 | 18 |
| IIE80011 | 5 | 1 | 3 | 4 | 4 | 17 | 5 | 1 | 4 | 4 | 5 | 19 | 7 | 4 | 5 | 3 | 6 | 25 | 7 | 4 | 4 | 3 | 5 | 23 |
| JRBD004 | 6 | 1 | 3 | 3 | 4 | 17 | 5 | 1 | 3 | 2 | 3 | 14 | 6 | 3 | 2 | 4 | 3 | 18 | 5 | 3 | 3 | 4 | 3 | 18 |
| Control | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 1 | 1 | 2 | 3 | 9 | 0 | 0 | 0 | 0 | 0 | 0 |
| Total | 24 | 6 | 16 | 22 | 18 | 86 | 22 | 6 | 16 | 19 | 17 | 80 | 32 | 18 | 22 | 27 | 25 | 124 | 27 | 15 | 18 | 23 | 20 | 103 |

Note: A.*C.clementina* B.*C.limmitioides* C.*C.sinensis*
D.*C.aurantium* E. *C.mandarine* . T. Total

**Table 5.8.** Comparison of assessment methods for scoring in *Ph.citrophthora* virulence towards five *Citrus* cultivars

| Isolate | Direct inspection | | | | | | Sterile soil extract | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | A | B | C | D | E | T | A | B | C | D | E | T |
| IIE14003 | 3 | 4 | 4 | 3 | 4 | 19 | 4 | 4 | 5 | 3 | 6 | 22 |
| IIE14008 | 3 | 4 | 3 | 5 | 2 | 17 | 4 | 3 | 3 | 5 | 2 | 17 |
| FAR001 | 3 | 2 | 3 | 4 | 2 | 14 | 3 | 2 | 4 | 4 | 3 | 16 |
| FAR003 | 5 | 2 | 2 | 2 | 5 | 16 | 4 | 2 | 5 | 4 | 6 | 21 |
| TMD001 | 2 | 2 | 3 | 4 | 3 | 14 | 3 | 3 | 3 | 4 | 3 | 16 |
| TMD002 | 3 | 2 | 6 | 3 | 3 | 17 | 4 | 3 | 6 | 4 | 3 | 20 |
| TMD003 | 8 | 3 | 2 | 2 | 2 | 17 | 3 | 1 | 4 | 3 | 2 | 13 |
| TMD004 | 5 | 4 | 3 | 2 | 2 | 16 | 3 | 6 | 1 | 5 | 5 | 20 |
| TMD005 | 5 | 4 | 5 | 3 | 3 | 20 | 2 | 2 | 4 | 2 | 2 | 12 |
| TMD006 | 5 | 4 | 3 | 4 | 4 | 20 | 3 | 0 | 3 | 2 | 3 | 11 |
| JRNP001 | 6 | 3 | 4 | 3 | 4 | 20 | 5 | 4 | 6 | 3 | 3 | 21 |
| JRNP004 | 4 | 2 | 3 | 4 | 2 | 15 | 4 | 2 | 3 | 5 | 3 | 17 |
| JRNP005 | 5 | 2 | 5 | 3 | 3 | 18 | 4 | 1 | 4 | 2 | 3 | 14 |
| JRBD003 | 2 | 3 | 4 | 5 | 2 | 16 | 3 | 3 | 5 | 4 | 3 | 18 |
| JRBD004 | 7 | 3 | 5 | 4 | 3 | 22 | 5 | 1 | 5 | 2 | 5 | 18 |
| JRBD006 | 7 | 2 | 3 | 2 | 3 | 17 | 6 | 2 | 4 | 3 | 2 | 17 |
| Total(infected discs) | 73 | 46 | 58 | 53 | 48 | 278 | 60 | 34 | 70 | 55 | 54 | 273 |
| Total(discs used) | 128 | 128 | 128 | 128 | 96 | 1376 | 128 | 128 | 128 | 128 | 96 | 1376 |

Note: A -*C.clementina* B -*C.limmitioides* C -*C.sinensis*
D -*C.aurantium* E -*C.mandarine* T- Total

host susceptibility did not differ according to the method of assessment ($X^2$ = 2.84, P =0.58).

These results indicate the two inspection methods led to statistically indistinguishable assessment of virulence of *Ph.citrophthora* towards *Citrus* cultivars.

### 5.3.2 Effect of age on susceptibility

Table 5.9 shows the results of the assessment of the age effect on susceptibility to *Ph.citrophthora*.

There were no significant differences between plants in their reaction towards isolates ( $X^2$ = 5.7849, $X^2$ critical = 5.9).

Pooling over all plants and isolates, there was a significant difference in the reaction of isolates towards leaf discs of the three age groups ( $X^2$ = 17.14, $X^2$ critical = 5.9).

The contribution from effects on old leaves was responsible for most of the variation. These results indicate that age of leaves has a significant effect on the susceptibility of discs to infection.

### 5.3.3 Fidelity of test procedure

The pathogenicity scores of 12 randomly chosen isolates observed by SSE method on two separate trials are given in Table 5.10 in relation to five hosts. Table 5.11 gives the log linear chi-square analysis and indicates that isolates did not differ in their pattern of response to the host in either of the two trials. Pooling scores over all hosts and isolates did however reveal that pathogenicity was on average higher in the second trial than the first ( $X^2$ = 4.35, $P < 0.05$).

Table 5.9. The effect of *Citrus* leaf age on susceptability to *Ph.citrophthora* .

| Isolate | *C.limmitioides* | | | | | | *C.aurantium* | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Seedling1 | | | Seedling 2 | | | Seedling 1 | | | Seedling 2 | | |
| | Y* | M | O | Y | M | O | Y | M | O | Y | M | O |
| IIE80011 | 2 | 3 | 1 | 1 | 2 | 0 | 4 | 3 | 1 | 4 | 2 | 2 |
| IB13002 | 3 | 2 | 1 | 4 | 3 | 1 | 4 | 3 | 1 | 4 | 3 | 1 |
| IIE12001 | 4 | 4 | 2 | 4 | 3 | 2 | 4 | 2 | 2 | 4 | 2 | 1 |
| IB4002 | 1 | 0 | 0 | 0 | 0 | 0 | 4 | 3 | 2 | 4 | 1 | 2 |
| JRBD004 | 1 | 0 | 0 | 1 | 0 | 1 | 4 | 3 | 2 | 4 | 3 | 2 |
| Total | 11 | 9 | 4 | 10 | 8 | 4 | 20 | 14 | 8 | 20 | 11 | 8 |

* Y - Young leaves
M - Middle age leaves
O - Old leaves.

Total = 20
( 4 / test )

**Table 5.10.** Pathogenicity of 12 randomly chosen isolates of *Ph.citrophthora* assessed using the SSE method to the five *Citrus* hosts in two independent trials.

| Isolate | First trial | | | | | | Second trial | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | A | B | C | D | E | T | A | B | C | D | E | T |
| IB13002 | 6 | 3 | 6 | 7 | 5 | 27 | 4 | 1 | 4 | 6 | 3 | 18 |
| IB13006 | 6 | 1 | 3 | 2 | 3 | 15 | 2 | 1 | 4 | 5 | 4 | 16 |
| IIE3003 | 1 | 2 | 6 | 5 | 4 | 18 | 5 | 1 | 3 | 5 | 5 | 19 |
| IIE7002 | 5 | 5 | 5 | 8 | 2 | 25 | 4 | 3 | 5 | 5 | 5 | 22 |
| IIE80011 | 6 | 0 | 1 | 6 | 1 | 14 | 7 | 2 | 4 | 6 | 6 | 25 |
| IIE11003 | 4 | 2 | 4 | 5 | 3 | 18 | 6 | 1 | 6 | 7 | 3 | 23 |
| IIE12001 | 4 | 1 | 3 | 2 | 3 | 13 | 7 | 6 | 7 | 6 | 6 | 32 |
| FAR003 | 4 | 2 | 5 | 4 | 6 | 21 | 8 | 1 | 5 | 6 | 4 | 24 |
| TMD003 | 3 | 1 | 4 | 3 | 2 | 13 | 5 | 2 | 7 | 6 | 3 | 15 |
| TMD006 | 3 | 0 | 3 | 2 | 3 | 11 | 4 | 3 | 1 | 5 | 2 | 15 |
| JRNP001 | 5 | 4 | 6 | 3 | 3 | 21 | 5 | 1 | 6 | 4 | 4 | 22 |
| JRBD004 | 5 | 1 | 5 | 2 | 5 | 18 | 5 | 1 | 7 | 6 | 6 | 26 |
| Total(infected discs) | 52 | 22 | 51 | 49 | 40 | 214 | 62 | 23 | 59 | 70 | 51 | 265 |
| Total(discs used) | 96 | 96 | 96 | 96 | 72 | 456 | 96 | 96 | 96 | 96 | 72 | 456 |

Note: A - *C.clementina* B - *C.limmitioides* C -*C.sinensis*
D - *C.aurantium* E - *C.mandarine* T - Total

Table 5.11. Log likelihood analysis of the pattern of virulence responses of 12 randomly chosen isolates of *Ph.citrophthora* to 5 *Citrus* hosts in two independent trials.

| Source | DF | Chi-square | $P_{(HO)}$ |
|---|---|---|---|
| Trial | 11 | 10.47 | 0.4882 |
| Host | 44 | 21.21 | 0.9985 |
| Trial X Host | 44 | 20.69 | 0.9989 |
| Likelihood ratio | 0 | 0.00 | 1.0000 |

Table 5.12. Total number of leaf discs (pooled over isolates) of each *Citrus* host with disease symptoms observed by the SSE.

| *Citrus* cultivar | Total SSE | Discs used for the assessment |
|---|---|---|
| *C.clementina* | 230 | 413 |
| *C.limmitioides* | 116 | 413 |
| *C.sinensis* | 224 | 413 |
| *C.aurantium* | 225 | 413 |
| *C.mandarine* | 179 | 333 |
| Total | 974 | 1985 |

The data also suggests that isolates different in their virulence and that host susceptibility varied with *C.limmitioides* being the least susceptible and *C.clementina* and *C.aurantium* the most.

The two trials were conducted on 26th April, and 23rd June 1989, and the lack of difference observed between the trials indicates that the test procedure employed was reliable.

### 5.3.4 Variation in susceptibility of *Citrus* hosts

Table 5.12 shows the total number of leaf discs pooled over isolates of each *Citrus* host with disease symptoms observed by the SSE assessment method.

The total number of diseased leaf discs of each *Citrus* host showed statistical differences in host susceptibility ( $X^2 = 34$, $P < 0.05$).

The least susceptible host was *C.limmitiodes* where 27% of the tested discs were infected, while the other 4 hosts were of similar susceptibility level, *C.sinensis* 52.2%, *C.aurantium* 52.4%, *C.clementina* 53.6% and *C.mandarine* 53.8%, noting that only 6 discs were used for assessing the last host instead of 8 discs for the others.

### 5.3.5 The differential response of isolates to the host range

Table 5.13 gives the pattern of infection response to the five *Citrus* hosts for all 63 isolates examined. Isolates 1-15 were excluded from analysis owing to the preponderance of zeroes in the data set. Log linear analysis indicated that there were no significant differences in the pattern of responses to hosts.

Considering isolates individually, a variation can be noticed in the response of some of the isolates. As example, isolates 56 (TMD005) and

**Table 5.13.** The number of infected leaf discs which produced sporangia as an indication of infection using SSE assessment method for virulence of 63 *Ph.citrophthora* isolates towards five *Citrus* hosts.

| | | *Citrus* cultivers | | | | | |
|---|---|---|---|---|---|---|---|
| | Isolate | A | B | C | D | E | Total |
| 1. | IB2001 | 1 | 0 | 0 | 1 | 0 | 2 |
| 2. | IB4001 | 0 | 0 | 1 | 1 | 0 | 2 |
| 3. | IB4002 | 0 | 0 | 2 | 0 | 0 | 2 |
| 4. | IB6001 | 0 | 0 | 2 | 0 | 2 | 4 |
| 5. | IB7001 | 1 | 0 | 1 | 1 | 1 | 4 |
| 6. | IB7002 | 1 | 0 | 1 | 0 | 0 | 2 |
| 7. | IB7003 | 0 | 0 | 0 | 1 | 1 | 2 |
| 8. | IB7004 | 0 | 0 | 1 | 1 | 1 | 3 |
| 9. | IB7005 | 0 | 0 | 0 | 1 | 1 | 2 |
| 10. | IB8001 | 2 | 0 | 1 | 1 | 1 | 5 |
| 11. | IB8002 | 0 | 0 | 0 | 0 | 0 | 0 |
| 12. | IB11004 | 1 | 0 | 1 | 1 | 2 | 5 |
| 13. | IB12002 | 2 | 0 | 1 | 1 | 2 | 6 |
| 14. | IB12004 | 0 | 0 | 1 | 0 | 0 | 1 |
| 15. | IB12005 | 0 | 0 | 0 | 1 | 0 | 1 |
| 16. | IB12006 | 7 | 1 | 5 | 6 | 4 | 23 |
| 17. | IB12007 | 3 | 2 | 5 | 5 | 3 | 18 |
| 18. | IB120010 | 5 | 3 | 6 | 6 | 6 | 28 |
| 19. | IB120012 | 3 | 2 | 8 | 7 | 3 | 23 |
| 20. | IB13001 | 7 | 3 | 4 | 4 | 6 | 24 |
| 21. | IB130026 | 3 | 7 | 7 | 5 | 2 | 28 |
| 22. | IB13005 | 6 | 1 | 3 | 5 | 4 | 19 |
| 23. | IB13006 | 6 | 1 | 3 | 2 | 3 | 15 |

| | Isolate | A | B | C | D | E | Total |
|---|---|---|---|---|---|---|---|
| 24. | IB13007 | 4 | 2 | 5 | 6 | 3 | 20 |
| 25. | IB13008 | 5 | 3 | 5 | 3 | 4 | 20 |
| 26. | IIE3003 | 1 | 2 | 5 | 6 | 4 | 18 |
| 27. | IIE5001 | 4 | 4 | 3 | 5 | 2 | 18 |
| 28. | IIE7001 | 3 | 2 | 5 | 7 | 1 | 18 |
| 29. | IIE7002 | 5 | 5 | 5 | 8 | 2 | 25 |
| 30. | IIE7003 | 6 | 2 | 6 | 5 | 3 | 22 |
| 31. | IIE7004 | 4 | 4 | 5 | 3 | 3 | 19 |
| 32. | IIE8001 | 8 | 2 | 4 | 4 | 5 | 23 |
| 33. | IIE8004 | 5 | 3 | 4 | 5 | 3 | 20 |
| 34. | IIE8005 | 5 | 4 | 5 | 6 | 4 | 24 |
| 35. | IIE8008 | 5 | 2 | 3 | 6 | 5 | 21 |
| 36. | IIE8009 | 7 | 3 | 3 | 4 | 4 | 21 |
| 37. | IIE80011 | 6 | 0 | 1 | 6 | 1 | 14 |
| 38. | IIE80014 | 7 | 2 | 3 | 5 | 4 | 21 |
| 39. | IIE80015 | 7 | 4 | 6 | 7 | 5 | 29 |
| 40. | IIE9001 | 6 | 4 | 3 | 5 | 3 | 21 |
| 41. | IIE9003 | 6 | 3 | 3 | 4 | 4 | 20 |
| 42. | IIE11003 | 4 | 2 | 4 | 5 | 3 | 18 |
| 43. | IIE11004 | 5 | 2 | 5 | 4 | 4 | 20 |
| 44. | IIE112001 | 4 | 1 | 3 | 2 | 3 | 13 |
| 45. | IIE12003 | 3 | 2 | 4 | 3 | 4 | 16 |
| 46. | IIE14001 | 4 | 4 | 6 | 3 | 4 | 21 |
| 47. | IIE14002 | 6 | 4 | 3 | 5 | 2 | 20 |
| 48. | IIE14003 | 4 | 4 | 5 | 3 | 6 | 22 |
| 49. | IIE14005 | 4 | 3 | 3 | 5 | 2 | 17 |
| 50. | FAR001 | 3 | 2 | 4 | 4 | 3 | 16 |

| | Isolate | A | B | C | D | E | Total |
|---|---|---|---|---|---|---|---|
| 51. | FAR003 | 4 | 2 | 5 | 4 | 6 | 21 |
| 52. | TMD001 | 3 | 3 | 3 | 4 | 3 | 16 |
| 53. | TMD002 | 4 | 3 | 6 | 4 | 3 | 20 |
| 54. | TMD003 | 3 | 1 | 4 | 3 | 2 | 13 |
| 55. | TMD004 | 3 | 1 | 6 | 5 | 5 | 20 |
| 56. | TMD005 | 2 | 2 | 4 | 2 | 2 | 12 |
| 57. | TMD006 | 3 | 0 | 3 | 2 | 3 | 11 |
| 58. | JRNP001 | 5 | 4 | 6 | 3 | 3 | 21 |
| 59. | JRNP004 | 4 | 2 | 3 | 5 | 3 | 17 |
| 60. | JRNP005 | 4 | 1 | 4 | 2 | 3 | 14 |
| 61. | JRBD003 | 3 | 3 | 5 | 4 | 3 | 18 |
| 62. | JRBD004 | 5 | 1 | 5 | 2 | 5 | 18 |
| 63. | JRBD006 | 6 | 2 | 4 | 3 | 2 | 17 |

Note 1. A -*C.clementina* B -*C.limmitioides* C -*C.sinensis*
D -*C.aurantium* E -*C.mandarine* .

2. For assessments of isolates 1-15 , three discs of each cultivar were used making a total of 15 discs for each isolate, while for the other 48 isolates in the assessment 8 discs were used from each cultivar (only 6 discs from *C.mandarine* ) making a total of 38 discs

57 (TMD006) were the least virulent isolates in the assessment as only 12 and 11 discs (respectively) were infected, while isolates 18 (IB120010), 21 (IB13002) AND 39 (IIE80015) were the most virulent when 28, 28 and 29 discs (respectively) were infected. However, this could not be related to the geographic distribution or origin (isolation method) of the isolates.

## 5.4. Discussion

The large number of isolates available made an exhaustive examination impossible within the time available. All *Ph.citrophthora* isolates from subsite B of Site I, subsite E of Site II and those from the Other Sites were therefore assessed.

The *Citrus* host species were expected to vary in their susceptibility to *Phytophthora* . Of the hosts available *C.josephine* showed very low seed germination and thus only five species were used.

Leaf age affected susceptibility to *Ph.citrophthora* infection, with old leaves showing more resistance than young or middle aged leaves. Thus mixing leaf ages in virulence assessment would be a misleading factor.In technique, leaf inoculation would have been possible if there had been a sufficient number of uniform middle aged leaves (Section 5.3.2.). As this was not the case, leaf discs of a uniform size were chosen from "middle aged" leaves and used in the assessment in order to reduce likely additional sources of variation in the virulence assessment. Eight seedlings from each species (6 from *C.mandarine* , due to the lower germination rate, slow growth of seedlings and smaller surface area of leaves) were used in the assessment of virulence.

Surface sterilization of leaf discs, which was efficient after 20 to 30 min exposure in 10% Domestos solution, did not have an effect on the

virulence of isolates towards sterile leaf discs.

Zoospore suspensions were as suitable as mycelial plugs for assessment of virulence, as shown in section 5.3.1.3. However, both techniques did not show variation amongst the isolates in virulence towards *Citrus* species.

The concentration of the zoospore inoculum was a critical factor in the assessment of virulence. A standard suspension containing 5,000 zoospores was adopted to allow some differentiation between *Citrus* hosts. This concentration is less than that used by Caten (1970) on inoculating potato leaves in studying virulence of *Ph.infestans* , where 20,000 per leaf were used to assess variation which was the least number of zoospores capable of showing clear variation, However, in the present study when 50,000 zoospores were used, all discs were infected. This would have made it difficult to assess variation and may be due to difference between the pathogenic *Phytophthora* and host plant pathogens.

The direct inspection method of disease assessment has been used by other workers ( e.g. Caten, 1970; Ann, 1984; Ho *et al* ., 1984). Results from this method were indistinguishable from those using the soil extract method.

Repeatability of the virulence test procedure was indicated by no significant differences between two trials ( Table 5.10). However, a closer examination of the results showed some differences in the ranking between the first and the second trial which might be attributed to the decrease or increase in virulence of some isolates over time or the effect of seasonality on some seedlings in the assessment( Matheron & Matejka, 1986).

Statistical differences in host susceptibility were detected among *Citrus* hosts. This varied between 28% in the overall infection of the *C.limmitioides* leaf discs to around 50% of the other hosts. In the

contrasting reports in the literature on the ranking of resistance in *Citrus* species (Table 5.1), two reports were found to rank *C.limmitioides* (Mendel, 1971; Sawant *et al* ., 1984) . Both ranked this cultivar as susceptible to invasion by *Ph.citrophthora* , either on a par with *C.aurantium* (Sawant *et al* .,1984) or as more susceptible. This is an indication of difficulty in ranking the susceptibility of the species in relation to other reports in the literature.

No statistically significant differences were found among the virulence of the isolates. However, differences can be seen on examining individual isolates. This variation do not follow any specific pattern in relation to geographic location or method of recovery from the field. This may be due to the absence of significant differences in the genetic structure of the isolates inspite of the geographic differences between the Permanent Sites and the Other Sites in the survey.

If this assessment was extended to include virulence towards the whole seedling, in a different environment, more variation may have resulted among the isolates. *Ph.nicotianae* whch was not assessed for virulence in this study could also be examined for variation in the same way.

Absence of variation indicates uniformity in the pathogen population as regards this fitness character, indicting that no changes in virulence have taken place over time inspite of the great differences between the environmental conditions within the sampling sites.

# CHAPTER SIX

## Protein and isozyme variability

## 6.1. Introduction

The determination of soluble protein patterns by gel electrophoresis is one of the promising physiologic methods which can be applied to classification and population genetics of *Phytophthora* species (Faris *et al* ., 1986). It can be less difficult and time consuming than serologic methods (Gallegaly, 1983), provide support for other methods of species identification ( Ribiero, 1978) and give evidence of intraspecific variation (Hansen *et al* ., 1986). Gene products which are protein in nature can be separated using this technique by either using starch or polyacrylamide gels (Hames & Rickwood, 1981). Using these methods for native proteins leads to fractionation on the basis of size and/or charge with preservation of subunit interaction, protein conformation and biological activity. Some studies employ electrophoresis using a denaturing buffer system to dissociate proteins into their individual polypeptide subunits, by heating in the presence of sodium dodecyl sulphate and a reducing agent, leading to denaturation and stoichiometric binding of the SDS to the polypeptides which unfold but remain intact (Kowit & Maloney, 1982). This can provide good estimates of molecular weight.

The gels can be stained with histochemical stains for proteins using amidoblack (Erselius & Shaw, 1982), coomassie blue (Bielnin *et al* ., 1988), colloidal acid violet (Patestos *et al* ., 1988), silver (Blum *et al* ., 1987) or double staining with coomassie blue and silver (Egly & Garaurd, 1984).

Enzyme activity of native proteins in the total protein extracts can be detected in the gel after electrophoresis by using specific stains. A particular protein generally possesses a characteristic electrophoretic mobility under defined conditions. However, a protein sequence is dependent on its DNA coding. Individual enzymes may be coded by more than one genetic locus or allele which result in different amino acid contents or sequences of the molecules. These can be detected as enzyme bands of differing electrophoretic mobility. Due to polymorphism in enzyme systems , allozyme phenotypes (appearance of several isozymes) displayed by particular populations can be significant criteria in placing isolates within a particular taxa (Barrett, 1986). The electrophoretic patterns of soluble proteins in the cell will give a direct manifestation of its genetic constitution and these patterns can be of taxonomic value (Clare *et al* ., 1968). The advantage over simply measuring enzyme activity in protein suspensions is that isozymes, multiple forms of an enzyme, can be readily resolved and detected. Species and individuals differ in isozymes.

Gel electrophoresis has been used with a wide range of fungi including *Mortierella* (Peberdy & Turner, 1968), *Fusarium* (Meyer& Reynard, 1969), *Mucor* (Stout & Shaw, 1974), *Puccinia* (B urden & Marshall, 1981), dermatophytes (Jones & Noble, 1982), *Saprolegnia* (Beaks & Ford, 1983), *Rhizopus* (Seviour *et al* ., 1985), *Sclerotinia* (Tariq *et al* ., 1985) and *Ascosphaera* (Maghrabi & Kish, 1985). The enzyme patterns in these and other related studies have been used to quantify genetic variation of specific fungi.

### 6.1.1. Electrophoretic analysis of *Phytophthora*

Both proteins and enzyme patterns have been examined in *Phytophthora* (e.g. Clare & Zentmyer, 1966; Hall *et al* .,1969). The major interest has been in their value in identification, particularly for " difficult" species, like those in the *palmivora* group, and as genetic markers. Early work showed that total protein patterns could distiguish between species, but the small numbers of isolates examined did not initially allow detection of intraspecific variation. Clare and Zentmyer (1966) differentiated between *Ph.cinnamomi* , *Ph.palmivora* and *Ph.citrophthora* on the basis of their protein patterns. Similarly, *Ph.cactorum* , *Ph.fragrarae* and *Ph.sojae* species could be delimited (Gill & Powell, 1968 a). Indeed, in a later study the species *Ph.fragrarae* could be subdivided into 8 groups on the basis of total protein patterns (Gill & Powell, 1968 b). Other members of the *palmivora* group have also been distinguished on the basis of protein patterns, with subspecific grouping possible on the basis of enzyme patterns ( *Ph.cinnamomi* , *Ph.cactorum* and *Ph.palmivora* ; Hall et al ., 1969; *Ph.cinnamomi*, *Ph.capsici* and *Ph.palmivora* ; Kaosiri & Zentmyer, 1980).

In studies aimed at species deliniation, protein electrophoresis has been used as one means of identification (*Ph.citricola* , Zentmyer *et al* ., 1974; *Ph.psudoptogea* , Hamm & Hansen , 1982; *Ph.megasperma* , Hansen *et al* ., 1986; and Nygard *et al* ., 1989).

Extensive studies on the use of enzymes as genetic markers have been made in *Ph.infestans* , resulting in the identification of some suitable loci ( Whittaker, 1989; Castro, 1989; El-Korany and Sattar, 1989; Spielman, 1989).

Previous studies of *Ph.citrophthora* and *Ph.nicotianae* by protein electrophoresis have revealed both inter and intra specific variation in these species.

Valavieille and Erselius (1984) were able to divide *Ph.citrophthora* from *Citrus* into two groups depending upon their protein profiles, esterases and acid phosphatase using isoeletcric focusing, and isozyme analysis. Oudemans, 1989 using cellulose acetate as a supportive medium was able to divide *Ph.citrophthora* into two groups which were obtained from cocoa and *Citrus*.

Some studies concerning zymograms have failed to reveal any bands and/or the banding pattern was inconsistant. Examples are, hexokinase and xanthine dehydrogenase (Nygaard *et al* .,1989), leucine amino peptidase (Erselius & Shaw, 1982; Tooley *et al* ., 1985), alkaline phosphatase (Erselius & Shaw, 1982) and esterase ( Tooley *et al*..,1985). Staining for other enzymes was successful, e.g. glucophospho isomerase, malate dehydrogenase, lactate dehydrogenase and malic enzyme ( Tooley *et al* ., 1985; Nygaard *et al* ., 1989).

The aim of this study was to extend the assessment of variation within *Ph.citrophthora* isolates and between *Ph.citrophthora* and *Ph.nicotianae* using electrophoretic patterns of SDS protein profiles and zymograms for acid phosphatase, alkaline phosphatase, esterase, lactate dehydrogenase, malate dehydrogenase, xanthine dehydrogenase, malic enzyme, tetrazolium oxidase, hexokinase, leucine amino peptidase and glucophospho isomerase. Variation with respect to sampling site was also of interest. Sample preparation methods will be assessed first, followed by the electrophoretic conditions, namely gel preparation, electrophoresis

conditions and staining procedures. Then the preliminary studies will follow on mycelium age, culture medium, extraction method and use of gel under various electrophoresis conditions.

## 6.2. Materials and Methods

### 6.2.1. Preparation of protein extracts

Erlenmeyer flasks (250 $cm^3$) containing 100 $cm^3$ of DR were inoculated with 3 x $10^5$ zoospores, incubated on a Gallenkamp orbital shaker at 100 rpm, at 23±2°C for 5 days. Zoospore suspensions were prepared as described in section 5.2.4. Mycelial mats were harvested by filtration through gauze mounted on a Buchner funnel under vacuum, washed thrice with 100 $cm^3$ of chilled distilled water followed by a final wash with 100 $cm^3$ of chilled 25 mM HEPES buffer, pH 7.0. The mycelia were weighed and 2 g (wet weight) were used for protein extraction.

A pestle and mortar were prechilled with liquid nitrogen, the mycelium added with an excess of liquid nitrogen and this was triturated carefully until no intact mycelium was visible on microscopic examination. The fine powder was collected in an ice chilled Potter homogenizer, 4 $cm^3$ of chilled 25 mM HEPES buffer (pH 7.0) added and homogenized to a uniform slurry. The slurry was then transferred to 15 $cm^3$ plastic tubes, centrifuged in a Baird and Tatlock Mark IV refrigerated centrifuge at 3 °C, at 5000 rpm for 30 min. The middle layer was collected using a pasteur pipette, avoiding the top lipid layer, transfered dropwise to an expanded polystyrene cup containing liquid nitrogen to form frozen pellets instantly. These were stored at -20 °C until required. The protein concentration

concentration was measured using the method of Bradford (1976), as described below, with bovine albumin as a standard. Glucose and protein concentrations of all isolates are shown in Appendix 5.

**Bradford protein assay -micro method** (after Bradford, 1976)

**Solution**

| | |
|---|---|
| Coomassie Brilliant Blue G-250 | 50 mg |
| ethanol 95% | 25 $cm^3$ |
| phosphoric acid | 50 $cm^3$ |

The stain was dissolved in the methanol, the phosphoric acid added, then the volume was diluted to 500 ml with distilled water and filtered.

**Procedure**

A volume of 1 $cm^3$ colour reagent was added to protein extracts containing in the range of 1 - 10 $\mu g$ protein in a volume of 0.1 $cm^3$ (adjusted with 0.25 mM HEPES buffer of pH 7.0). The absorbance at 595 nm was measured using an LKB Ultrospec 4050 spectrophotometer after at least 3 min, but not more than 1 h. The sample blank constituted a volume of 1 $cm^3$ colour reagent added to 0.1 $cm^3$ buffer reference( or distilled water when using the standard protein). The standard protein was prepared from bovine serum albumin (Sigma) used as above.

## 6.2.2.Electrophoretic conditions

### 6.2.2.1.Gel preparation

Three different types of gel system were used, namely homogenuous native, SDS homogeneous and gradient native polyacrylamide gels.

**A. Homogeneous polyacrylamide gel (12.5%) (after Davis,1964)**

**a. Separation gel and plug**

| | |
|---|---|
| 1M tris HCl buffer pH 8.9 | 7.50 $cm^3$ |
| acrylamide45% | 9.75 $cm^3$ |
| (acrylamide: bis-acrylamide 45 :1.2) | |
| water | 39.75 $cm^3$ |
| ammonium persulphate (10 mg $cm^{-3}$) | 3.00 $cm^3$ |
| TEMED | 60.00 $\mu m^3$ |
| Total | 60.00 $cm^3$ |

For the homogeneous and SDS gels, the acrylamide and bis-acrylamide were weighed, dissolved in water, made up to 100 $cm^3$ and filtered through Whatman number 1 filter paper.

The buffer and water were added to the acrylamide, then divided into two (28.5 $cm^3$ each) and 30 $\mu m^3$ TEMED were added to each half. Finally 1.5 $cm^3$ of ammonium persulphate were added to each portion when ready to pour. One half was used to seal the gel cassette and the other as a separation gel.

b. Stacking gel

| | |
|---|---|
| 1M tris orthophosphoric acid buffer pH 6.9 | 2 $cm^3$ |
| (6.4 $cm^3$ 0.1 M orthophosphoric acid :1.43g tris) | |
| acrylamide10% | 2 $cm^3$ |
| (acrylamide: bisacrylamide10: 2.5 ) | |
| water | 2 $cm^3$ |
| ammonium persulphate (10 mg $cm^{-3}$) | 2 $cm^3$ |
| TEMED | 20 $\mu m^3$ |
| Total | 8.0 $cm^3$ |

c. Electrophoresis buffer (pH 8.3)

| | |
|---|---|
| 25 mM tris-HCl | 3.0 g |
| 192 mM glycine | 14.4 g |
| Water | 1000 $cm^3$ |

## B. SDS Acrylamide Gels

a. Stock solutions

| | |
|---|---|
| 1M tris HCl pH 8.8 | 60.57 g tris/500 $cm^3$ |
| 0.5M tris HCl pH 6.8 | 30.285 g tris/500 $cm^3$ |

| | |
|---|---|
| 10% SDS | 50 g /500 $cm^3$ |
| 30% acrylamide, filtered | 30 g acrylamide + 0.8 g bis-acrylamide/100 $cm^3$ |
| ammonium persulphate ( freshly prepared ) | 150 mg / 10 $cm^3$ |

**b. Disaggregating buffer**

| | |
|---|---|
| 0.5M tris HCl buffer, pH 6.8 | 3.74 $cm^3$ (0.187 M tris/HCl) |
| SDS | 0.6 g (6 %) |
| glycerol | 3.0 $cm^3$ (30%) |
| β-mercaptoethanol | 1.5 $cm^3$ (15%) |
| water | 1.4 $cm^3$ |
| bromophenol blue | 1-3 mg |
| total volume | 10 $cm^3$ |

( Prepared in a volumetric flask )

Half volume disaggregating buffer was added to each sample, mixed and boiled in a water bath for 2 mins.

**c. SDS separation gel and sealant (12.5%)**

| | |
|---|---|
| 1.0M tris HCl, pH 8.8 | 18.75 $cm^3$ |
| 10% SDS | 0.5 $cm^3$ |
| 30% acrylamide | 20.8 $cm^3$ |

| | | |
|---|---|---|
| water | 4.9 | $cm^3$ |
| ammonium persulphate | 5.0 | $cm^3$ |
| TEMED | 100 | $\mu m^3$ |
| total volume | 50 | $cm^3$ |

Gel solutions were prepared, minus TEMED. Ten or 20 $cm^3$ were used as a sealent , after adding 10 $\mu m^3$ TEMED to10 $cm^{-3}$ of the gel solution to initiate polymerization. The rest of the TEMED was then added to the gel solution which was poured into the glass casette and allowed to set for use as a separation gel . Both the sealent and the separation gel required 25 - 30 min to set.

**d. Stacking gel**

| | |
|---|---|
| 0.5 M tris/ Hcl, pH 6.8 | 5.0 $cm^3$ |
| 10% SDS | 0.2 $cm^3$ |
| 30% acrylamide | 4.0 $cm^3$ |
| water | 8.5 $cm^3$ |
| ammonium persulphate | 2.0 $cm^3$ |
| TEMED | 40 $\mu m^3$ |
| total volume | 19.7 $cm^3$ |

The TEMED was added when ready to pour and after removal of the water layer from the top of the separation gel.

**e. Electrophoresis buffer ( pH 8.3 )**

| | |
|---|---|
| 25mM tris base | 3 g |
| 192mM glycine | 15 g |
| 0.1% SDS | 1 g |
| water | 1000 $cm^3$ |

The chemicals were dissolved in 1000 $cm^3$ of distilled water and the pH adjusted to 8.3 using NaOH and HCl.

## C. Gradient polyacrylamide gel

### a. Preparation of 5% gel

| | |
|---|---|
| acrylamide45% (acrylamide: bis acrylamide45:1.2) | 1.6 $cm^3$ |
| tris HCl Buffer (pH8.9) | 2.0 $cm^3$ |
| ammonium persulphate (10mg.$cm^3$) | 0.5 $cm^3$ |
| water | 10.9 $cm^3$ |
| TEMED | 10 $\mu m^3$ |
| Total | 15.0 $cm^3$ |

### b. Preparation of 25% gel

| | |
|---|---|
| acrylamide 45% (acrylamide:bisacrylamide 45:1.2) | 8.25 $cm^3$ |

| | |
|---|---|
| 1M Tris HCl Buffer (pH8.9) | 2.0 $cm^3$ |
| ammonium persulphate (10mg $cm^{-3}$) | 2.0 $cm^3$ |
| water | 2.75 $cm^3$ |
| total volume | 15.0 $cm^3$ |

**c. Sealant gel**

| | |
|---|---|
| acrylamide 45% (acrylamide: bis acrylamide 45:1.2 ) | 2.5 $cm^3$ |
| 1M tris HCl buffer pH8.9 | 1.0 $cm^3$ |
| ammonium persulphate (10mg $cm^{-3}$) | 1.0 $cm^3$ |
| water | 2.5 $cm^3$ |
| TEMED | 10 $\mu m^3$ |
| total volume | 7.0 $cm^3$ |

**d. Stacking gel**

| | |
|---|---|
| acrylamide 10% (acrylamide:bisacrylamide 10:2.5) | 2.5 $cm^3$ |
| 1M tris orthophosphoric acid buffer pH 6.9 | 1.5 $cm^3$ |
| ammonium persulphate (10mg $cm^{-3}$) | 2.0 $cm^3$ |
| water | 1.5 $cm^3$ |
| TEMED | 10 $\mu m^3$ |
| total volume | 7.5 $cm^3$ |

e. Gradient polyacrylamide gel preparation

The gel cassette was sealed with water proof tape, and additionally with a concentrated , rapidly setting gel solution. Two further solutions were prepared, one containing 5% acrylamide and a few crystals of bromophenol blue and the other 25% acrylamide only. The gradient maker consisted of two glass cylinders joined by flexible rubber tubing. The tubing was initially closed by a haemostat clip. The 5% gel cylinder was raised slightly with respect to the 25% gel so that the solution would flow out easily once the clip was removed. An even flow could be observed by the bromophenol blue passing along the connection tube from the 5% to the 25% cylinder. The 25% gel was stirred continuously with a small magnetic stirrer. The gradient gel was run into the centre of the cassette and allowed to set once it was within a few centimetres of the top. The stacking gel was then poured into the cassette, a comb inserted and then left to set. The samples were finally injected into the wells using an automatic pipette or a microsyringe after fixing the cassette into the electrophoretic apparatus.

### 6.2.2.2. Electrophoresis conditions

The electrophoresis time was 16 h, applying 70 volts at 25 mA for single gel using the Studier apparatus. However, the voltage was increased to 120 volts for 16 h when using 4 gels together in the Hoeffer apparatus and the gels were cooled by circulating tapwater.

### 6.2.2.3. Staining procedure

## A. Enzyme stains

### 1. Acid phosphatase (modified from Nealson & Garber, 1967)

| | |
|---|---|
| Na α- naphthyl acid phosphate | 100 mg |
| fast blue RR salt | 100 mg |
| 0.2 M acetate buffer pH5.8 | 100 $cm^3$ |

Prepared as:

(0.2 M Sodium acetate 70 $cm^3$)

(0.2 M Acetic acid 30 $cm^3$)

1. The gel was first soaked in 100 $cm^3$ of 0.2 M acetate buffer for 15 - 20 minutes.

2. The acetate buffer was replaced by the stain and incubated at 37 °C for two hours with occasional agitation.

Acid phosphatase bands appeared as dark brown bands against a yellow background.

### 2. Alkaline phosphatase (after Nealson & Garber, 1967)

| | |
|---|---|
| Na α-naphthyl phosphate | 25 mg |
| fast red TRN | 100 mg |
| 0.6M acetate buffer, pH 5 | 100 $cm^3$ |

The materials were first dissolved in the buffer, added to the gel, then the gel was incubated at 22± 2°C for one hour. Alkaline phosphatase bands were expected to appear as dark brown bands against a colourless background.

**3. Esterase activity** (modified from Rice & Crowden, 1987)

| | |
|---|---|
| α-naphthyl acetate | 10 mg |
| β-naphthyl acetete | 10 mg |
| fast blue RR salt | 50 mg |
| 0.2 M phosphate buffer, pH 6.4 | 200 $cm^3$ |

To prepare 100 $cm^3$ of the 0.2 M phosphate buffer at pH 6.4, 13.25 $cm^3$ of 0.2 M $Na_2HPO_4.12H_2O$ were added to 36.75 $cm^3$ of 0.2 M $NaH_2PO_4.2H_2O$. The volume was completed to 100$cm^3$ with water.

The gel was soaked in the phosphate buffer for 15 minutes with constant gentle agitation. Meanwhile, the α - and β - naphthyl acetate were dissolved in 2 ml of 50% acetone.(Solution B)

The fast blue RR salt was dissolved in 10 ml 0.2 M phosphate buffer. ( Solution C).

A volume of 87 ml 0.2 M phosphate buffer( pH 6.4 ) was added to solution C with constant stirring, then solution B was added with further stirring for 10 minutes. The mixture was finally filtered.

The buffer was replaced by the stain and the gel incubated at room temperature for 30 minutes with constant gentle agitation until brown bands appeared against an orange yellow background.

The stain was washed in distilled water and then the gel was soaked and preserved in 5% acetic acid.

**4.Leucine aminopeptidase** ( a modification of Rice & Crowden, 1987)

| | |
|---|---|
| L-leucyl -β-naphthyl amide HCl | 40 mg |
| fast black salt | 40 mg |
| $MgCl_2.6H_2O$ | 10 mg |
| 0.5 M boric acid | 100 $cm^3$ |
| 0.2 M tris maleate buffer, pH 5.3 | 25 $cm^3$ |

The tris maleate buffer was prepared by dissolving 24.2 g tris HCl and 23.2 g maleic acid in 1000 $cm^3$ of water.

The stains were dissolved in the buffer , then the boric acid was added. The gel was soaked before staining in 0.5 M boric acid for one hour, then replaced with the staining reagent and incubated at 22±2 °C for another one hour. The bands appeared black against a colourless background.

**5. Malic enzyme** (a modification of Siciliano & Shaw, 1976 )

| | |
|---|---|
| L- malic acid | 60 mg |
| β - NADP | 10 mg |
| $MgCl_2.6H_20$ | 10 mg |
| MTT | 7 mg |
| PMS | 5 mg |
| tris HCl buffer at pH 8.0 | 30 $cm^3$ |

All materials were dissolved in 30 $cm^3$ of the buffer then added to the gel. The gel was stained in the dark with gentle agitation for 2 hours or until dark violet bands appeared against a pale violet background ( about 30 min).

**6. Lactate dehydrogenase** ( a modification of Rice and Crowden, 1987)

| | | |
|---|---|---|
| 0.1 M tris HCl pH 8.0 | 100 | $cm^3$ |
| sodium lactate* | 10 | $cm^3$ |
| $NAD^+$ | 25 | mg |
| MTT | 15 | mg |
| PMS | 5 | mg |
| total | 110 | $cm^3$ |

* Prepared as:

| | |
|---|---|
| DL-lactic acid 85% | 10.6 $cm^3$ |
| sodium carbonate 1M | 49.0 $cm^3$ |

Each component was dissolved sequentially in 100 $cm^3$ of water and finally adjusted to pH 7.0.

The gel was covered with the stain and incubated in the dark at room temperature (PMS is light sensitive) for 30 min. Violet bands appeared on a pale violet background .

**7.Malate dehydrogenase** ( a modification from Rice & Crowden, 1987)

| | | |
|---|---|---|
| 0.1 M tris HCl pH 8.0 | 100 | $cm^3$ |
| sodium malate* | 10 | $cm^3$ |
| NAD | 25 | mg |

| | |
|---|---|
| MTT | 15 mg |
| PMS | 5 mg |
| total | 110 $cm^3$ |

* Prepared as:

| | |
|---|---|
| malic acid | 13.4 g |
| sodium carbonate | 10.6 g |

Each was dissolved sequentially in 100 $cm^3$ of water , and adjusted to pH 7.0.

The gel was covered with the stain and incubated in the dark at room temperature( PMS is light sensitive) for 30 min. Violet bands appeared on a pale violet background .

## 8. Xanthine dehydrogenase (after Shaw & Prasad, 1970)

### a. Substrate (1 M hypoxanthine)

| | |
|---|---|
| hypoxanthine | 68 mg |
| 1 M KOH | 1 $cm^3$ |
| water | 4 $cm^3$ |

### b. Staining materials

| | |
|---|---|
| PMS | 2 mg |
| $NAD^+$ | 60 mg |
| NBT | 30 mg |
| 1 M hypoxanthine | 3 $cm^3$ |
| 0.5 M tris HCl, pH 7.1 | 20 $cm^3$ |
| water | 77 $cm^3$ |

The PMS, $NAD^+$, NBT were added to the Tris buffer, then the substrate was added and the stain completed to 100 $cm^3$ with water.

The gel was covered with the stain and incubated at 37°C for 2 h. The enzyme appeared as violet bands against a blue background.

**9. Glucophospho isomerase** ( a modification of Rice & Crowdon, 1987)

| | |
|---|---|
| 0.1M Tris HCl buffer (pH 7.5) | 94 $cm^3$ |
| Magnesium chloride (10 %) | 6 $cm^3$ |
| Fructose -6- phosphate | 10 mg |
| Glucose-6-phosphate dehydrogenase | 10 units |
| $NADP^+$ | 5 mg |
| PMS | 5 mg |
| MTT | 10 mg |

The gel was covered with the stain, incubated in the dark for 2 hours or until the appearance of violet bands against the light violet background.

**10. Hexokinase** ( after Eaton *et al* . , 1966)

| | |
|---|---|
| Glucose | 90 mg |
| Magnesium chloride | 20 mg |
| ATP | 25 mg |
| $NADP^+$ | 25 mg |
| PMS | 3 mg |
| NBT | 20 mg |
| glucose -6- phosphate dehydrogenase | 0.04 mg |
| 0.5 M tris HCl buffer, pH 7.1 | 10 $cm^3$ |
| water | 90 $cm^3$ |

The materials were dissolved in the buffer then the water was added. The gel was stained at 37°C in the dark for 30 min, or until the appearance of violet bands against a light violet background.

**11. Tetrazolium oxidase** (after Siciliano & Shaw, 1976)

| | |
|---|---|
| NADP | 15 mg |
| NBT | 15 mg |
| PMS | 5 mg |
| Magnesium chloride | 50 mg |
| 0.2 M tris HCl, pH 8.0 | 10 $cm^3$ |
| water | 40 $cm^3$ |

The materials were dissolved in the buffer, then the water was added. The gel soaked in the stain for 30 min at 22±2 °C in the dark for 30 min. Zones of enzyme activity appeared as white bands against a blue background.

**B. Stains for native and SDS proteins**

**1. Coomassie blue** ( after Wilson, 1979)

**a. Staining solution**

| | |
|---|---|
| Coomassie blue 250R | 0.5 g |
| methanol | 12.5 $cm^3$ |
| acetic acid, glacial | 25 $cm^3$ |
| water | 100 $cm^3$ |

**b. Destaining solution**

| | |
|---|---|
| methanol | 50 $cm^3$ |

| | |
|---|---|
| acetic acid, glacial | 100 $cm^3$ |
| water | 850 $cm^3$ |

**c. Staining procedure**

The gel was stained at 22±2 °C for 30 min, then the staining solution was replaced with an excess of the destaining solution and was shaken gently with several changes until the bands were visible. The stained gel was then placed in 5% acetic acid solution for storage.

## 2. Silver staining (after Wray *et al* ., 1981)

**a. Stock solutions**

| | |
|---|---|
| ammonium hydroxide | 14.8 M |
| sodium hydroxide | 36 % |
| citric acid | 1 % |
| formaldehyde | 38 % |
| methanol | 50 % |

**b. Staining procedure**

The gel was soaked overnight in 50% methanol. Freshly prepared silver nitrate (0.8 g/4 $cm^3$) was added dropwise with stirring to a mixture of 21 $cm^3$ sodium hydroxide and 1.4 $cm^3$ ammonium hydroxide. This volume was increased to 100 $cm^3$ with water and used within 5 min. If a brown colour or precipitate developed the reagents were discarded and the preparation repeated. At a later stage it was found that titrating 2 - 5 $cm^3$ (of sodium ammonium hydroxide mixture prepared above) into the brown material dissolved the precipitate and gave equivalent staining results to using the normal

staining reagent. The gel was covered with the stain, incubated in the light with gentle shaking for 15 min. The gel was then washed and flooded with distilled water and placed back on the shaker for 10 min during which time the developer solution was prepared. The developing solution was prepared by mixing 2.5 $cm^3$ of citric acid and 0.25 $cm^3$ of formaldehyde stock solutions and then making the volume up to 500 $cm^3$ with water. The water was poured off the gel and replaced with the developer. It was then placed back on the shaker until dark brown stained bands appeared, within 20-30 min. The developer was replaced immediately with water, followed by two further washings then flooded with 50% methanol to stop the reaction and fix the stain. The gel was later stored in distilled water.

### 6.2.3. Establishment of routine procedure

#### 6.2.3.1.Efficiency of protein extraction method

Two *Ph.citrophthora* isolates (IA14002 and IC13008) were used to assess the efficiency of the protein extraction method. Two grams of mycelia from each of the isolates grown as described in section 6.2.1. were ground in a liquid nitrogen chilled pestle and mortar, homogenised with 4 $cm^3$ of 25 mM HEPES buffer (pH 7) and centrifuged at 3 °C, at 5000 rpm for 30 min. The middle layer was collected and kept as the protein of the extraction attempt. This was followed by two more extractions from the sedimented mycelium paste. The volume of HEPES buffer used for these attempts was 2:1 the weight of the pellet.

Protein concentrations of the three extraction attempts were measured and the results compared.

### 6.2.3.2.Optimising growth conditions

The aim of this experiment was to discover the effect of culture age on the protein and enzyme patterns.

A series of 12 erlenmeyer flasks (250 $cm^3$) containing 100 $cm^3$ of DR were inoculated with 3 x $10^5$ zoospores from each of *Ph.citrophthora* ID4001 and *Ph.nicotianae* IA7007. The flasks were incubated in the dark on a shaker (100rpm) for 5, 10, 15 and 20 days. Three flasks were harvested at the end of each period.

Growth was assessed by fresh weight and residual glucose measurements using the method of Miller, (1959 ) as described below. Proteins were extracted and assayed as described in section 6.2.3.1.

The samples were analysed for enzyme patterns (acid phosphatase, esterase, lactate dehydrogenase, malate dehydrogenase, glucophospho isomerase and xanthine dehydrogenase), using 12.5% homogeneous gels. In addition to that the samples were analysed for total protein patterns using SDS homogeneous gels, stained with silver.

**Assay method for reducing sugars** (after Miller, 1959)

**Materials**

| | |
|---|---|
| 3,5,dinitrosalicylic acid | 10.0 g |
| phenol | 2.0 g |
| sodium sulphite | 0.5 g |
| sodium hydroxide | 10.0 g |
| sodium potassium tartarate | 200.0 g |

The chemicals were dissolved in one litre of distilled water, and refrigerated in an amber bottle until use.

Procedure

A volume of 3 $cm^3$ DNS reagent were added to 1 $cm^3$ of the growth medium (containing 0.1 - 0.6 mg glucose or equivalent) in a 16 mm large test tube and heated in a boiling water bath for 15 min then cooled under a stream of cold tap water. The optical density was measured using an LKB Ultrospec 4050 spectrophotometer at 575 nm.

### 6.2.3.3.Choice of growth medium

Two media were chosen initially for this study, DR and V8. DR is a totally synthetic medium whereas V8 is based on natural materials (See Appendix 2).

Fifteen isolates were chosen randomly from each of Site I (IB4002, IB8002, IB11004, IB13002, IB13006), Site II (IIE3003, IIE7002, IIE80011, IIE11003, IIE120010, and the Other Sites (FAR003, TMD003, JRPN001, JRBD004, TMD006). These isolates were inoculated into a set of flasks containing 100 $cm^3$ of DR and another containing 100 $cm^3$ V8, incubated shaken at $23\pm2°C$ and harvested after 5 days.

The wet weight of the mycelia and the protein content of the extracts prepared as in 6.2.1. were measured. Electrophoretic patterns for enzymes were obtained using gradient gels. All the isolates were analysed for the V8 medium but only 3 of each of the Permanent Sites and 4 of the Other Sites from the DR medium.

### 6.2.3.4.Comparison of electrophoresis apparatus

Two makes of electrophoretic apparatus were compared. The Studier apparatus was built and used as described in Hames and Rickwood (1981)

to house a single slab gel with 10 wells. The Hoeffer (SE600, Hoeffer Scientific Instruments, San Francisco) apparatus was used following the manufacturers' instructions and held a minimum of 1 and a maximum of 4 slab gels with 15 wells in each. Thus it was possible to run 60 samples simultaneously and under the same conditions with a possibility of staining 15 samples for 4 enzymes at one time.

The same protein samples were run on gels in both systems to examine reproducibility, resolution and ease of use. Gels were run and stained for enzymes as described in section 6.2.2.

### 6.2.4.Interspecific variation between *Ph.citrophthora* and *Ph.nicotianae*.

To assess protein and isozyme profiles of *Ph.citrophthora* and *Ph.nicotianae* 6 isolates of each species were used ( *Ph.citrophthora* IB7005, IB8002, IE5002, IIA2005, IMI 129906; *Ph.nicotianae* IB13004, IC8oo1, ID11003, IID3006, IIA10004, IIC130026). Protein samples were prepared as described in section 6.2.1. from V8 grown mycelia (5 days incubation) and electrophoresis conditions were as described in section 6.2.2. using the gradient gels for enzymes and the SDS homogenous gels for silver stained protein profiles.

### 6.2.5.Intraspecific variation within *Ph.citrophthora*

*Ph.citrophthora* isolates constituted 86.6% of the total number of identified isolates obtained during the orchard survey. All *Ph.citrophthora* isolates from 2 subsites, one in each orchard (IB and IIE) and all isolates from the Other Sites were examined for their electrophoretic patterns.

These constituted a total of 63 isolates, 25 from IB, 23 from IIE and 13 from the Other Sites. The reasons for choosing these subsites are described in section 5.2.5.

Protein samples were prepared as described in section 6.2.1. and electrophoresis conditions were as described in section 6.2.2. using gradient gels for enzymes and SDS homogenous gels for silver stained protein profiles.

**Recording of enzyme patterns**

The zymograms were recorded in relation to the bromophenol blue indicator front, considering band numbers and positions mainly rather than the band thickness and intensity. However, the assessments were made on the basis of the first two parameters.

**Recording of protein patterns**

Some bands were visible by eye but did not appear in photographs and thus a drawing was made for each isolate to show the position of all bands which were visible on direct examination of the gel .

A few very prominant protein bands shared by all isolates (except band 2 for isolates IB2001, IB4001, IB7001) were used as markers (Figure 6.1). The position of the other bands which were detectable in between the maker bands was not considered for the recording and no judgement was carried out to compare their similarity. However only the numbers of these bands were considered and were grouped referring to the marker band number which followed their position. As example in Figure 6.1, the first banding group was composed of 1 for isolate IB7005 and 3 for IE5002 indicating the number of bands between the origin and the first reference

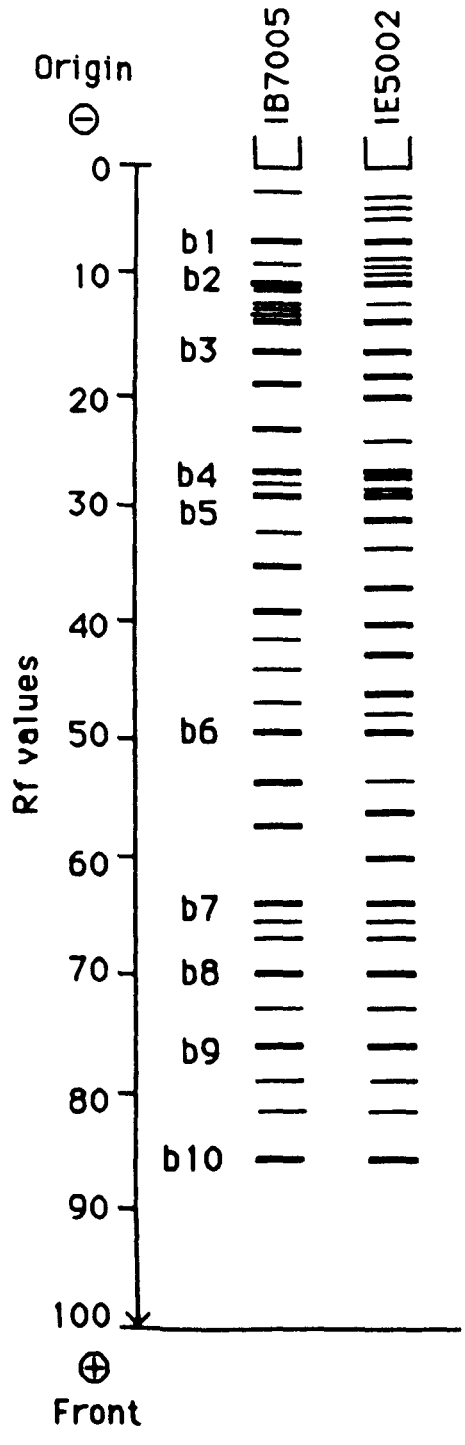



Figure 6.1. Marker bands shared by isolates of *Ph.citrophthora* used for grouping to apply pricipal component analysis

band, while the next group was composed of the same number of bands(1 and 3) again for the two isolates but in a different position and were referred to as band group 2 , which is the marker band that follows and thus separates these non similar bands from the third grouping within the profile and so on. This system was followed in order to allow assessment of variation among the isolates and to find out if a specific grouping pattern can be referred to sites or method of collection of the isolates.

A multivariate analysis method was used to assess variation among the isolates by relating these isolates together according to the shared bands and then separating them into groups according to the general banding pattern of each isolate. The principal component analysis was used as it would show the variation between isolates giving maximum importance to those banding patterns which form the major differences.

**Principal components analysis (PCA)**

PCA is a method of multivariate analysis that examine the relationships amongst observations described by several variables. The principle aim of the analysis is to present the inherent variation in a manner which requires a minimum number of axis for the display of variations. The analysis produces a partition of variation according to a series of axes which are orthogonal to one another and are chosen to account for successively diminishing sources of variation. The axes themselves can be seen as composed of " components " of variability from the original data variables. The method of construction of these axes is given in Williamson (1972) and Manly(1986) and involves calculations of eigenvalues and associated eigenvectors from the matrix of correlation

coefficients between all original variables. Procedure PRINCOMP (SAS, 1985) was used to compute the PCA.

## 6.3. Results

### 6.3.1.Efficiency of protein extraction method

Table 6.1 shows the protein concentration of three successive extracts for both isolates. It is clear that the first trial extracted about 72% of the total protein content soluble in buffer, whereas the subsequent ones yielded much smaller amounts.

**Table 6.1.** Concentration and percentage of total protein content from three successive extracts of two *Ph.citrophthora* isolates.

| Isolate | Extraction trials | | | | | | Total | |
|---|---|---|---|---|---|---|---|---|
| | first | % | second | % | third | % | Total | % |
| IA14002 | 0.97* | 72 % | 0.25 | 19% | 0.12 | 9% | 1.34 | 100% |
| IC13008 | 1.33 | 73% | 0.38 | 21% | 0.10 | 6% | 1.81 | 100% |

* Protein concentration in $mg.cm^{-3}$

### 6.3.2. Optimising growth conditions

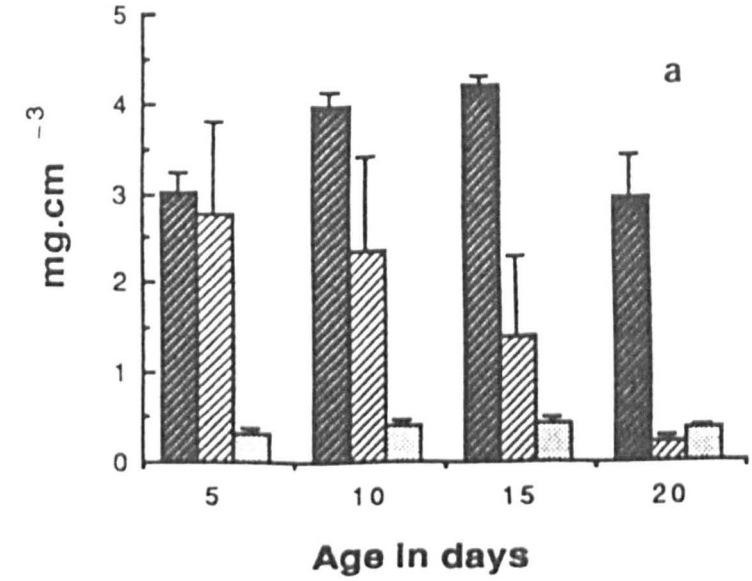
The effect of age on sugar consumption (measured as residual glucose: initial concentration is 9 $mg.cm^{-3}$), protein content and wet weight of mycelia is shown in Figure 6.2.a. for *Ph.citrophthora* and Figure 6.2.b. for *Ph.nicotianae* . The mycelium wet weight of both both species increased with age but showed a decline at the 20 days old harvest, while the residual glucose kept on decreasing all through the assessment. However, the protein content extracted from 2 g of mycelia remained the same over time for both species.

No differences were found between any of the enzyme patterns of the three replicates of each age group of both species. The general pattern for both species can be seen in section 6.3.5.. However, SDS protein profiles displayed differences between the 5 day old mycelia and the rest of the age groups of both species. Two marker bands (3 and 7) were missing from the protein profiles of the three age groups other than the 5 day old mycelia of *Ph.citrophthora* , as well as band 5 for *Ph.nicotianae* as shown in Figure 6.3.

### 6.3.3.Choice of growth medium

Both DR and V8 media were capable of supporting enough mycelial growth to obtain sufficient protein for electrophoresis.

The results of wet weight and protein contents are shown in Table 6.2. Figure 6.4. shows zymograms of *Ph.citrophthora* IB11004 grown in these two media. There was a difference between zymograms of acid phosphatase and glucophospho isomerase, namely an extra band from the



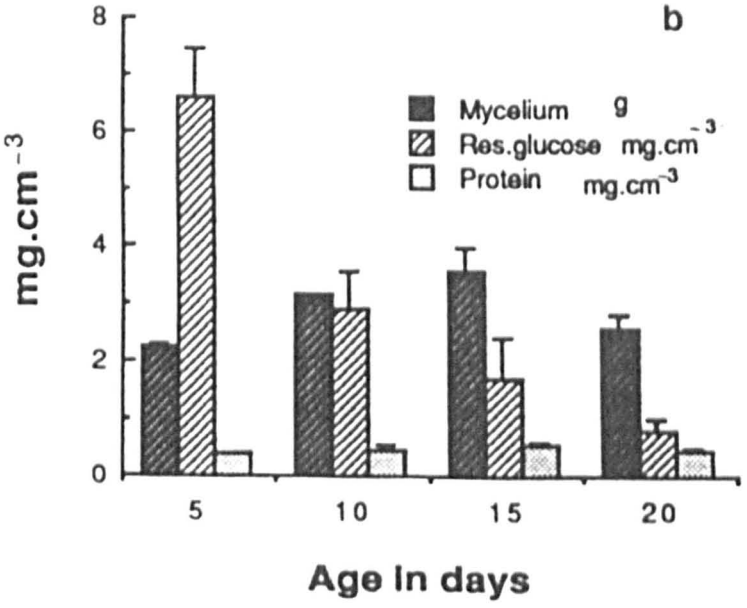




Figure 6.2.Effect of age on sugar consumption (measured as residual glucose ) , wet weight of mycelia and protein content (extracted from 2 g of mycelia). Bars indicate 2XSE. a. *Ph.citrophthora* . b. *Ph.nicotianae*

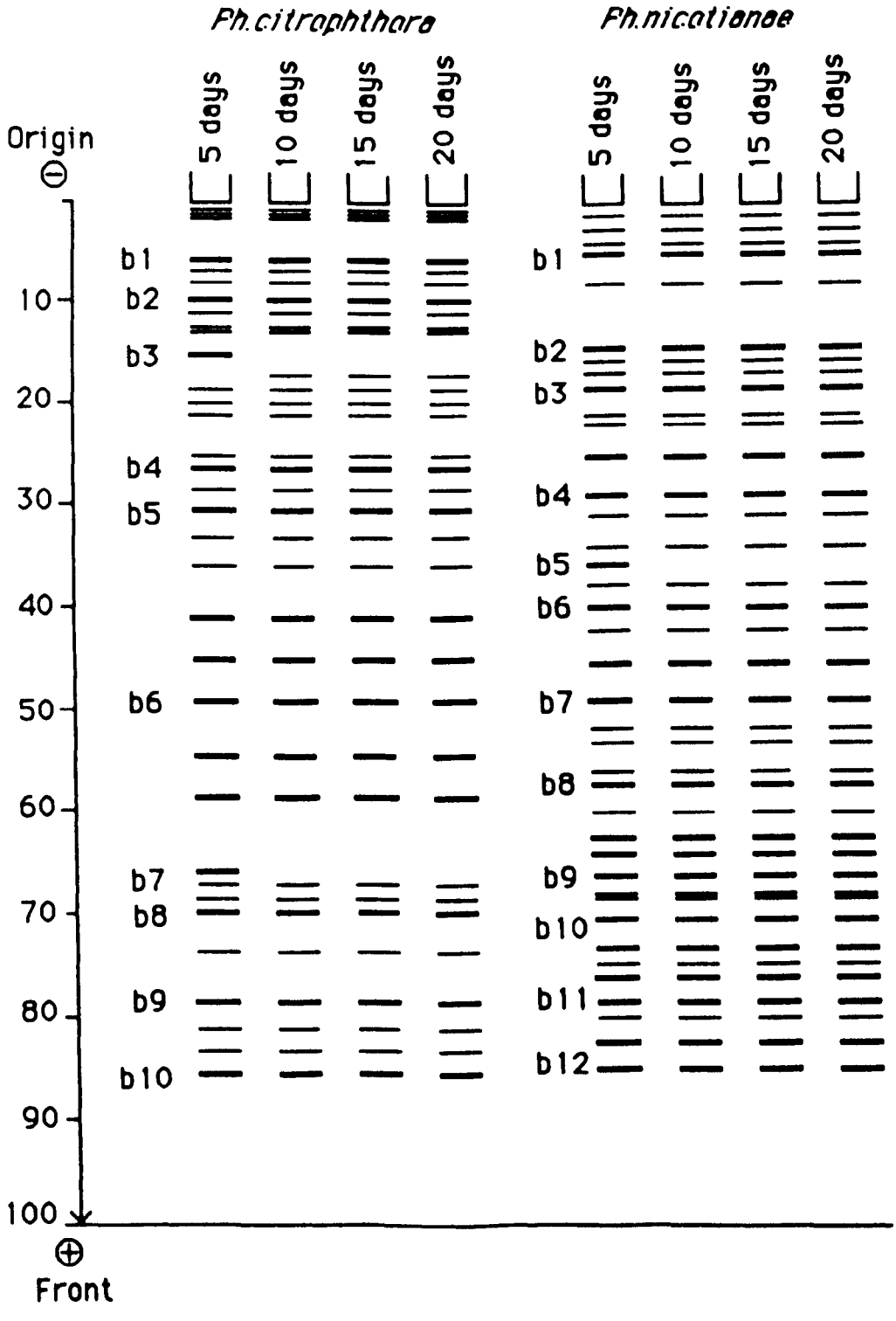



**Figure 6.3.** Effect of age on the protein profiles of *Ph.citrophthora* and *Ph.nicotianae*

**Table 6.2.** A comparison between DR and V8 for wet weight and protein concentration of extracts from mycelia.

| Isolate | Wet weight g | | Protein content $mg.cm^{-3}$ | |
|---|---|---|---|---|
| | DR | V8 | DR | V8 |
| IB4002 | 2.72 | 3.04 | 0.57 | 2.0 |
| IB8002 | 2.58 | 3.84 | 0.72 | 1.7 |
| IB11004 | 2.36 | 2.61 | 0.56 | 1.9 |
| IB13002 | 2.17 | 4.82 | 0.50 | 1.3 |
| IB13006 | 2.83 | 6.12 | 0.57 | 1.7 |
| IIE3003 | 2.58 | 4.43 | 0.46 | 1.6 |
| IE7002 | 2.92 | 3.61 | 0.47 | 1.8 |
| IIE80011 | 1.85 | 5.39 | 0.91 | 1.6 |
| IE11003 | 2.47 | 4.44 | 0.87 | 1.5 |
| IIE12001 | 2.33 | 2.95 | 0.64 | 1.9 |
| FAR003 | 4.65 | 5.10 | 0.80 | 1.5 |
| TMD003 | 2.62 | 4.03 | 0.47 | 1.5 |
| TMD006 | 4.06 | 6.59 | 0.60 | 1.7 |
| JRNP001 | 2.58 | 3.34 | 0.68 | 1.7 |
| JRBD004 | 2.67 | 3.29 | 0.65 | 1.9 |

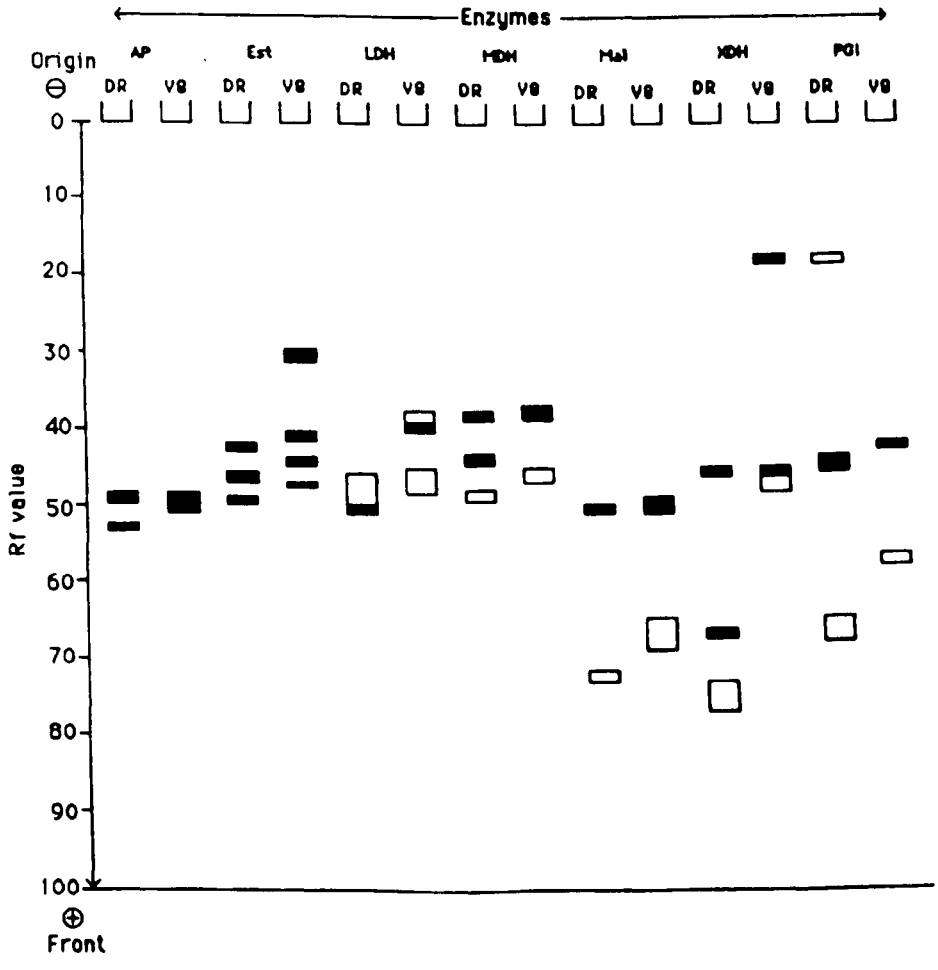



**Figure 6.4. Differences in zymogram resolution using DR and V8 in *Ph.citrophthora* .**

Black blocks - Stained bands of various enzymes

white blocks - Tetrazolium oxidase bands

protein extracted from the DR grown mycelia. In addition, there is an extra band in the esterase and lactate dehydrogenase zymograms from the V8 grown mycelia. However, no change in the enzymatic loci of xanthine dehydrogenase or malic enzyme was detected.

The SDS protein profiles for 4 isolates (IB11004, IIE7002, IIE11003, FAR003) were identical for both media as shown in Plate 6 compared with IMI129906 and a mixture of standard proteins.

### 6.3.4.Comparison of electophoretic apparatus

A comparison between enzymatic patterns obtained from protein extracts of isolate IB8001 using the Hoeffer and Studier apparatus for DR grown mycelia is shown in Figure 6.5.

The Studier revealed bands of enzymes (acid phosphatase, esterase, lactate dehydrogenase, malic enzyme and xanthine dehydrogenase ) with many bands present in different positions. Enzymatic patterns for malate dehydrogenase and glucophospho-isomerase were the same in number for both systems, but positions were different.

These results indicate that the Studier apparatus was more suitable but due to its limited capacity (only 10 samples at one time) and the ability of the Hoeffer to reveal clearly (even though with less numbers) the enzymatic pattern, the latter was used in order to cover maximum number of enzymes and isolates.

### 6.3.5.Interspecific variation between *Ph.citrophthora* and *Ph.nicotianae*

Staining for LDH, MDH, XDH, esterase and acid phosphatase revealed enough differences to distiguish the two species. Malic enzyme and PGI

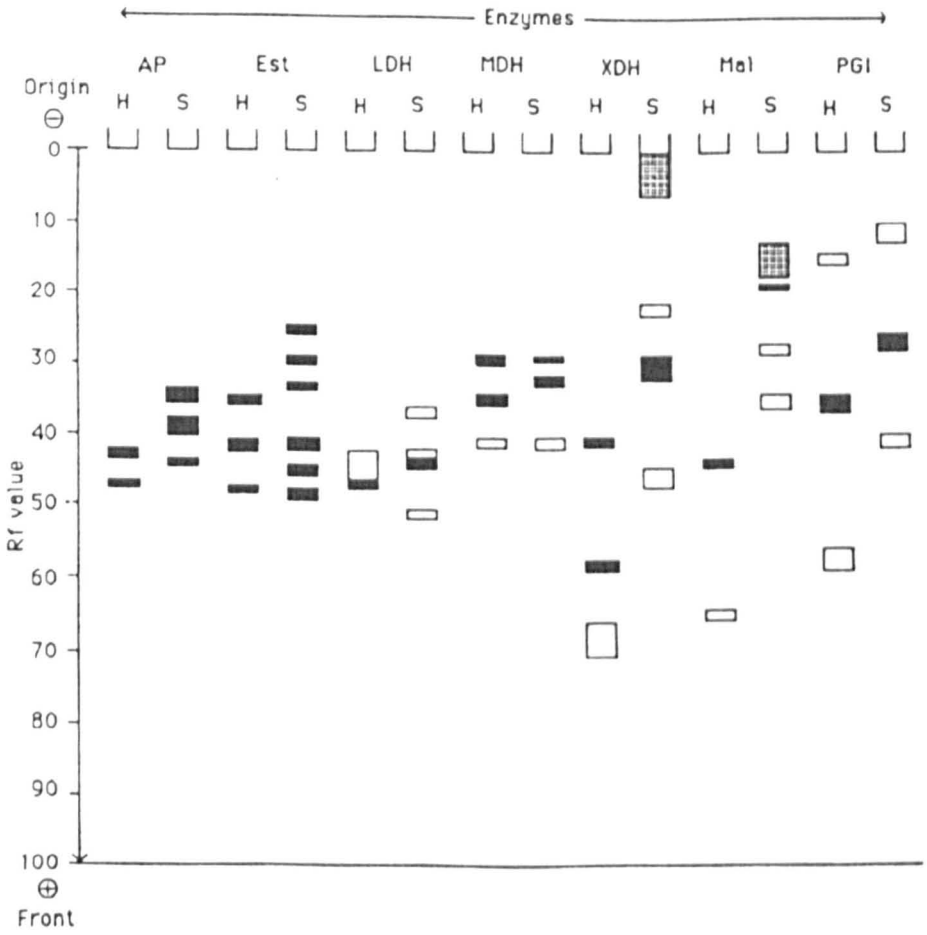



**Figure 6.5.** Comparison of zymograms using Hoeffer (H) and Studier (S) apparatus for DR protein extracts of *Ph.citrophthora*

Black blocks - Stained bands of various enzymes

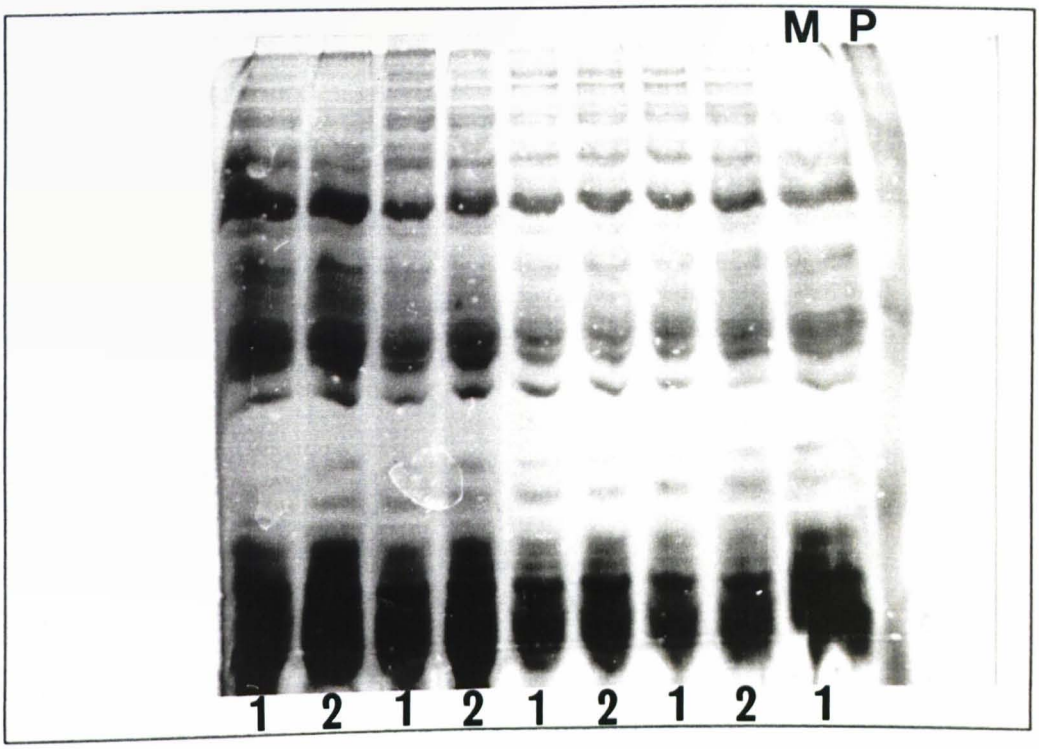
Shaded blocks - Faintly stained bands

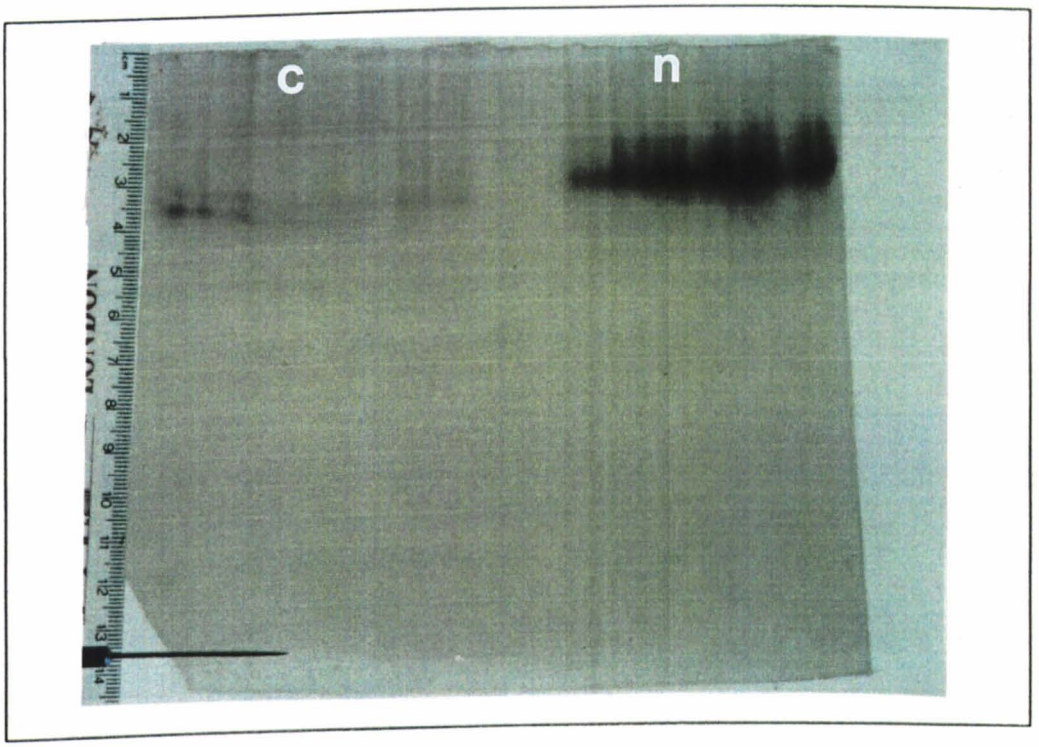
white blocks - Tetrazolium oxidase bands

Plate 6. SDS protein profiles comparing the patterns of four isolates of *Ph.citrophthora* grown on two media with an authentic isolate IMI 129906 (M) and a mixture of standard proteins( P) of 30-200 kD .

1. V8 juice 2. Double Ribiero liquid medium.

Plate 7. Esterase zymograms of five representative isolates from each of (c)*Ph.citrophthora* and (n) *Ph.nicotianae* showing 4 bands for the former and two for the latter.


M P
1 2 1 2 1 2 1 2 1


c
n

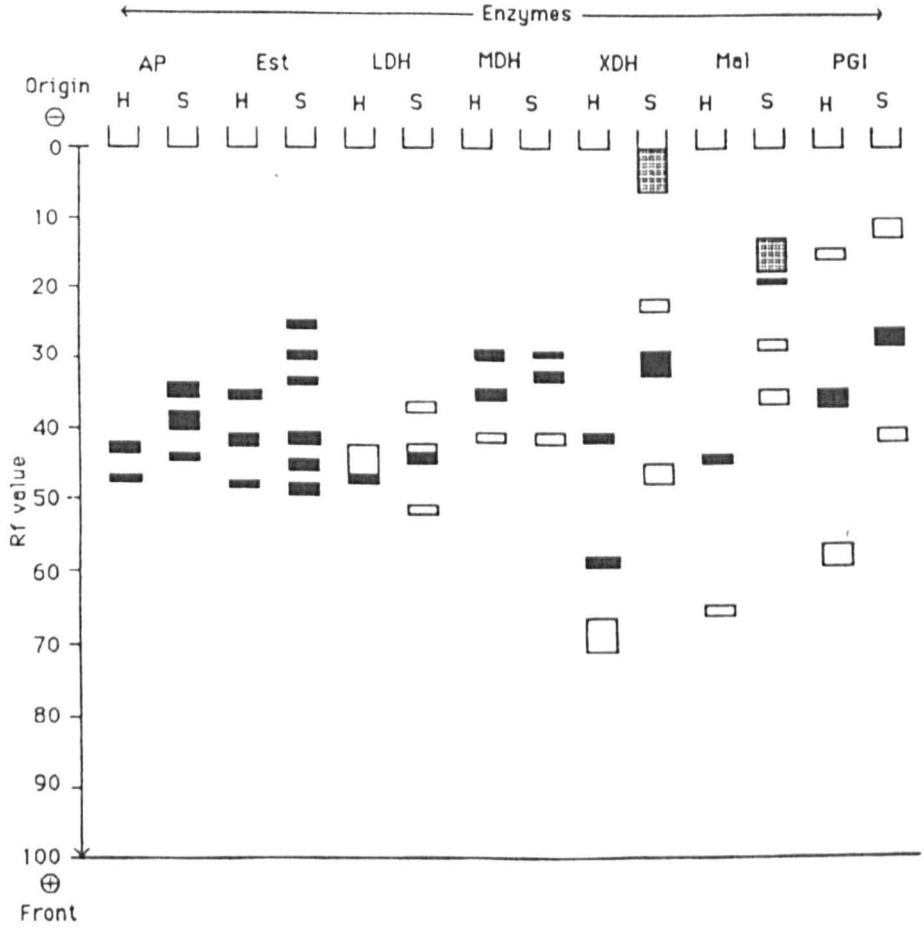



**Figure 6.5.** Comparison of zymograms using Hoeffer (H) and Studier (S) apparatus for DR protein extracts of *Ph.citrophthora*

Black blocks - Stained bands of various enzymes

Shaded blocks - Faintly stained bands

white blocks - Tetrazolium oxidase bands

zymograms of both species shown in Figure 6.6. for representative isolates showing positions of these enzymes drawn as rates of flow (Rf) in comparison with the indicator front, while Plate 7 shows the differences in the esterase zymograms. There are clear differences between the species.

The protein profiles shown in Figure 6.7. also indicate a clear separation of the two species. No bands were revealed between 10 and 17% Rf values in the distribution of marker bands. However, 4 marker bands were shared by isolates from both species, namely 5, 7, 9 and 10 of *Ph.citrophthora* were of the same Rf value as bands 4, 9, 11 and 12 of *Ph.nicotianae* .

### 6.3.6.Intraspecific variation within *Ph.citrophthora*

No intraspecific variation could be detected in the zymograms for the 63 isolates and a representative isolate as shown in Figure 6.8 , while Plates 8 and 9 show zymograms for esterase and MDH of 10 randomly chosen isolates. Staining for two enzymes (alkaline phosphatase and leucine amino peptidase) was unsuccessful.

The protein patterns of the SDS treated extracts indicated clear variation among isolates (Figure 6.9 and Plates 10 and 11). All of isolates shared 10 fixed bands, which were used as marker bands (see section 6.2.5.for explanation). All the three replicates of each sample resulted in the same protein pattern apart from four isolates (IB7002, IIE80011, IIE14002, JRBD003) in which one replicate differed from the other two.

Variations were seen in higher molecular weight proteins represented in the first 7 groups, while none were seen in the 8th to 10th groupings. The groupings are shown in Appendix 6.

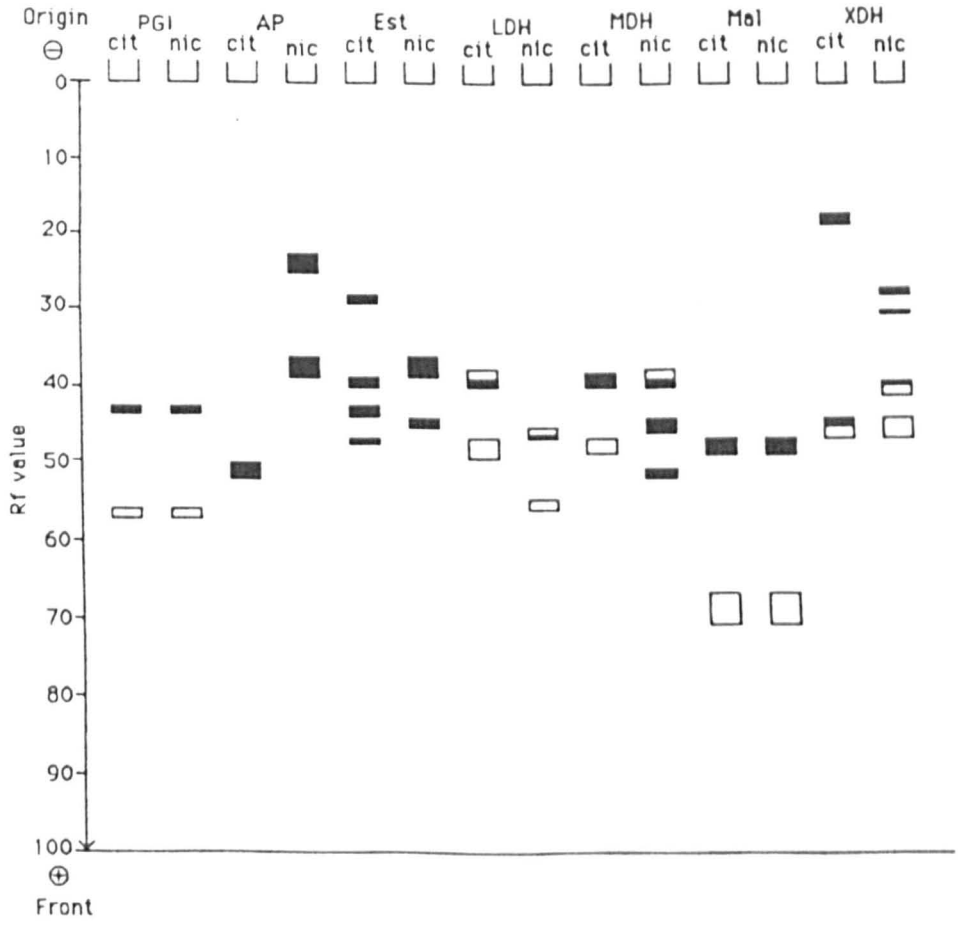



**Figure 6.6** - Intraspecific variation in zymograms of *Ph.citrophthora* (cit) and *Ph.nicotianae* (nic).

Black blocks - Stained bands of various enzymes

white blocks - Tetrazolium oxidase bands

Table 6.3. Variation in band numbers of enzyme sites for *Ph.citrophthora* and *Ph.nicotianae* grown on V8.

| Enzyme | Species | |
|---|---|---|
| | *Ph.citrophthora* | *Ph.nicotianae* |
| Acid phosphatase | 1 | 2 |
| Esterase | 4 | 2 |
| MDH | 2 | 4 |
| LDH | 3 | 3 |
| XDH | 3 | 5 |
| Malic enzyme | 2 | 2 |
| PGI | 2 | 2 |

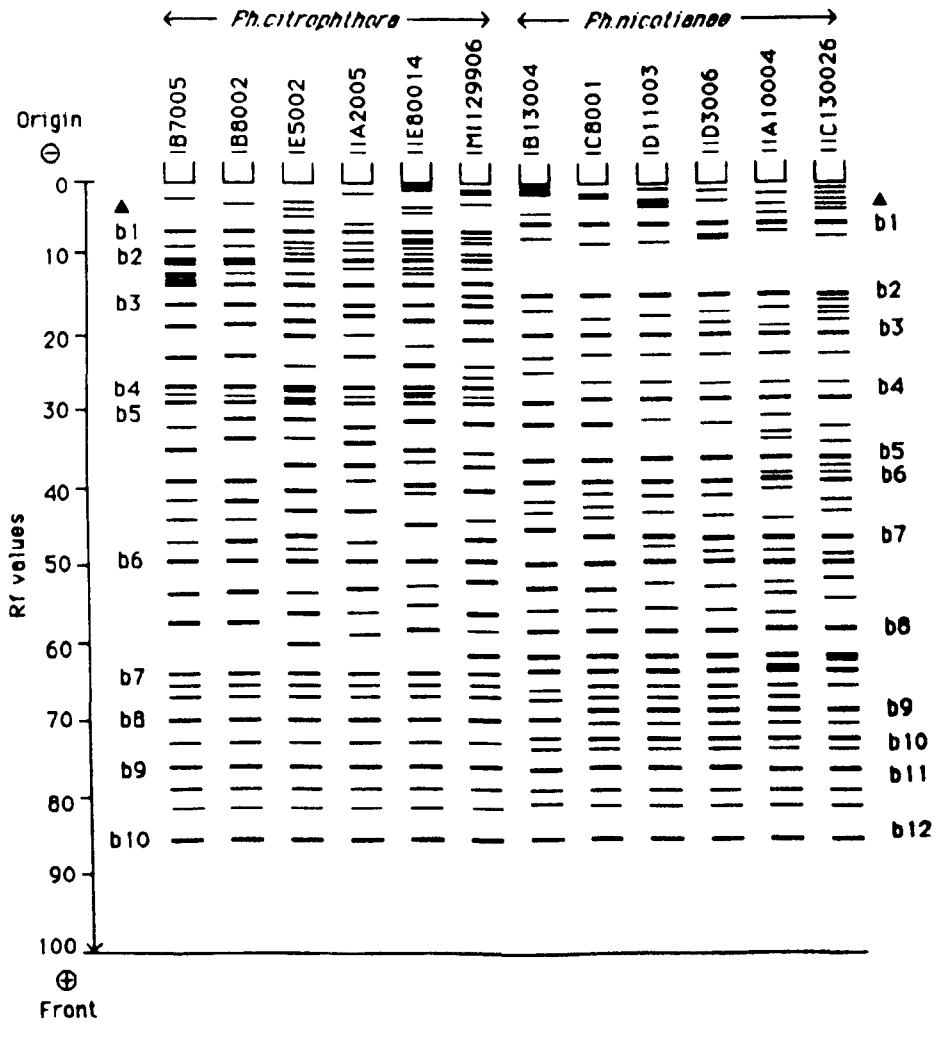



**Figure 6.7.** Interspecific variation in protein profiles between *Ph.citrophthora* and *Ph.nicotianae*.

▲ Shared bands among isolates of the same species.

**Plate 8.** Malic dehydrogenase zymograms of 10 representative isolates from *Ph.citrophthora*

**Plate 9.** Esterase zymograms of 15 representative isolates from *Ph.citrophthora*

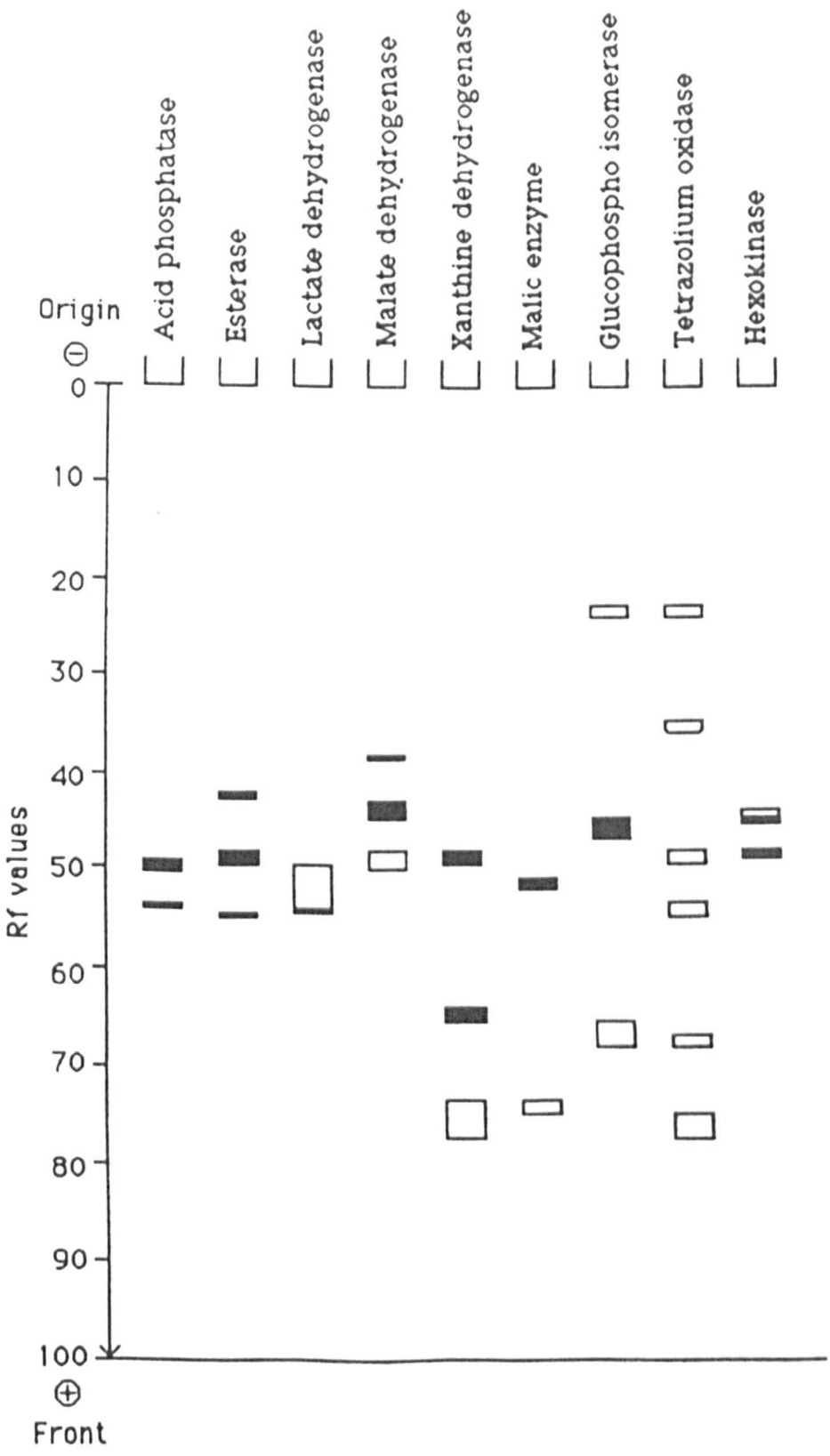


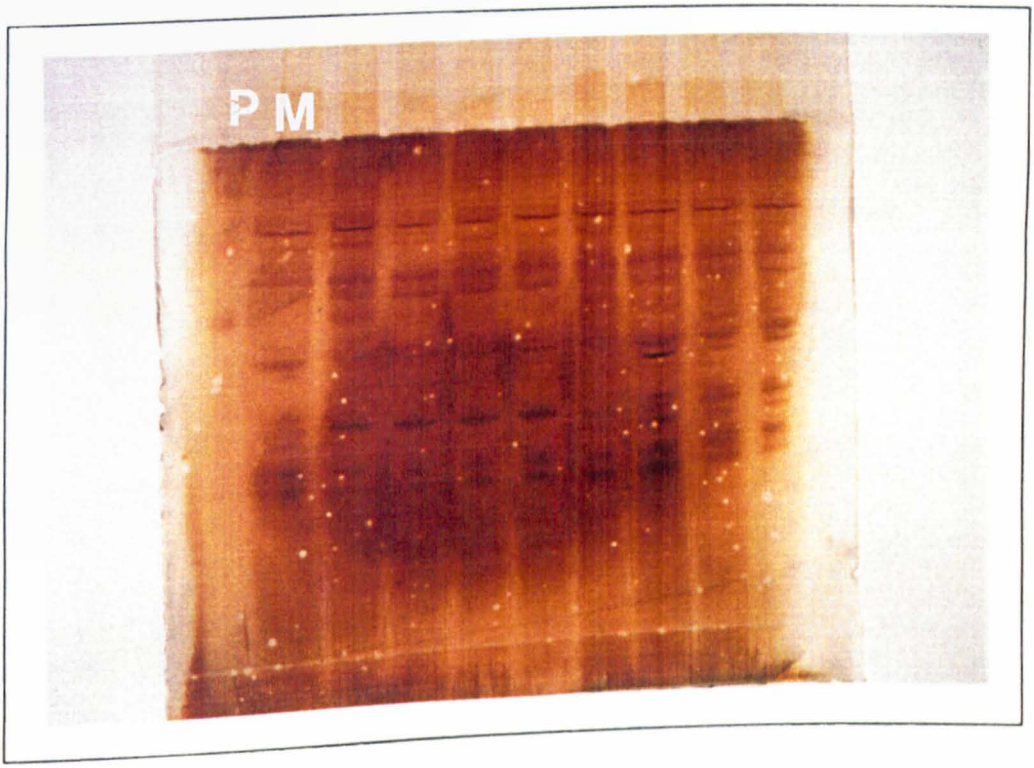

**Figure 6.8.** Zymograms of *Ph.citrophthora* isolate IB2001

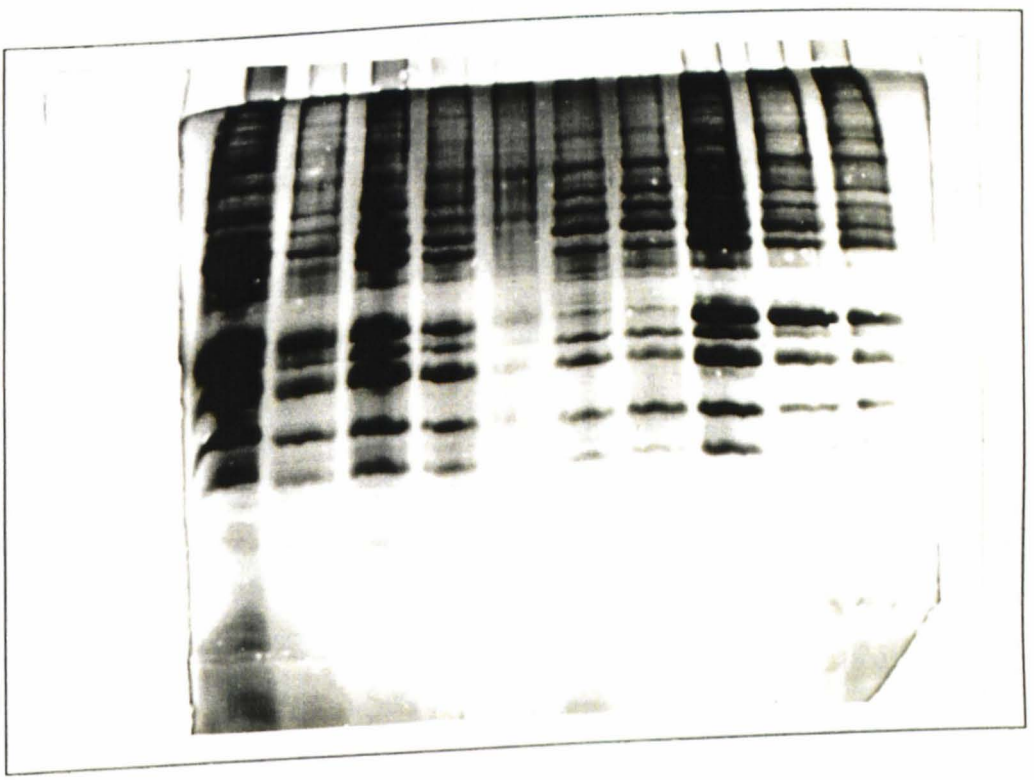
Black blocks - Stained bands of various enzymes

white blocks - Tetrazolium oxidase bands

Plate 10. SDS protein profiles showing 8 representatives from *Ph.citrophthora* and an authentic isolate IMI 129906(M) with a mixture of standard proteins (P) of 30-200kD

Plate 11. SDS protein profiles showing the patterns of 15 representatives from *Ph.citrophthora*


P M



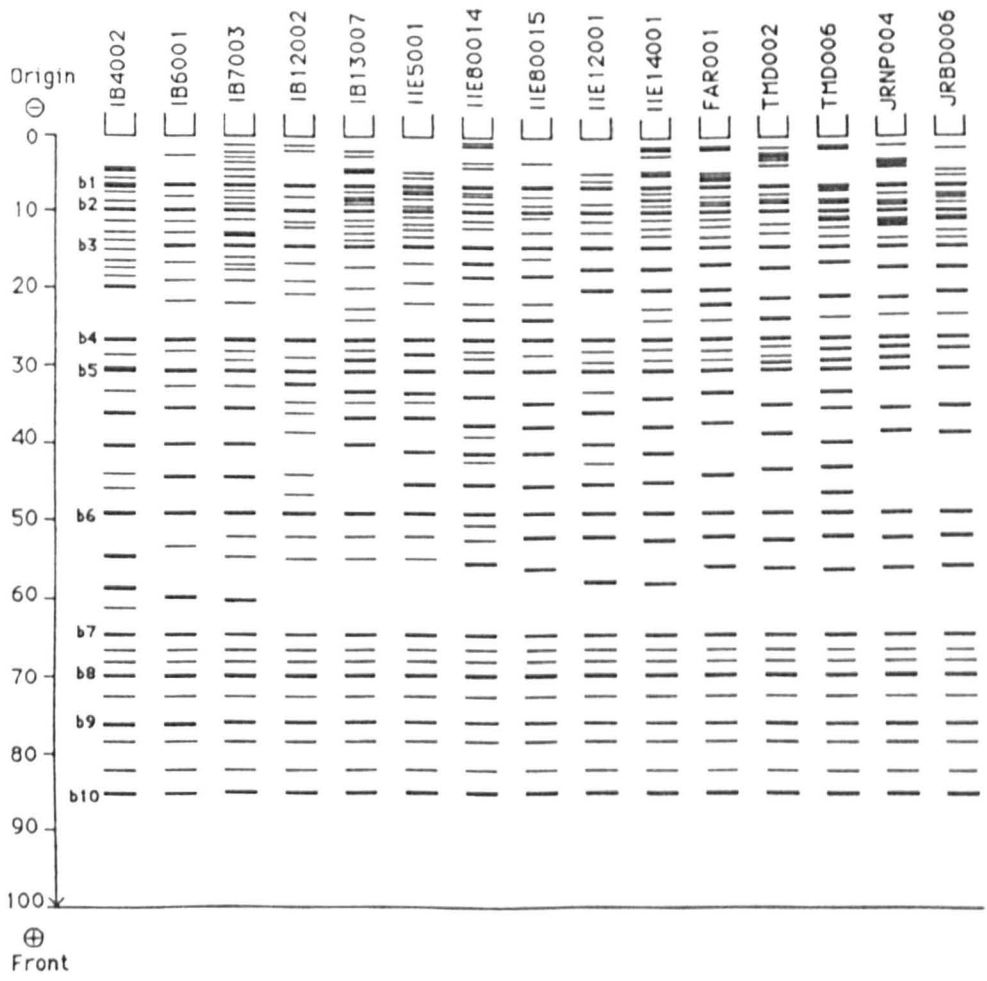



**Figure 6.9.** Protein profiles of 15 randomly selected *Ph.citrophthora* isolates from Sites I,II and the Other sites.

A summary of the PCA is given in Table 6.4. Application of principal component analysis for 60 isolates (3 isolates from the 63 were not included in this analysis due to one missing marker band as shown in Appendix 6), in which the variation among isolates took the form of 7 groups to form 7 axes (indices). These axes are the principal components of the protein profiles. It was found that the first 3 axes accounted for 76% of the variance. Examining the data for clustering based on either geographic origin (Figure 6.10) or isolation method (Figure 6.11) failed to reveal significant grouping. Inspection of the other four plots (axis) also yielded uninterpretable patterns.

## 6.4. Discussion

Electrophoretic analysis has provided an important additional tool in the characterization of fungi already defined by conventional morphological and pathological methods which are still the usual basis of identification. The use of electrophoresis by Clare (1963) to separate soluble proteins of the genus *Pythium* spurred extensive work in this direction in *Phytophthora* (see section 6.1.1.)

The present study has demonstrated that :

1. The growth media have a marked effect on the zymograms.

2. Age of the mycelium (which grew from fixed numbers of zoospores) has no effect on the zymograms but has on the protein profiles.

3. *Ph.citrophthora* and *Ph.nicotianae* can be separated on the

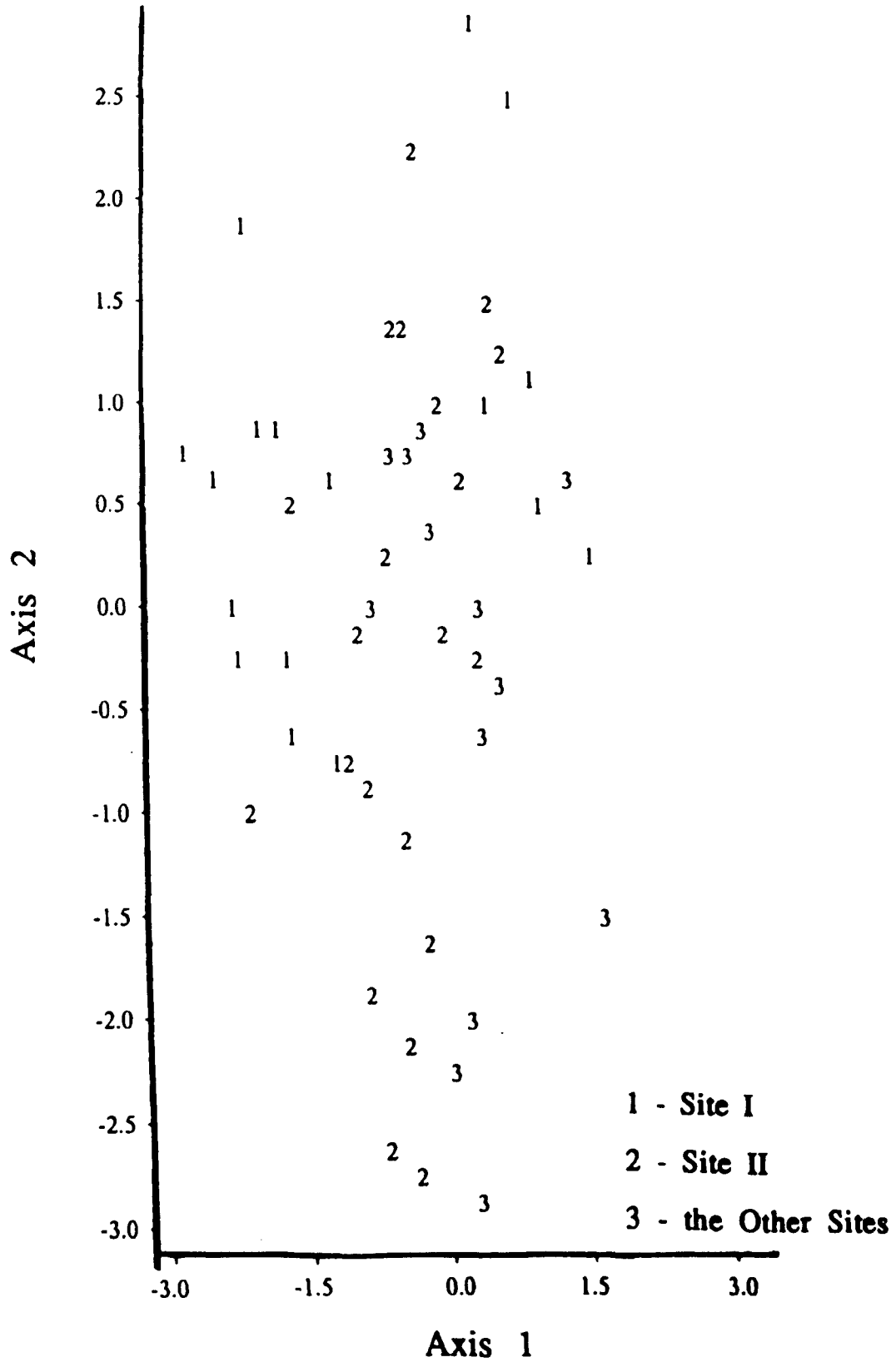



**Figure 6.10.** Position of 60 *Ph.citrophthora* isolates on axes 1-2 of principal components valued according to site.

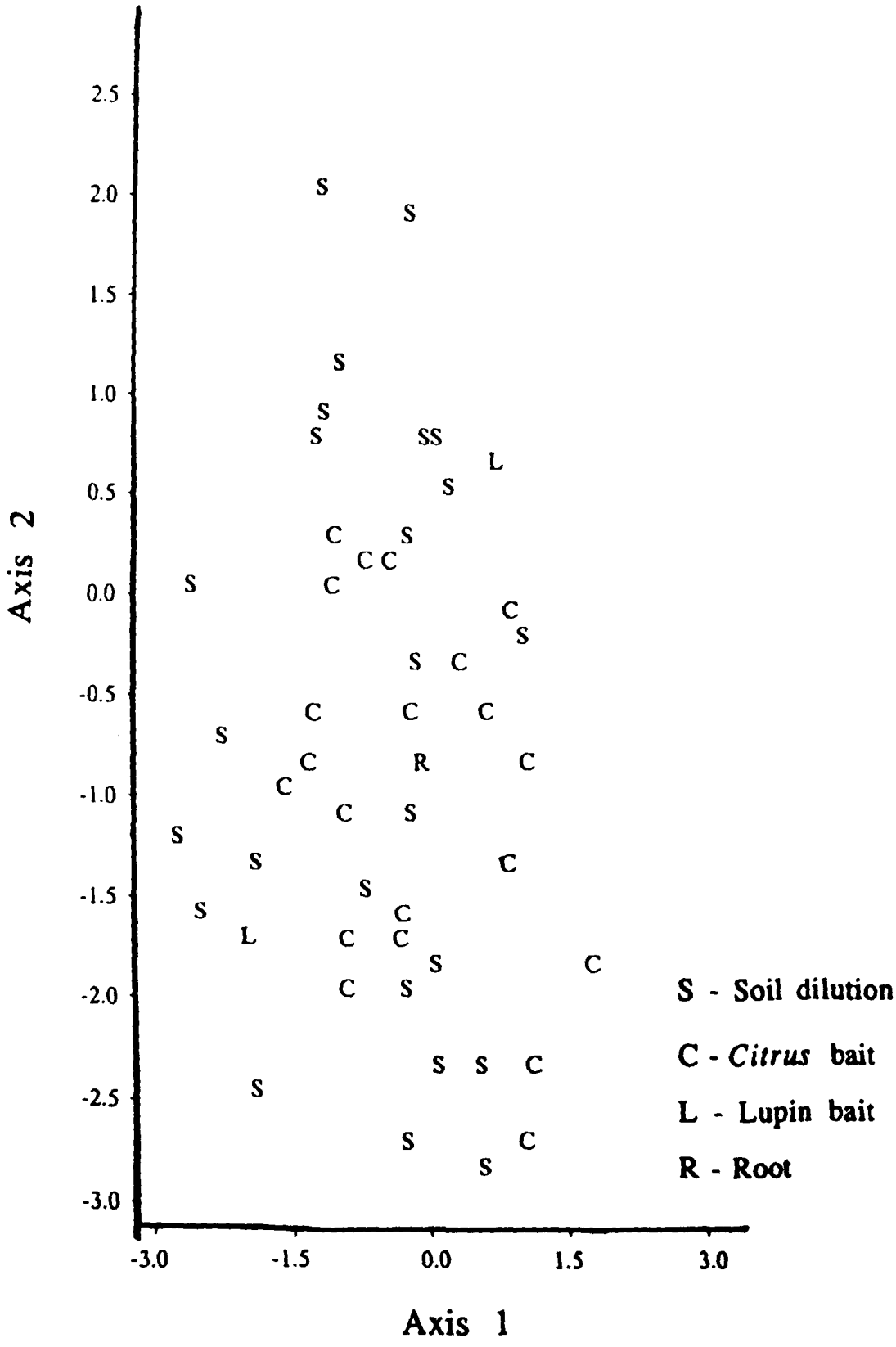



**Figure 6.11.** Position of 60 *Ph.citrophthora* isolates on axes 1-2 of principal components valued according to origin.

**Table 6.4.** Principal component analysis of 7 characters measured on banding groups of SDS treated protein extracts of 60 *Ph.citrophthora* isolates. Eigenvector loadings for each character on the first three principal components are presented with total eigen values and the percentage of the total variation accounted for by successive components.

| Character | | component | | |
|---|---|---|---|---|
| | | I | II | III |
| Banding group | 1 | 2.0101 | 0.5830 | 0.1389 |
| " " | 2 | 0.5830 | 1.1344 | 0.3807 |
| " " | 3 | 0.1389 | 0.3807 | 0.8259 |
| " " | 4 | -0.0152 | 0.2843 | 0.3056 |
| " " | 5 | 0.5355 | 0.2610 | 0.2000 |
| " " | 6 | -0.1716 | -0.1716 | 0.0163 |
| " " | 7 | 0.2203 | 0.0960 | 0.0564 |
| Eigen value | | 2.6268 | 1.4688 | 0.9686 |
| % of variation | | 39.5 | 22.1 | 14.5 |
| Cumulative% | | 39.5 | 61.6 | 76.1 |

basis of their zymograms and / or protein profiles.

4. *Ph.citrophthora* isolates from the West Bank are not related to the geographical location and in this study they did not vary in their enzymatic patterns. However, protein profiles can help to detect variation within the isolates of the species, but these isolates do not group according to their geography or isolation method.

The use of zymograms showed differences between *Ph.citrophthora* and *Ph.nicotianae* in at least four enzyme systems, namely acid phosphatase, esterase, malate dehydrogenase and xanthine dehydrogenase. No differences can be detected in other enzymes, namely lactate dehydrogenase, malic enzyme and glucophospho isomerase. These results agree with Vallavieille and Erselius (1984) regarding acid phosphatase and Erselius and Vallavieille (1984) regarding acid phosphatase and esterase patterns. The variation in protein profiles which is confirmed by this study has been previously detected between species of *Phytophthora* in several studies e.g., Gill and Powell (1968), Hall *et al* ., (1969), Gill and Zentmyer (1977), Erselius and Vallavieille (1984), Vallavieille and Erselius (1984) and Nygaard *et al* ., (1989).

The absence of variation amongst *Ph.citrophthora* isolates might be due to the failure in staining for enzymes which have displayed variability, especially since it was possible to illustrate variation when staining SDS treated total protein extracts. Faris *et al* .,(1986) found the protein patterns of *Ph.citrophthora* identical regardless of geographic location or host source which contradicts the findings here, which are supported by those of Erselius and Vallavieille (1984). However, the inability to relate the isolates to geographic or host source may be due to the heterogenity in their origin, or the inability to detect the faintly

stained bands especially in the first three banding groups which accounted for 73% of the variance in the principal component analysis.

It would be possible to follow this variation in protein profiles by looking again at the zymograms and to try to further modify the methods in the direction of additional enzyme stains, gel concentration and other electrophoresis conditions in general. As the culture medium plays a significant role, it might also be worth trying other media. The electrophoretic apparatus may also be a significant factor. It seems that the apparatus used in the assessment affects the zymograms and it is probable that using an alternative apparatus might reveal some zymogram differences. Two dimensional electrophoresis might also have an effect on revealing these differences.

# CHAPTER SEVEN

## General discussion

In this study both distribution and variation in *Phytophthora* species in *Citrus* plantations on the West Bank were investigated. Only two *Phytophthora* species were isolated during fortnightly sampling over a 9 month period from two sites, contrasting in their geographic location, topography, *Citrus* population and cultivation practices. Single samples were also obtained from 12 additional sites distributed throughout the West Bank. Only two *Phytophthora* species were identified namely *Ph.citrophthora* (Smith and Smith) Leonian and *Ph.nicotianae* van Breda de Haan.

The presence of only two species, in material from a range of sites examined in four ways for *Phytophthora* - soil dilution, *Citrus* and lupin baiting and direct isolation from roots - is of significance. Neither species has *Citrus* as its only host plant ( Waterhouse & Waterson, 1964 a,b; Ribiero, 1978) and up to 13 *Phytophthora* species have been isolated from *Citrus* world wide (Ann, 1984). Previous reports of *Phytophthora* from *Citrus* in the eastern shore of the Mediterranean and Near East also only found these two species (apart from a single report of *Ph.hibernalis* Carne, [Schiffmann, 1951]). The two species are readily separable on the basis of their colonial morphology, temperature tolerance and fructification production and dimensions as described in the taxonomic keys (Waterhouse, 1963, 1970; Newhook *et al* ., 1978). However, only about half of the isolates were obtained in pure culture to enable identification to species rather than generic level. There is no reason to believe that there is

a systematic difference between identified and unidentified isolates. *Ph.citrophthora* and *Ph.nicotianae* must therefore contain sufficient variation within each species to allow attack on a range of *Citrus* hosts, and growth over a range of geographic variables. This implies considerable genetic diversity or phenotypic plasticity. The fact that *Ph.citrophthora* , the predominant species, has only a very poorly documented sexual system makes this putative variation even more remarkable.

Considering the field isolation techniques, the initial isolation of *Phytophthora* using soil dilution and *Citrus* -baiting techniques were equally suitable for recovery from soil. However, soil dilution was more effective than *Citrus* in recovering *Ph.nicotianae* . Lupin bait and direct isolation from roots were poor at recovering *Phytophthora* .

The isolation medium VP3 (Ali-Shtayeh *et al* ., 1986) after improvements by removing rose Bengal and adding hymexazole and benlate was more suitable than PVPPHB (Papavizas *et al* ., 1982) not in terms of isolate numbers recovered but because the extra agar rendered the medium surface harder and thus colonies more uniform and easily detectable.

Fewer isolates were recovered from Site I than Site II, more were identified , but there was only half the number of *Ph.nicotianae* . The use of spot irrigation in Site I rather than the open irrigation system in Site II may have contributed to increase in isolate numbers from the latter, which could have disseminated propagules from the surrounding orchards through the irrigation water. This factor may influence the *Phytophthora* abundance, but have no effect on species diversity ( see later).

The absence of a detectable seasonal relationship in the fluctuations in propagule numbers is supported by the findings of other workers ( e.g. Timmer *et al* ., 1989) who also found no relation between site temperature and moisture and propagule numbers.

Fluctuations in mycelial growth and zoospore production may occur over a shorter time scale than that used in the sampling programme. For example as the study sites are subjected to sudden irrigation, this rapid environmental change may stimulate mycelial growth or sporulation. Similarly, cool nights and hot days or the arrival of leaf litter on the soil may all have effects on *Phytophthora* biomass. These could mask gross seasonal effects and could only be detected by much more intensive sampling and monitoring of environmental parameters.

Morphological parameters used to assess variation within the species showed that 20% of the assessed *Ph.nicotianae* isolates and 16% of the *Ph.citrophthora* exhibited isolate-environment interactions. This clearly indicates the presence of variation within populations detectable from overall growth parameters which may reflect the effects of large numbers of genes. Further study of the whole collection might expose more variation.

The study of sporangial dimensions did not show any clear variation among the few isolates studied and did not even differentiate *Ph.citrophthora* from *Ph.nicotianae* . The literature contains considerable contradictions regarding this character (see Figure 4.1).

Assessment of virulence was able to show variations in host susceptibility but not in isolate virulence. *C.limmitioidis* was clearly more resistant than the other hosts. The other *Citrus* species could not be differentiated in the same way.

The electrophoretic assessment for variation has indicated that the growth media, mycelial age and electrophoretic apparatus all contribute to variation in the enzymatic and protein patterns. *Ph.citrophthora* and *Ph.nicotianae* could be separated on the basis of zymograms and protein profiles. Variation within *Ph.citrophthora* could not be clearly detected when examining for 9 enzymes. Protein profiles did however allow detection of significant variation among the isolates.

If phenotypic plasticity is discounted as an explanation, the observations reported here tend to contradict each other. With the wide geographical distribution and habitat range in which *Ph.citrophthora* ( and *Ph.nicotianae* ) were found in this and other studies (e.g. Perlberger, 1936: Sneh & Katz, 1988) variability might be expected, yet relatively small amounts of variation was found within *Ph.citrophthora* .

A methodological explanation may lie in the possibility that the cultures are physical mixtures of genotypes, despite efforts to obtain single cultures at the field isolation stage. Evidence for this comes from the observations of sexual reproduction in *Ph.nicotianae* . This well documented heterothallic species did produce oospores in single cultures in about a third of isolates. Single zoospore isolates from these were unable to reproduce sexually unless paired with another isolate. Reports on oospore production in *Ph.nicotianae* are diverse and contradictory, questioning both the underlying genetics and experimental procedure involved (Boccas & Zentmyer, 1975; Ho & Jong, 1989).

Against this idea of a high proportion of physical mixtures is the fact that *Ph.citrophthora* and *Ph.nicotianae* cultures were clearly distinguishable, with no isolates of intermediate or variable morphology. In addition, the enzymatic patterns showed no variation at all, whereas random physical mixtures would not be expected to give a totally uniform response. Not all isolates will be mixtures, so that variation will be reduced not eliminated. Indeed the isolate-environment interaction data indicates that around 20% of isolates distinctly differ from the others.

An analysis of variation in single zoospore isolates, examining their total protein profiles, and genotype-environment interactions is the obvious next step in this study which might resolve the dilemmma.

## References


Afek, U. and Sztejnberg, A. (1988) Accumulation of scoparone, a phytoalexin associated with resistance of *Citrus* to *Phytophthora citrophthora* . Phytopathology, **78**: 1678-1682.

Ali-Shtayeh, M. S. , Lim-Ho, C. L. , and Dick, M. W. (1986) An improved method and medium for quantitative estimates of population of *Pythium* species from soil. Transactions of the British Mycological Society, **86**: 39-47.

Ann, P. J. (1984) Species mating types and pathogenicity of *Phytophthora* distribution in *Citrus* orchards in Taiwan. Transactions of the British Mycological Society. **82**: 631-634.

Ann, P. J. and Ko, W. H. (1980) *Phytophthora insolita* , a new species from Taiwan. Mycologia, **72**: 1180-1185.

Ann, P. J. and Ko, W. H. (1988) Hormonal heterothallism in *Phytophthora parasitica* : a novel model of sexual reproduction. Journal of General Microbiology, **134**: 2985-2992.

Apple, J. L. (1957) Pathogenic, cultural, and physiological variation within *Phytophthora parasitica* var *nicotianae* . Phytopathology, **47**: 733-740.

Apple, J. L. (1959) Sexuality of *Phytophthora parasitica* var *nicotianae* . Phytopathlogy, **49**: 37-43.

Ashbel, D. (1973) Climate of Israel. In: Israel pocket book of geography. Keter, Jerusalem. Pages 94-111

Ashby, S. F. (1928) The oospores of *Phytophthora nicotianae* Br. de Haan, with notes on the taxonomy of *Phytophthora parasitica* Dastur. Transactions of the British Mycological Society, **13**: 86-95.

Awuab, R. T. and Lorbeer, J. W. (1989) Role of light, temperature and method of propagation in cultural variability of *Fusarium oxysporum* f.sp. AP II Race 2. Mycologia, **81**: 278-283.

Bagga, D. K. and Salley, E. B. (1974) Variation in *Hypoxyolon pruinatum* in cultural morphology and varulence. Phytopathology, **64**:663-667.

Banihashemi, Z. (1983) Detection and isolation of *Phytophthora* species in *Citrus* soil and their distribution in *Citrus* growing areas of southern Iran. Proceedings of the 7th Plant Protection Congress of Iran; College of Agriculture. University of Tehran, Karaj, Iran.

Barr, D. J. (1986) *Allochytridium expandens* rediscovered: Morphology, physiology and zoospore ultrastructure. Mycologia, **78**: 439-448.

Barrett, J. (1986) Molecular variation and evolution. In: Evolutionary biology of fungi British Mycological Society Symposium, No.11. Editors: Rayner, A.D.M., Brasier, C.M. and Moore, D.,. Cambridge University Press. Pages 83-95

Beaks, G. and Ford, H. (1983) Esterase isozyme variation in the genus *Saprolegnia* with particular reference to fish pathogenic *Saprolegnia decline -parasitica* complex. Journal of General Microbiology, **192**: 2605-2619.

Benham, R. W. (1931) Certain monilias parasitic on man, their identification, by morphology and by agglutination. Journal of Infectious Diseases, **49**: 183-187.

Bielenin, A., Jeffers, S. N., Wilcox, W. F., and Jones A. L. (1988) Separation by protein electrophoresis of six species of *Phytophthora* associated with deciduous fruit crops. Phytopathology, **78**: 1402- 1408.

Blum, H., Brier, H. and Gross, H. J. (1987) Improved silver staining of plant proteins, RNA, and DNA in polyacrylamide gels. Electrophoresis, **8**: 93-99.

Boccas , B. and Zentmyer, G. A. (1976) Genetical studies with interspecific crosses between *Phytophthora cinnamomi* and *Phytophthora parasitica* . Phytopathology, **66**: 477-484.

Boccas , B. and Lavielle, E. (1978) Les manladies a *Phytophthora* desagrumes. Institute de Recherches sur les fruits et Agrumes (IRFA). 162 pages.

Borsuk,Z., Gat, Z. and Hermony, Z. (1976) A geotopoclimatic study in the Jordan Valley. The Meteorological survey of Israel, Bet Dagan. 54 pages.

Bourdoen, J. J. and Marshall, D. R. (1981) Isozyme variation between species and forma speciales of the genus *Puccinia*. Canadian Journal of Botany, 59:2628-2634.

Bradford, M.M. (1976) A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Analytical Chemistry, **72**: 248-254.

Brasier, C. M. (1969) The effect of light and temperature on reproduction *in vitro* in two tropical species of *Phytophthora* . Transactions of the British Mycological Society, **52**: 105-113.

Brasier, C. M. (1971) Induction of sexual reproduction in single A2 isolates of *Phytophthora* species by *Trichoderma viride* . Nature New Biology, **231**: 283.

Brasier, C. M.(1972) Observation on the sexual mechanism in *Phytophthora palmivora* and related species. Transactions of the British Mycological Society, **58**: 237-251.

Brasier, C. M. (1975 a) Stimulation of sex organ formation in *Phytophthora* by antagonistic species of *Trichoderma* . I. The effect *in vitro* . New Phytologist, **74**: 183-194.

Brasier, C. M. (1975 b) Stimulation of sex organ formation in *Phytophthora* by antagonistic species of *Trichoderma* . II. Ecological implication . New Phytologist, **74**: 195-198.

Broadbent, P. (1969) Observation on the mode of infection of *Ph.citrophthora* in resistant and susceptible *Citrus* roots.Proceedings of the First International *Citrus* Symposium, **3**: 1207-1210.

Broadbent, P., Fraser, L. R. and Waterworth, Y. (1971) The reactions of seeds of *Citrus* and related species to *Ph.citrophthora*. Proceedings of the Linnean Society of New South Wales, **96**: 119-127.

Bumbieries, M. (1974) Characteristics of two *Phytophthora* species. Australian Journal of Botany, **22**: 655-660.

Burdon, J. J. and Marshall, D. R. (1981) Isozyme variation between species and forma specialis of the genus *Puccinia* . Canadian Journal of Botany, **59**: 2628-2634.

Burdon, J. J., Marshall, D. R. and Graves, R. H. (1980) Isozyme variation in *Chondrilla juncea* L. in Australia. Australian Journal of Botany, **28**: 193-198.

Cameron, H. R. (1966) Variation in the genus *Phytophthora* . II. Effects of vitamin on growth. Phytopathology, **56**: 812-815.

Cameron, H. R. and Milbrath, G. M. (1965) Variability in the genus *Phytophthora* . I. Effects of nitrogen sources and pH on growth. Phytopathology, **55**: 653-657.

Carder, J. H. (1989) Distinction between cellulose isozyme patterns of five plant pathogenic *Verticillum* species. Mycological Research, **92**: 297-301.

Castro, C. (1989). Studies on Brazilian isolates of *Phytophthora infestans* . In: *Phytophthora* ; an International Symposium, Ireland (Abstract) Pages 43-44

Caten, C. E. (1970) Spontaneous variability of single isolates of *Phytophthora infestans* . II. Pathogenic variation. Canadian Journal of Botany, **48**: 897-905.

Chee, K. H. and Newhook, F. J. (1965) Improved methods for use in studies on *Phytophthora cinnamomi* Rands and other *Phytophthora* species. New Zealand Journal of Agricultural Research, **9**: 32-43.

Christ, B. J., Person, C. O. and Pope, D. D. (1987) The genetic determination of variation in pathogenicity. In: Populations of plant pathogens, their dynamics and genetics. Editors: Wolfe, M. S. and Caten, C. E. Blackwell Scientific Publications, Oxford. Pages 7-19

Christensen, M. (1981) Species diversity and dominance in fungal communities. In: The fungal community. Editors: Wicklow, D. and Carroll, G. Marcel Decker, New York. Pages 201-232.

Christie, T. (1958) Nutritional studies of *Phytophthora cactorum* (Leb. and Cohn) Schroet. Part I: Utilization of nitrogen and carbon compounds. New Zealand Journal of Science, **1**: 83-90.

Cinar, A. and Tuzcu, O. (1976) Resistance study of the *Citrus* rootstocks to *Phytophthora citrophthora* (Smith and Smith) Leonian. II. The use of a new method in selecting resistant *Citrus* rootstocks to *Phytophthora citrophthora* (Smith and Smith) Leonian. Journal of Turkish Phytopathology, **5**: 99-104.

Cinar, A., Tuzcu, O. and Goksedef, M. O. (1976) Resistance study of *Citrus* rootstocks to *Phytophthora citrophthora* (Smith and Smith) Leonian. Journal of Turkish Phytopathology, **5**: 49-59.

Clare, B.G. and Zentmyer, G.A. (1966) Starch gel electrophoresis of proteins from species of *Phytophthora* . Phytopathology, **56**:1334-1335.

Clare, B. G., Flentje, N. T. and Atkinson, M.R. (1968) Electrophoretic patterns of oxidoreductases and other proteins as criteria in fungal taxonomy. Australian Journal of Biological Sciences, **21**: 275-295.

Cowling, E. B. and Kelman, A. (1964) Influence of temperature on growth of *Fomes annosus* isolates. Phytopathology, **54**: 373-378.

Cuypers, B. and Hahlbrock, K. (1988) Immunohistochemical studies of compatible and incompatible interactions of potato leaves with *Phytophthora infestans* and of the non host response *Phytophthora megasperma* . Canadian Journal of Botany, **66**: 700-705.

Dance, M. H., Newhook, F. J. and Cole J. S. (1975) Bioassay of *Phytophthora* species in soil. Plant Disease Reporter, **59**: 523-527.

Davis, B. J. (1964) Disc electrophoresis II. Methods and application to human serum proteins. Annals of the New York Academy of Science, **121**: 404-427.

Day, P. R.(1960) Variation in Phytopathogenic fungi. Annual Review of Phytopathology, **14**: 1-16.

Day, P. R. and Wolfe, M. S. (1987) The genetics of epidemics. In: Population of Plant pathogenes, their dynamics and genetics. Editors: Wolfe, M. S. and Caten, C. E. Blackwell Scientific Publications, Oxford. Pages 3 - 6.

de Bary, A. (1876). Research into the nature of the potato fungus *Phytophthora infestans* . Journal of the Royal Agricultural Society, England, **12**: 239-269.

Dimitman, J. E. and Klotz, L. J. (1960) Effect of stem girdling of *Citrus* seedlings on size of *Phytophthora* gummosis lesion. Phytopathology, **50**: 83.( Abstract).

Doepel, R. F.(1966) *Phytophtora* species on *Citrus* in Western Australia. Plant Disease Reporter, **50**:494-496.

Doster, M. A. and Bostock, R. M. (1988) Incidence, distribution, and development of pruning wound cankers caused by *Phytophthora syringae* in almond orchards in California. Phytopathology, **78**:468-472.

Duniway, J. M. (1979) Water relations of water molds. Annual Review of Phytopathology, **17**: 431-460.

Eaton, G. M., Brewer, G. J., and Tashian, R. E. (1966) Hexokinase isoenzyme patterns of human erythrocytes and leukocytes. National Meeting on Hematology, (Abstract).

Eberhart, S. A. and Russel, W. A. (1966) Stability parameters for comparing varieties. Crop Science, **6**: 36-40.

Eckert, J. W. and Tsao, P.H. (1960) A preliminary report on the use of pimaricin in the isolation of *Phytophthora* species from root tissues. Plant Disease Reporter, **44**: 660-661.

Eckert, J. W. and Tsao, P. H. (1962) A selective medium for isolation of *Phytophthora* and *Pythium* from plant roots. Phytopathology, **52**: 771-777.

Egly, C. R. and Garaud, C. S. (1984) Selective detection of calmodulin in polyacrylamide gels by double staining with coomassi blue and silver. Electrophoresis, **5**: 285-288.

El-Korany, A. and Sattar, M. A. (1989) *Phytophthora infestans* in Egypt. In: *Phytophthora* ; an Intrnational Symposium, Ireland. (Abstract) Page 53

Erselius, L. J. and Shaw, D. S. (1982) Protein and enzyme differences between *Phytophthora palmivora* and *Phytophthora megasperma* : Evidence for self fertilization in pairings of the two species. Transactions of the British Mycological Society, **78**: 727-738.

Erselius, L. J. and Vallavieille, C. (1984) Variation in protein profiles of *Phytophthora* : comparison of six species. Transactions of the British Mycological Society, **83**: 463-472.

Erwin, D. C. (1983) Variability within and between species of *Phtyophthora* . In: *Phytophthora* : its biology, taxonomy and pathology. Editors: Erwin, D. C., Batrinicki - Garcia, S. and Tsao, P. H. American Phytopathological Society, St. Paul, Minnesota. pages 149-165.

Faris, M. A., Sabo, F. E. and Cloutier, Y. (1986) Intraspecific variation in gel electrophoresis patterns of soluble mycelial proteins of *Phytophthora megasperma* isolated from alfalfa. Canadian Journal of Botany, **64**: 262-265.

Fatemi, J. (1980) The role of *Phytophthora* species in almond decline in Iran. Phytopathologische Zeitschrift, **99**: 97-100.

Fawcett, H. S. (1923) Gummosis of *Citrus* . Part I. Gummosis due to *Pythesystis citrophthora* . Journal of Agricultural Research **26**: 191-236.

Fawcett, H. S. and Bitacourt, A. A.( 1940) Occurrence, pathogenicity and temperature relations of *Phytophthora* species on *Citrus* in Brazil and other South American countries. Arquivos do Instiuto Biologica, **11**: 107-118.

Favaloro, M. and Summarco, G. (1973) Investigations on collar rot of *Citrus* . *Phytophthora* species detected in *Citrus* orchards of eastern Sicily. Phytopathologique Mediterraneen, **9**: 54-56.

Feichtenberger, E., Zentmyer, G. A. and Menge, J. A. (1984) Identity of *Phytophthora* isolated from milk weed vine. Phytopathology, **73**: 50-55.

Feld, S. J. , Menje, J. A. and Pehrson, J. E. (1989) Brown rot of Citrus . A review of the disease. Citrograph, **64**: 101 - 106.

Finlay, K. W. and Wilkinson, G. N. (1963) The analysis of adaptation in plant- breeding programme. Australian Journal of Agricultural Research, **14**: 742-754.

Flentje, N. T. (1970) Genetical aspects of pathogenic and saprophytic behaviour of soil-born fungi: Bsidiomycetes with special reference to*Thanatephorous cucumeris* . In: Root disease and soil-born plant pathogens. Editors: Toussoun, T. A., Bega, R.V. and Nelson, P. E. University of California press, Berkley. Pages 45-49.

Flowers, R. A. and Hendrix, J. W. (1969) Gallic acid in a procedure for isolation of *Phytophthora parasitica* var *nicotianae* and *Pythium* from soil. Phytopathology, **59**: 725-731.

Follin, J. C. (1971) L'utilisation du benomyl pour l'isolement selectif des Pythiacees . Coton Fibres Trop. (Fr. ed.) **26**: 467.

Fraser, L. (1942) *Phytophthora* root rot of *Citrus* . The Journal of Australian Institute of Agricultural Science, **8**: 101-105.

Freeman, G. H. and Perkins, J. M. (1971) Environmental and genotype-environmental components and measures of the environments. Heredity, **27**: 15-23.

Fripp, Y. J. (1972) Genotype - environment interactions in *Schizophyllum commune* .II. Assessing the environment. Heredity, **28**: 223-238.

Fripp , Y. J. and Caten, C. E. (1971) Genotype - environment interactions in *Schizophyllum commune* .I. Analysis and character. Heredity, **27**: 393-407.

Fripp , Y. J. and Caten, C. E. (1973) Genotype - environment interactions in *Schizophyllum commune* . III. The relationship between gene expression and sensitivity to change in the environment. Heredity, **30**: 341-349.

Furr, J. R. and Carpenter, J. B. (1961) Program of breeding *Citrus* rootstocks tolerant to *Phytophthora* root rot. Proceedings of Florida State Horticultural Society, **74**: 18-23.

Gallegly, M. E. (1983) New criteria for classifying *Phytophthora* and critique existing approaches.. In: *Phytophthora* : its biology, taxonomy, ecology and pathology. Editors: Erwin, D. C., Bartinicki-Garcia, S.B., and Tsao, P. H. The American Phytopathological Society, St. Paul. pages 167-172 .

Gill, H. S. and Powell, D. (1968 a) Polyacrylamide gel (disc) electrophoresis of physiologic races A1 to A8 of *Phytophthora fragrarae*. Phytopathology, **58**: 722-723.

Gill, H. S. and Powell, D. (1968 b) The use of polyacrylamide gel electrophoresis in delimiting three species of *Phytophthora* . Phytopathology, **63**: 23-29.

Gisi, U., Oertli, J. J. and Schwinn, F. J. (1977) Water and salt relations of sporangia of *Phytophthora cactorum* (Leb. et Cohn) Schroet. *in vitro* . Phytopathology Z., **89**: 261-284.

Goodwin, P. H., Kirkpatrick, B. C. and Duniway, G.M. (1989) Clonal DNA probes for identification of *Ph.parasitica*. Phytopathology, **79**:716-721.

Green, R. J., and Pratt, R. G. (1970) Rot rot of black walnut seedlings caused by *Phytophthora citricola* . Plant Disease Reporter, **54**: 39-43.

Gregory, P. H. (1983) Some major epidemics caused by *Phytophthora* . In: *Phytophthora* : its biology, taxonomy and pathology. Editors: Erwin, D. C., Batrnicki - Gracia, S. and Tsao, P. H. American Phytopathological Society, St. Paul, Minnesota. Pages 271-278.

Grimm, G. R. and Alexander, A.F. (1973) *Citrus* leaf pieces as traps for *Phytophthora parasitica* from soil slurries. Phytopathology, **63**: 540-541.

Grimm, G. R. and Hutchison, D. J. (1973) A procedure for evaluating resistance of *Citrus* seedlings to *Phytophthora parasitica* . Plant Disease Reporter, **57**: 669-672.

Grimm, G. R. and Hutchison, D. J. (1977) Evaluation of *Citrus* species, relatives and hybrids for resistance to *Phytophthora parasitica* Daster. Proceedings of the International Society of Citriculture, **3**: 863-865.

Grimm, G. R. and Whidden, R. (1962) Range of pathogenicity of Florida cultures of the foot rot fungus. Proceedings of the Florida State Horticultural Society, **75**: 73-74.

Hall, R., Zentmyer, G. A. and Erwin, D. C. (1969) Approach to taxonomy of *Phytophthora* through acrylamide gel electrophoresis of proteins. Phytopathology, **59**: 770-774.

Hames, B. D. and Rickwood, D. (1981) Gel electrophoresis of proteins. ILR, Oxford, 290 pages.

Hamm, P. B. and Hansen, E.M. (1982) Single spore isolate variation: the effect on varietal designation in *Phytophthora megasperma* . Canadian Journal of Botany, **60**: 2931-2938.

Hansen, E. M., Brassier, C. M. ; Shaw, D. S. and Hamm, P. B. (1986) The taxonomic structure of *Phytophthora megasperma* : Evidence for emerging biological species groups. Transactions of the British Mycological Society, **87**: 557-573.

Hartman, G. (1969) Investigations on *Citrus* gummosis in Lebanon. 2e Congres de lUnion Phytopathologique Mediterranee, **1**: 181-183.

Hartman, G. and Nienhaus, F. (1974 a) *Phytophthora citrophthora* and *Hendersonula toruloidea* causing brown rot gummosis of *Citrus limon* in Lebanon. I. Identification and symptomatology. Proceedings of the International Society of Citriculture, **81**: 269-286.

Hartman, G. and Nienhaus, F. (1974 b) *Phytophthora citrophthora* and *Hendersonula toruloidea* causing brown rot gummosis of *Citrus limon* in Lebanon. II. Seasonal influences. Proceedings of the International Society of Citriculture, **81**: 434-457.

Hass, J. H. (1964) Isolation of *Phytophthora megasperma* var *sojae* in soil dilution plates. Phytopathology, **54**: 894. (Abstract).

Hassan M. S., El-Bahadli, A. H. and Al-Saadawi, I. S. (1989) *Citrus* replant problems in Iraq. I. Possible role of soil fungi and nematodes. Plant and Soil, **116**: 151-155.

Hawksworth, D. L., Sutton, B. C. and Ainsworth, G.C. (1983) Dictionary of the fungi. Commonwealth Mycological Institute, Surrey. 7th edition.

Hill, J. (1975) Genotype- environment interactions, a challenge for plant breeding. Journal of Agricultural Science, **85**: 477-493.

Ho, H. H. (1981) Synoptic keys to the species of *Phytophthora* . Mycologia, **73**: 705-714.

Ho, H. H. (1982) Affinity groups among plant pathogenic species of *Phytophthora* in culture. Mycopathologia, **79**: 141-146.

Ho, H. H. and Jong, S. C. (1989) *Phytophthora nicotianae* (*P.parasitica* ). Mycotaxon, **35**: 243-276.

Ho, H. H., Liang, Z. R., Zhuang, W. R. and Yu, Y. Y. (1984) *Phytophthora* species from rubber tree plantations in Yunnan Province of China. Mycopathologia, **86**: 121-124.

Hussain, M.I.(1980) Taxonomy and variation in *Phytophthora* . Ph.D. thesis, University of Liverpool.

Itavaara, M. (1988) Identification of Shiitake strains and some other basidiomycetes: protein profiles, esterase and acid phosphatase zymograms as an aid in taxonomy. Transactions of the British Mycological Society, **91**: 295-304.

Jeffers, S. N.; Aldwinkle, H. S.; Burr, T. J. and Anderson, P. A. (1982) *Phyttophthora* and *Pythium* species associated with crown rot in New York apple orchards.Phytopathology, **72**: 533-538.

Jennings, D. H. (1987) The medium is the message. Transactions of the British Mycological Society, **89**: 1-11.

Jones, M. G. and Noble, W. C. (1982) An electrophoretic study of enzymes as a tool in the taxonomy of dermatophytes. Journal of General Microbiology, **128**: 1101-1107.

Kannaiyan, J., Ribiero, O. K., Erwin, D. C. and Nene, Y. L. (1980) *Phytophthora* blight of pigeon pea in India. Mycologia,**72**: 169-181.

Kaosiri, T. and Zentmyer, G. A. (1980) Protein, esterase and peroxidase patterns in *Ph.palmivora* complex fromcocoa.Mycologia,**72**:988-999.

Karmon, Y. (1971) Israel, a regional geography. Wiley- Interscience, London. 345 pages.

Kellam, M. K. and Zentmyer, G. A. (1986) Morphological, physiological, ecological and pathological comparisons of *Phytophthora* species isolated from *Theobroma cacao* . Phytopathology, **76**: 159- 164.

Khaki, I. A. and Shaw, D. S. (1974) The inheritance of drug resistance and compatibility type of*Ph.drechsleri*. Genetical Research, **23**: 75-86.

Kliejunas, J. T. and Nagata, J. T. (1979) *Ph.cinnamomi* in Hawaiian forest soils: seasonal variation in population levels.Phytopathology, **69**: 1268-1272.

Kliejunas, J. T., Scharpf, R. F. and Smith Jr, R. S.(1977) The occurence of *Phytophthora cinnamomi* in Hawaii in relation to Ohio forest sites and edaphic factors. Plant Disease Reporter, **61**: 280-293.

Klotz, L. J. and Dewolfe, T. A. (1959) Studies on *Phytophthora* root rot of *Citrus* . Proceedings of the 9th International Botanical Congress, **2**: 194-195.( Abstract).

Klotz, L. J. and Dewolfe, T. A. (1960) The production and use of zoospore suspensions of *Phytophthora* species for investigations on diseases of *Citrus* . Plant Disease Reporter, **44**: 572-573.

Klotz, L. J. , Dewolfe, T. A. and Baines, R. C. (1969) Resistance of trifoliate orange stocks to gummosis. California Citrograph, **52**: 387-388.

Klotz, L. J. , Dewolfe, T. A. and Wong, P. P. (1958) Decay of fibrous roots of *Citrus* . Phytopathology, **48**: 616-622.

Ko, W. H. (1988) Hormonal heterothallism and homothallism in *Phytophthora* . Annual Review of Phytopathology, **26**: 57-73.

Kowit, J. D. and Maloney, J. (1982) Protein cleavage by boiling in sodium dedocyl sulphate prior to electrophoresis. Analytical Biochemistry, **123**: 86-93.

Laviola, C. and Gallegly, M. E. (1967) Heterothallism in *Phytophthora citrophthora* . Phytopathology, **57**: 646.( Abstract).

Leonian, L. H. (1985) Physiological studies on the genus *Phytophthora*. American Journal of Botany, **12**: 444-495.

Leonian, L. H.(1934) Identification of *Phytophthora* species. Agricultural Experimental Station Bulletin, **262**: 1-36.

Leonian, L. H. and Lilly, V. G. (1937) Partial purification of a vitamin like substance which stimulates reproduction of certain fungi. American Journal of Botany, **24**: 700-702.

Longe, K., Fraser, L., Bacon, P. and Broadbent, P. (1977) The benton citrange: A promising resistant rootstock for *Citrus* trees. Proceedings of the International Society of Citriculture, **2**: 541-544.

Maghrabi, H. A. and Kish, L. P. (1985) Isozyme characterization of *Ascospherales* associated with bees: II. *Ascosphera major* , *Ascosphera atra* and *Ascosphera asterophora*. Mycologia, **77**: 366-372.

Manly, B. ( 1986) Multivariate statistical methods. Chapman and Halls, London. pp 159.

Mansoori, B. and Fasssihiani, A. (1985) Root and crown rot of *Citrus* Trees in Kerman and Hormozgan provinces of Iran. Iran Journal of Plant Pathology, **21**: 19-20.

Marks, G. C., Fagg, P. C. and Kassaby, F. Y. (1975) The distribution of *Phytophthora cinnamomi* in forests of Eastern Grippsland, Victoria. Australian Journal of Botany, **23**: 263-275.

Marks, G. C. and Kassaby, F. Y. (1974) Pathogenicity of *Pythium* and *Phytophthora drechsleri* to *Eucalyptus* species. Australian Journal of Botany, **22**: 661-668.

Marks, G. C., Kassaby, F. Y. and Reynolds, S. T. (1972) Die back in mixed wood forests of eastern Victoria: A preliminary report. Australian Journal of Botany, **20**: 141-154.

Massago, H., Yoshikawa, M., Fukada, M. and Nakanishi, N. (1977) Selective inhibition of *Pythium* species on a medium for direct isolation of *Phytophthora* species from soils and plants. Phytopathology, **67**: 425-428.

Mather, K. and Jinks, J. L.(1971) Biometrical genetics, 2nd edition. Chapman and Hall Ltd. London. 382 pages.

Matheron, E. and Matejka, J. C. (1986) Seasonal fluctuations in susceptability of six *Citrus* rootstocks to *Phytophthora parasitica* and *Phytophthora citrophthora* . Phytopathology, **76**, 1065. (Abstract).

Matheron, M. E. and Mircetich, S. M. (1985) Influence of flooding duration on development of *Phytophthora* root and crown rot of *Juglus hindsii* and Paradox walnut rootstocks. Phytopathology, **75**: 973-976.

Mc Cain, A. H., Holtzman, O. U. and Trujillo, E. E. (1967) Concentration of *Phytophthora cinnamimi* chlamydospores by soil sieving. Phytopathology, **57**: 1134-1135.

Mendel, K. (1971) "Poorman" A promising rootstock for Israeli *Citrus* . Journal of Horticultural Science, **6**: 45-46.

Meyer, J. A. and Renard, J. L. (1969) Protein and esterase patterns of two formae speciales of *Fusarium oxysporum* . Phytopathology, **59**: 1409-1411.

Michales, J. A.; Bonde, M. R. and Peterson, G. L. (1986) The use of isozyme analysis in fungal taxonomy and genetics. Mycotaxon, **27**: 405-449.

Middleton, J. (1985) Potentially pathogenic *Phytophthora* isolates in irrigation. Ph.D. thesis, Botany Department, University of Reading.

Miller, G. L. (1959) Use of dinitrosalycylic acid reagent for determination of reducing sugar. Analytical Chemistry, **31**: 426-428.

Mircetich S. M. and Matheron, M. E. (1976) *Phytophthora* root and crown rot of cherry trees. Phytopathology, **66**: 549-558.

Mircetich S. M. and Matheron, M. E. (1983) *Phytophthora* root and crown rot of walnut trees. Phytopathology,**73**: 1481-1488.

Morton, D. J. and Dukes, P. D. (1967) Serological differentiation of *Pythium aphanidermatum* from *Phytophthora parasitica* var *nicotianae* and *Phytophthora parasitica* . Nature **213**: 923.

Mulder, D. (1969) The pathogenicity of several *Pythium* species to rootlets of apple seedlings. Netherlands Journal of Plant Pathology. **75**: 178-181.

Muller, E. (1979) Factors inducing sexual and asexual sporulation in fungi (mainly Ascomycetes). In: The whole fungus, the sexual and asexual analysis. Proceedings of the second International Mycological Conference. Editor: Kendrick, B. National Museum of Natural Science, Ottawa. Pages 265-282, .

Nealson, K. H. and Garber (1967) An electrophoretic survey of esterases, phosphatases and leucine amino peptidases in mycelial extracts of the species of *Aspergillus*. Mycologia, **59**: 330-336.

Newhook, F. J. and Jackson, G. V. H. (1977) *Phytophthora palmivora* in cocoa plantation soils in the Solomon Islands. Transactions of the British Mycological Society, **69**: 31-38.

Newhook, F. J.; Waterhouse, G. M. and Stamps, D. J. (1978) Tabular key to the species of *Phytophthora* De Bary. Mycological papers, **143**, CMI 20 pages.

Nygaard, S. L., Elliot, C. K., Cannon, S. J. and Maxwell, D. P. (1989) Isozyme variability among isolates of *Phytophthora megasperma* . Phytopathology, 79: 773-788.

Ocana, G. and Tsao, P. H. (1966) A selective agar medium for direct isolation and enumeration of *Phytophthora* in soil. Phytopathology, 56: 1070. (Abstract).

Otrosina, W. J. and Marx, D. H. (1975) Populations of *Phytophthora cinnamomi* and *Pythium* species under shortleaf and lobolly pines in little leaf disease sites. Phytopathology, 65: 1224-1229.

Oudemans, P. (1989) Relationship between *Phytophthora* species: Evidence from isozyme analysis. In: *Phytophthora* ; an International Symposium, Ireland. (Abstract) Page7.

Oxenham, B. L. and Stone, Y. N. (1969) *Citrus* brown rot in Queensland. Queensland Journal of Agriculture and Animal Sciences, **26**: 173-180.

Panabieres, F. Marais, A., Trenten, F., Bonnet, P. and Ricci, P. (1989) Repetitive DNA polymorphism analysis as a tool for identifying *Phytophthora* species. Phytopathology, **79**: 1105-1109.

Papavizas, G. C., Bowers, J. H. and Johnson, S. A. (1981) Selective isolation of *Phytophthora capsici* from soils. Phytopathology, **71**: 129-133.

Parmeter, Jr. J. R. (1970) Mechanism of variation in culture and soil. In: Root disease and soil- borne pathogens. Editors: Toussoun, T. A., Bega, R. V. and Nelson, P. E. University of California Press, Berkley. Pages 63-74 .

Patestos, N., Fauth, M. and Radola, B. J. (1988) Fast and sensitive staining with colloidal acid violet 17 following isoelectric focusing in carrier ampholyte generated and immobilized pH gradients. Electrophoresis, **9**: 488-496.

Peberdy, J. F. and Turner, M. (1968) The esterase of *Mortierella ramanniana* in relation to taxonomy. Journal of General Microbiology, **51**: 305 - 312.

Perkins, J. M. and Jinks, J. L. (1968) Environmental and genotype-environmental components of variability. III. Multiple lines and crosses. Heredity, **30**: 111-126.

Perlberger, J. (1936) *Phytophthora* stem and tip blight of *Citrus* seedlings. Hadar, **9**: 145-150.

Pittis, J. E. and Colhoun, J. (1984) Isolation and identification of pythacious fungi from irrigation water and their pathogenicity to *Antrihinum* , tomato and *Chamaecyparis lawsonianae* . Phytopathologische Zeitschrift, **110**: 301-318.

Ponchet, J., Recci, P., Andereoli, C. and Auge, G. (1972) Methodes selectives d'isolement du *Phytophthora nicotianae* f.sp. *parasitica* ( Daster) Waterh. a partir du sol. Annals of Phytopathology, **4**: 97-108.

Prosser, J. I. (1983) Hyphal growth patterns. In: Fungal differentiation. ed: Smith, J. E., Marcel Dekker Inc. New York. Pages 357-396.

Purseglove, J. W. (1968) Tropical crops, Dicotyledons 2. Longmans: London, pp 719.

Raabe, R. D. (1967) Variation in pathogenicity and virulences in *Armillaria mella* , Phytopathology, **57**: 73-75.

Rebour, S. E. (1966) Assessment of resistance of four *Citrus* cultivars to *Ph.citrophthora* . Iran Journal of Plant Pathology, **2**: 115-123.

Reeves, R. J. and Jackson, R. M. (1974) Stimulation of sexual reproduction in *Phytophthora* by damage. Journal of General Microbiology, **84**: 303-310.

Reichert, I. (1932) A new rot of Citrus in Palestine. Hadar, **5**: 254-256.

Reichert, I. and Littauer, F. (1931) The causal agent of gummosis disease in Palestine. Hadar, **4**: 145-150.

Ribiero, O. K. (1978) A source book of the genus Phytophthora. Cramer, Vaduz, Liechtenstein. pp 417.

Rice, E. L. and Crowden, P.K. (1987) An improved method of extraction and electrophoresis of proteins and active enzymes from fucalean macroalgae (Phaeophyta), Phycologia, **26**: 235-246.

Ridings, W. H., Schoulties, C. L., El-Gholl, N. E. and Mitchell, D. J. (1977) The milkweed vine pathotype of *Ph.citrophthora* as a biological control agent of *Morrenia odorata*. Proceedings of the International Society of Citriculture, **3**: 877-881.

Rosenan, N. (1976) Rainfall map of Israel. In: Atlas of Israel IV/2. Survey of Israel, Ministry of Labour, Jerusalem and Elsevier Publications Company. Amsterdam.

Rosettie, V. (1947) Susceptibility of different species of *Citrus* to some species of *Phytophthora* . Arquivos do Instituto Biologico, **18**: 97-124.

Rural Studies Centre Annual Report (1987) Rural Studies Research Centre, An-Najah National University, Nablus.

Sansome, E. (1965) Meiosis in diploid and polyoloid sex organs of *Phytophthora* and *Achyla* . Cytologia, **30**: 103-117.

SAS Institute Incorporated (1982). SAS user's guide: Statistics. Cary, North Carolina.

SAS Institute Incorporated (1985). SAS user's guide: Statistics Version 5. Cary, North Carolina. pp 956.

Savage, E. J. , Vlayton, C. W. , Hunter, J. H. , Brenneman, J. A. , Laviola, C. and Gallegly, M. E. (1968) Homothallism, heterothallism and interspecific hybridization in the genus *Phytophthora* . Phytopathology, **58**: 1004-1021.

Sawant, D. M. , Lande, P. S. and Chandari, K. G. (1984) Reaction of *Citrus* rootstock to *Phytophthora* diseases. Indian Journal of Mycology and Plant Pathology, **14**: 211-218.

Schiffmann N. M. (1951) L'influence de la temperature et de la pluviosite sur l'apparition des differentes especes de *Phytophthora* Revue de Pathologie et d'Entomologie agricole de France, **30**: 232-237.

Schiffman N. M. and Cohen, E. (1969) The incubation period of *Phytophthora citrophthora* in *Citrus* fruits stored at different temperatures. Phytopathology, **59**: 237-238.

Schofield, R. K. and Taylor, M. L. (1955) The measurement of soil pH. Proceedings of the American Soil Society, **19**: 164-167.

Sivour, R. J. , Petheca, L. M. , Soddell, J. A. and Pitt, D. E. (1985) Electrophoretic patterns of sporangispore proteins of *Rhizopus* isolates as taxonomic characters. Transactions of the British Mycological Society, **84**: 701-708.

Shattock R. C. (1989) Mating type, metalaxyl sensitivity and isozyme patterns among isolates of *Phytophthora infestans* from England and Wales. 1985-1988. In: *Phytophthora* ; an International Symposium, Ireland (Abstract). Page 45.

Shaw, C.R. and Prasad, R. (1970) Starch gel electrophoresis of enzymes- a compliation of recipes. Biochemical Genetics, **4**: 297-320.

Shepherd, C. J. (1978) Mating behaviour of Australian isolates of *Phytophthora* species. I. Inter- and inra- specific mating. Australian Journal of Botany, **26**: 123-138.

Siciliano, M. J. and Shaw, C. R. (9176) Separation and visualisation on gels. In: Chromatographic and electrophoretic techniques. Editor: Smith, I. William Heinman Medical Books Ltd. London. Pages 185-209,

Sleeth, B. (1945) Agar medium and technique for isolating *Pythium* free from bacteria. Phytopathology, **35**: 1030-1031.

Smillie, R. , Grant, B. R. and Guest, D. (1989) The mode of action of phosphite: Evidence for both direct and indirect modes of action on three *Phytophthora* species in plants. Phytopathology, **79**: 921-926.

Smith, R. E. and Smith E. H. (1906) A new fungus of economic importance. Botanical Gazette, **42**: 215-221.

Sneh, B. and Katz, D. A. (1988) Behaviour of *Phytophthora cirophthora* and *Phytophthora nicotianae* in soil and differences in their tolerance to antimicrobial compounds of selective media used for isolation of *Phytophthora* species. Phytopathology, **122**: 208-221.

Solel, Z. and Pinkas, Y. (1984) A modified selective medium for detecting *Phytophthora cinnamomi* from avocado roots. Phytopathology, **84**: 506-508.

Spileman, L. (1989) Molecular approaches to the genetics and sytematics of the Oomycetes: Phytophthora and Pythium. Phytophthora News Letter, **15**: 1 (Abstract)

Stamps, D. J. (1953) Oospore production in paired cultures of *Phytophthora* species. Transactions of the British Mycological Society, **36**: 255-259.

Stolzy, L. H. , Letey, J., Klotz, L. J. and Labanauskas, C. K. (1965) Water and aeration as factors in root decay of *Citrus sinensis* . Phytopathology, **55**: 270-275.

Stout, D. L. and Shaw, C. R. (1974) Genetic distance among certain species of *Mucor* . Mycologia, **66**: 969-977.

Talbot, P. H. (1971) Principles of fungal taxonomy. Macmillan, London. 274 pages .

Tariq, N. V. , Gutterbrige, C. S. and Jeffries, P. (1985) Comparative studies of cultural and biochemical characteristics used for distinguishing species of *Sclerotina* . Transactions of the British Mycological Society, **84**: 381-397.

Timmer, L. W. (1972) Management of soil- born diseases of *Citrus* in the lower Rio Grande Valley. Journal of Rio Grande Valley Horticultural Society, **27**: 44-48.

Timmer, L. W., Sandler, H. A. , Graham, J. H. and Zitko, S. E. (1988) Sampling *Citrus* orchards in Florida to estimate populations of *Phytophthora parasitica* . Phytopathology, **78**: 940-944.

Timmer, L. W. , Zitko, S. E. , Sandler, H. A. and Graham, J. H. (1989) Seasonal and spatial analysis of populations of *Ph. parasitica* in *Citrus* orchards in Florida. Plant Disease, **73**: 810-813.

Tooley, P. W. , Fry, W. E. and Gonzalez, M. J. V. (1985) Isozyme characterization of sexual and asexual *Phytophthora infestans* populations . The Journal of Heredity, **76**: 431-435.

Tooley, P. W. , Sweigard, J. A. and Fry, W. E. (9186) Fitness and virulence of *Phytophthora infestans* isolates from sexual and asexual populations. Phytopathology, **76**: 1209-1212.

Tsao, P. H. (1960) A serial dilution end point method for estimating disease potential of *Citrus Phytophthora* s in soil. Phytopathology, **50**: 717-724.

Tsao, P. H. (1983) Factors affecting isolation and quantitation of *Phytophthora* from soil. In: *Phytophthora* : its biology, taxonomy and pathology. Editors: Erwin, D. C., Batrinicki-Garcia, S. and Tsao, P. H. American Phytopathological Society, St. Paul, Minnesota. Pages 219- 236.

Tsao, P. H. and Menyonga, J. M. (1966) Response of *Phytophthora* species and soil microflora to antibiotics in the pimaricin- vancomycin medium. (Abstract) Phytopathology, **56**: 152.

Tsao, P. H. and Ocana, G. (1969) Selective isolation of species of *Phytophthora* from natural soils on an improved antibiotic medium. Nature, **223**: 636-638.

Tsao, P. H. , Ugale, R. , Hobbs, S. and Farih, A. (1980) Control of homothallic oospore formation in *Phytophthora parasitica* by culture manipulations. Transactions of the British Mycological Society, **75** :153-156.

Tucker, C. M. (1931) Taxonomy of the genus *Phytophthora* de Bary. Missouri University Agricultural Experiment Station Research Bulletin, **153**: 208 pages.

Tuzcu, O. , Cinar, A. , Gokesedef, M. O. Ozsan, M. and Bicici, M. (1984) Resistance of *Citrus* rootstocks to *Phytophthora citrophthora* during winter dormancy. Plant Disease, **68**: 502-505.

Uppal, B. N. and Kamat, M. N. (1936) Gummosis of *Citrus* in Bombay. Indian Journal of Agricultural Science, **4**: 803-822.

Vallavieille, C. and Erselius, L. J. (1984) Variation in protein profiles of *Phytophthora* : Survey of a composite population of three species on *Citrus* . Transactions of the British Mycological Society, **83** :473-479.

Van Breda de Haan, J. (1896) Dbibitzeikte in de Deli-tabakveroorzkt door Phytophthora nicotianae . Meded. 'S Land Platinium, **25**:1-107.

Vanderplank, J. E. (1982) Host - pathogen interactions in plant disease. Academic Press, New York. 207 pages.

Vanderweyen, A. (1983) Contribution a letude de la gommose a *Phytophthora* de agrumes au Maroc. Fruits, **38**: 83 -118.

Vardi, A. (1983) *Citrus* - genetics, breeding and use of tissue culture. (Abstract), Israel Journal of Botany, **32**: 39

Walker, J. (1975) Mutual responsibilities of taxonomic mycology and plant pathology. Annual Review of Plant Pathology, **13**: 335-355.

Waterhouse, G. M. (1963) Key to the species of *Phytophthora* de Bary. Mycological papers, number **92**. CMI, 22 pages.

Waterhouse, G. M. (1970) The genus *Phytophthora* de Bary. Diagnosis ( or descriptions) from the original papers. Mycological papers, number **112**. CMI, 59 pages.

Waterhouse, G. M. and Blackwell, E. M. (1954) Key to the species of *Phytophthora* recorded in the Brirish Isles. Mycological papers, number **57**. CMI, 9 pages.

Waterhouse, G. M. and Waterson, J. M. (1964 a). *Phytophthora hibernalis* . Descriptions of pathogenic fungi and bacteria, number **31**. CMI, 2 pages

Waterhouse, G. M. and Waterson, J. M. (1964 b). *Phytophthora citrophthora* . Descriptions of pathogenic fungi and bacteria, number **33**. CMI, 2 pages.

Waterhouse, G. M. and Waterson, J. M. (1964 c). *Phytophthora nicotianae var parasitica* . Descriptions of pathogenic fungi and bacteria, number **35**. CMI, 2 pages.

Waterhouse, G. M. and Waterson, J. M. (1964 d). *Ph. nicotianae* var *nicotianae* . Descriptions of pathogenic fungi and bacteria, number **34**. CMI, 2 pages.

Waterhouse, G. M. , Newhook, F. J. and Stamps, J. (1983) Present criteria for classification of *Phytophthora* . In: *Phytophthora* : its biology, taxonomy and pathology. Editors: Erwin, D. C., BatrnicKi - Garcia, S. and Tsao, P. H. American Phytopathological Society, St. Paul, Minnesota. Pages 139- 147

Weber, J. R. (1977) Soil properties, herbicide sorption and model soil system. In : Research methods in weed science , second edition. Editor: Southern, T. Weed Science Society, USA. Page 59 - 72.

Weste, G. (1983) Population dynamics and survival of *Phytophthora* . In: *Phytophthora* : its biology, taxonomy and pathology. Editors: Erwin, D. C., Batrinicki - Garcia, S. and Tsao, P. H. American Phytopathological Society, St. Paul, Minnesota. Pages 237- 257.

Weste, G. and Ruppin, P. (1975) Factors affecting the population density of *Phytophthora cinnamomi* in native forests of Brisbane Ranges, Victoria. Australian Journal of Botany, **23**: 77-85.

Weste, G. and Ruppin, P. (1977) *Phytophthora cinnamomi* : population density in forest soils. Australian Journal of Botany, **25**: 461-475.

Weste, G. and Vithange, K. (1977) Microbial populations of three forest soils, seasonal variation and changes associated with *Phytophthora cinnamomi* . Australian Journal of Botany, **25**: 337-383.

Weste, G. and Vithange, K. (1978) Seasonal variation in numbers of chlamydospores in Victoria forest soils infected with *Phytophthora cinnamomi* . Australian Journal of Botany, **26**: 657-662.

Weste, G. and Vithange, K. (1979) Survival of chlamydospores of *Phytophthora cinnamomi* in several non- sterile, host free forest soils and gravels at different soil water potentials. Australian Journal of Botany, **27**: 1-9.

Westerman, J. M. (1971) Genotype - environment interactions and developmental regulation in *Arabidopsis thaliana* . II. Inbred lines; analysis. Heredity, **26**: 93-106.

Westerman, J. M. and Lawrence, M. J. (1970) Genotype - environment interactions and developmental regulation in *Arabidopsis thaliana* . I. Inbred lines; description. Heredity, **25**: 609-627.

Wheeler, J. E. and Boyle, A. M. (1971) Identification of four *Phytophthora* isolates previously unreported from Arizona. Phytopathology, **61**: 1293-1295.

Whiteside, J. O. (1974) Zoospore inoculation techniques for determining the relative susceptibility of *Citrus* root stocks to foot rot. Plant Disease Reporter, **58**: 313-317.

Whittaker, S. L. (1989) Isozyme markers for *Phytophthora infestans* : co-resolution of peptidase and glucose phosphate isomerase using ONE starch gel.( Abstract) *Phytophthora* Newsletter, **15**: 32.

Williamson, M. (1972) The analysis of biological populations. Edward Arnold, 180 pages.

Wilson, C. M. (1979) Studies and critique of amido black 10 B, coomassie blue R, and fast green FCF as stains for proteins after polyacrylamide gel electrophoresis. Analytical Boichemistry, **96**: 263-278.

Wong, T. K. and Vorghese, G. (1966) Assessing the susceptability of local *Citrus* species to *Phytophthora* root rot in Malaya. Experimental Agriculture, **2**: 305-308.

Wray, W. , Boulikas, T. , Wray, V. P. and Hancock, R. (1981) Silver staining of proteins in polyacrylamide gels. Analytical Biochemistry, **118**: 197-203.

Zak, B. (1961) Aeration and other factors affecting Southern pines as related to little leaf disease. U.S. Department of Agriculture Technical Bulletin, **1248**: 30 pages.

Zentmyer, G. A. (1974) Variation, genetics and geographic distribution of mating types. Page 89-102, in: *Phytophthora* disease of cocoa. Editor: Gregory, P. H., Longman, London. 342 pages.

Zentmyer, G. A. (1976) *Phytophthora* plant destroyer. Bioscience, **26**:686-689.

Zentmyer, G. A. (1979) Stimulation of sexual reproduction in the mating type of *Phytophthora cinnamomi* by a substance in avocado roots. Phytopathology, **69**: 1129-1131.

Zentmyer, G. A. , Jefferson, L. , Hickman, C. J. and Chang H. Y. (1974) Studies of *Phytophthora citricola* , isolated from *Persea americana* Mycologia, **66**: 830-845.

Zimmer, D. E. and Urie, D. E. (1967) Influence of irrigation and soil infestation with strains of *Phytophthora drechsleri* on root rot of safflower. Phytopathology,**57**: 1056-1059.

Zohary, M. (1942) The vegetative aspect of Palestine soil. Palestine Journal of Botany, **2**: 200-246.

## Appendix One

Isolates recovered from all sampling sites in the West Bank of the River Jordan during the survey period from 4th of June 1987 to 22nd January 1988.

The numbers indicate the following: Example IA7001

I indicates the sampling site.

A " the subsite within the orchard.

7 " the sampling occasion.

00 " used as a separater

1 " the isolate number taken at the specific sampling occasion

All numbers not listed in Appendix 1 refer to isolates that could not be obtained in pure culture and thus remained unidentified up to the species level.

### *Ph.citrophthora*

### Site I

#### Subsite A

| Isolate number | Date | Isolation method | Source |
|---|---|---|---|
| IA1000 | 4.6.87 | Soil dil./ VP3HB | Soil |
| IA1003 | " | " | " |
| IA1004 | " | " | " |
| IA1005 | " | " | " |
| IA1006 | " | " | " |

| Isolate number | Date | Isolation method | Source |
|---|---|---|---|
| IA7001 | 1.9.87 | Soil dil./ VP3HB | Soil |
| IA7004 | " | " | " |
| IA7009 | " | " | " |
| IA70011 | " | " | " |
| IA70012 | " | " | " |
| IA70014 | 1.9.87 | *Cit* . root/ VP3HB | Roots |
| IA11002 | 28.10.87 | *Cit* . bait/ " | Soil |
| IA11005 | " | " | " |
| IA11006 | " | Lupin bait/ " | " |
| IA11007 | " | " | " |
| IA11009 | " | " | " |
| IA110010 | " | " | " |
| IA110011 | " | " | " |
| IA12003 | 7.11.87 | *Cit* . bait/ " | " |
| IA12007 | " | " | " |
| IA120010 | " | " | " |
| IA13002 | 21.11.87 | Soil dil./ " | " |
| IA13009 | " | " | " |
| IA130011 | " | " | " |
| IA130013 | " | " | " |
| IA130015 | " | " | " |
| IA130020 | " | " | " |
| IA130021 | " | " | " |
| IA130022 | " | " | " |
| IA130023 | " | " | " |
| IA130024 | " | " | " |
| IA130025 | " | " | " |
| IA130026 | " | " | " |
| IA130027 | " | " | " |
| IA130028 | " | *Cit* . bait/ " | " |
| IA130030 | " | " | " |
| IA130031 | " | " | " |
| IA130032 | " | " | " |
| IA130033 | " | " | " |
| IA130034 | " | " | " |
| IA14001 | 9.12.87 | Soil dil./ " | " |
| IA14002 | " | *Cit* . bait/ " | " |
| IA14003 | " | " | " |
| IA14004 | " | " | " |

| Isolate number | Date | Isolation method | Source |
|---|---|---|---|
| IA14007 | 9.12.87 | Soil dil./VP3HB | Soil |
| IA14009 | " | " | " |
| IA15002 | 23.12.87 | " | " |
| IA15003 | " | " | " |
| IA15004 | " | " | " |
| IA15006 | 23.12.87 | Soil dil./ " | " |
| IA15007 | " | *Cit* . bait/ " | " |
| **Subsite B** | | | |
| IB2001 | 17.6.87 | Soil dil./ " | " |
| IB4001 | 16.7.87 | " | " |
| IB4002 | " | " | " |
| IB6001 | 13.8.87 | " | " |
| IB7001 | 1.9.87 | " | " |
| IB7002 | " | " | " |
| IB7003 | " | " | " |
| IB7004 | " | " | " |
| IB7005 | " | " | " |
| IB8001 | 24.9.87 | Lupin bait/ " | " |
| IB8002 | " | *Cit* . root/ " | Root |
| IB11004 | 28.10.87 | Lupin bait/ " | Soil |
| IB12001 | 7.11.87 | Soil dil./ " | " |
| IB12002 | " | " | " |
| IB12004 | " | *Cit* . bait/ " | " |
| IB12005 | " | " | " |
| IB12006 | " | " | " |
| IB12007 | " | " | " |
| IB120010 | " | " | " |
| IB120012 | " | " | " |
| IB13001 | 21.11.87 | Soil dil./ " | " |
| IB13002 | " | " | " |
| IB13005 | | " | " |
| IB13006 | " | " | " |
| IB13007 | " | " | " |
| IB13008 | " | " | " |

| Isolate number | Date | Isolation method | Source |
|---|---|---|---|
| **Subsite C** | | | |
| IC3003 | 29.6.87 | Soil dil./ VP3HB | Soil |
| IC4001 | 16.7.87 | " | " |
| IC4002 | 16.7.87 | " | " |
| IC4003 | " | " | " |
| IC4004 | " | " | " |
| IC4005 | " | " | " |
| IC4006 | " | " | " |
| IC4007 | " | " | " |
| IC4008 | " | " | " |
| IC40011 | " | " | " |
| IC40012 | " | " | " |
| IC40013 | " | " | " |
| IC40014 | " | " | " |
| IC40015 | " | " | " |
| IC40016 | " | " | " |
| IC40017 | " | " | " |
| IC40018 | " | " | " |
| IC40019 | " | " | " |
| IC40021 | " | " | " |
| IC40022 | " | " | " |
| IC40023 | " | " | " |
| IC4oo24 | " | " | " |
| IC40026 | " | " | " |
| IC4oo31 | " | *Cit* . root/ " | Roots |
| IC40032 | " | " | " |
| IC6003 | 13.8.87 | Soil dil./ " | Soil |
| IC6005 | " | " | " |
| IC6006 | " | " | " |
| IC6007 | " | " | " |
| IC6008 | " | " | " |
| IC9001 | 20.9.87 | " | " |
| IC9003 | " | *Cit* . root/ " | Roots |
| IC10001 | 13.10.87 | Soil dil./ " | Soil |
| IC10002 | " | " | " |
| IC10003 | " | " | " |

| Isolate number | Date | Isolation method | Source |
|---|---|---|---|
| IC10004 | " | *Cit* . bait/ VP3HB | Soil |
| IC11001 | 28.10.87 | " | " |
| IC11002 | " | " | " |
| IC11003 | " | " | " |
| IC13002 | 21.11.87 | Soil dil./ " | " |
| IC13003 | " | *Cit* . bait/ " | " |
| IC13004 | " | " | " |
| IC13006 | 21.11.87 | " | " |
| IC13007 | " | " | " |
| IC13008 | " | " | " |
| IC13009 | " | " | " |
| IC130011 | " | " | " |
| **Subsite D** | | | |
| ID4001 | 16.7.87 | Soil dil./ " | " |
| ID4002 | " | " | " |
| ID8001 | 10.9.87 | Lupin bait/ " | " |
| ID8002 | " | *Cit* . bait/ " | " |
| ID9002 | 20.9.87 | *Cit* . root/ " | Roots |
| ID11006 | 18.10.87 | *Cit* . bait/ " | Soil |
| ID12005 | 5.11.87 | " | " |
| ID13002 | 20.11.87 | " | " |
| ID13004 | " | " | " |
| ID13005 | " | " | " |
| ID13006 | " | " | " |
| ID13007 | " | " | " |
| ID13009 | " | " | " |
| ID130010 | " | " | " |
| ID130011 | " | " | " |
| ID130016 | " | " | " |
| ID130017 | " | " | " |
| ID14001 | 9.12.87 | " | " |
| ID14003 | " | " | " |
| ID14005 | " | " | " |
| ID14006 | " | " | " |
| ID14007 | " | " | " |

| Isolate number | Date | Isolation method | Source |
|---|---|---|---|
| ID14008 | " | *Cit* . bait/ VP3HB | Soil |
| ID14009 | " | " | " |
| ID140010 | " | " | " |
| ID15001 | 23.12.87 | Soil dil. / " | " |
| ID15002 | 23.12.87 | *Cit* . bait/ VP3HB | Soil |
| ID16001 | 7.1.88 | " | " |
| ID16002 | " | " | " |
| ID16004 | 7.1.88 | " | " |
| ID16005 | " | " | " |
| ID16006 | " | " | " |
| ID16007 | " | " | " |
| ID16008 | " | " | " |
| ID16009 | " | " | " |
| ID160011 | " | " | " |
| **Subsite E** | | | |
| IE2000 | 17.6.87 | Soil dil. / " | " |
| IE2002 | " | " | " |
| IE2005 | " | " | " |
| IE2006 | " | " | " |
| IE2009 | " | " | " |
| IE20012 | " | " | " |
| IE20013 | " | " | " |
| IE20014 | " | " | " |
| IE20015 | " | " | " |
| IE20016 | " | " | " |
| IE20017 | " | " | " |
| IE3001 | 29.6.87 | " | " |
| IE3003 | " | " | " |
| IE3004 | " | " | " |
| IE5002 | 29.7.87 | *Cit* . root/ " | Roots |
| IE80011 | 10.9.87 | *Cit* . bait/ " | Soil |
| IE9001 | 20.9.87 | *Cit* . root/ " | Rots |
| IE12001 | 5.11.87 | *Cit* . bait/ " | Soil |
| IE12007 | " | " | " |

## Site II

### Subsite A

| Isolate number | Date | Isolation method | Source |
|---|---|---|---|
| IIA2005 | 6.7.87 | *Cit* . root/ VP3HB | Roots |
| IIA3001 | 23.7.87 | Soil dil./ " | Soil |
| IIA3002 | " | " | " |
| IIA3006 | " | " | " |
| IIA3007 | " | " | " |
| IIA30014 | " | " | " |
| IIA30026 | " | *Cit* . bait/ " | " |
| IIA30027 | " | " | " |
| IIA5002 | 2.8.87 | Soil dil/ " | " |
| IIA7001 | 4.9.87 | *Cit* . bait/ " | " |
| IIA8001 | 26.9.87 | " | " |
| IIA10001 | 26.10.87 | " | " |
| IIA10003 | " | " | " |
| IIA10006 | " | " | " |
| IIA11001 | 13.11.87 | " | " |
| IIA11002 | " | " | " |
| IIA11003 | " | " | " |
| IIA11004 | " | " | " |
| IIA13001 | 11.12.87 | Soil dil./ " | " |
| IIA13002 | " | " | " |
| IIA13003 | " | " | " |
| IIA13004 | " | " | " |
| IIA13006 | " | *Cit* .bait/ " | " |
| IIA13007 | " | " | " |
| IIA13008 | " | " | " |
| IIA13009 | " | " | " |
| IIA130010 | " | " | " |
| IIA14001 | 31.12.87 | " | " |
| IIA14003 | " | " | " |
| IIA14004 | " | " | " |
| IIA14006 | " | " | " |
| IIA14007 | " | " | " |
| IIA140010 | 31.12.87 | *Cit* . bait/ " | Soil |
| IIA140011 | " | " | " |

| Isolate number | Date | Isolation method | Source |
|---|---|---|---|
| **Subsite B** | | | |
| IIB8001 | 25.9.87 | Soil dil./ VP3HB | " |
| IIB10001 | 26.10.87 | *Cit* . bait/ " | " |
| IIB10002 | " | " | " |
| IIB10003 | " | " | " |
| IIB10004 | " | " | " |
| IIB10005 | " | " | " |
| IIB10007 | " | " | " |
| IIB10008 | " | " | " |
| IIB10009 | " | " | " |
| IIB100010 | " | " | " |
| IIB13002 | 11.12.87 | " | " |
| IIB13003 | " | " | " |
| IIB13006 | " | " | " |
| IIB13007 | " | " | " |
| IIB13008 | " | " | " |
| IIB13009 | " | " | " |
| IIB130010 | " | " | " |
| IIB130011 | " | " | " |
| IIB130012 | " | " | " |
| IIB130013 | " | " | " |
| IIB130014 | " | " | " |
| IIB130015 | " | Soil dil/ " | " |
| IIB130016 | " | " | " |
| IIB130017 | " | " | " |
| IIB130018 | " | " | " |
| IIB130023 | " | *Cit* . bait/ " | " |
| IIB14001 | 31.12.87 | " | " |
| IIB13002 | " | " | " |
| IIB13004 | " | " | " |
| IIB14005 | 31.12.87 | *Cit* . bait/ " | Soil |
| IIB13006 | " | " | " |
| IIB13008 | " | " | " |
| IIB13009 | " | " | " |
| IIB130010 | " | " | " |
| **Subsite C** | | | |
| IIC002 | 6.7.87 | Soil dil./ " | " |

| Isolate number | Date | Isolation method | Source |
|---|---|---|---|
| IIC4005 | 3.8.87 | Soil dil./ VP3HB | " |
| IIC60010 | 4.9.87 | *Cit* . root/ " | Root |
| II60011 | " | " | " |
| IIC8001 | 25.9.87 | Lupin bait/ " | Soil |
| IIC9003 | 9.10.87 | *Cit* . " / " | " |
| IIC9005 | " | " | " |
| IIC9006 | " | Lupin bait/ " | " |
| IIC10002 | 26.10.87 | *Cit* . " / " | " |
| IIC10004 | " | " | " |
| IIC10005 | " | " | " |
| IIC11007 | 17.11.87 | Soil dil./ " | " |
| IIC11008 | " | " | " |
| IIC11008(B) | " | " | " |
| IIC11009 | " | " | " |
| IIC110012 | " | *Cit* . bait/ " | " |
| IIC110016 | " | " | " |
| IIC12002 | 27.11.87 | Soil dil./ " | " |
| IIC12006 | " | *Cit* . bait/ " | " |
| IIC13001 | 11.12.87 | Soil dil. / " | " |
| IIC13002 | " | " | " |
| IIC13004 | " | " | " |
| IIC13005 | " | " | " |
| IIC13006 | " | " | " |
| IIC13007 | " | " | " |
| IIC13009 | " | " | " |
| IIC130011 | 11.12.87 | Soil dil. / VP3HB | soil |
| IIC130012(A) | " | " | " |
| IIC130013 | " | *Cit* .bait/ " | " |
| IIC130014 | " | " | " |
| IIC130016 | " | " | " |
| IIC130017 | " | " | " |
| IIC130018 | " | " | " |
| IIC130019 | " | " | " |
| IIC130020 | " | " | " |
| IIC130021 | " | " | " |
| IIC130022 | " | " | " |
| IIC130023 | " | " | " |
| IIC130024 | " | " | " |
| IIC130025(A) | " | " | " |

| Isolate number | Date | Isolation method | Source |
|---|---|---|---|
| IIC14001 | 31.12.87 | Lupin bait/ " | " |
| IIC13007 | " | *Cit* . bait / " | " |
| IIC13009 | " | " | " |
| **Subsite D** | | | |
| IID2003 | 6.7.87 | Soil dil. / VP3HB | Soil |
| IID2005 | " | " | " |
| IID2006 | " | " | " |
| IID7002 | 4.9.87 | *Cit* . root/ " | Root |
| IID11006 | 17.11.87 | Soil dil / " | Soil |
| IID110010 | " | *Cit* . bait / " | " |
| IID110011 | " | " | " |
| IID12004 | 27.11.87 | Soil dil / " | " |
| IID12005 | " | " | " |
| IID12006 | " | " | " |
| IID12009 | " | " | " |
| IID13001 | 11.12.87 | *Cit* . bait / " | " |
| IID13002 | " | " | " |
| IID13003 | " | " | " |
| IID13004 | " | " | " |
| IID13005 | " | " | " |
| IID13006 | 31.12.87 | *Cit* . bait/ VP3HB | Soil |
| IID13007 | " | " | " |
| IID13008 | " | " | " |
| IID13009 | " | " | " |
| IID130010 | " | " | " |
| IID130011 | " | " | " |
| IID130012 | " | " | " |
| IID14001 | 31.12.87 | " | " |
| IID14003 | " | " | " |
| IID14004 | " | " | " |
| IID14005 | " | " | " |
| IID14006 | " | " | " |
| IID14007 | " | " | " |
| IID14008 | " | " | " |
| IID14009 | " | " | " |
| **Subsite E** | | | |
| IIE3001 | 23.7.87 | Soil dil. / VP3HB | " |

| Isolate number | Date | Isolation method | Source |
|---|---|---|---|
| IIE3003 | 23.7.87 | Soil dil. / VP3HB | Soil |
| IIE3003 | " | *Cit* . bait / " | " |
| IIE5001 | 23.8.87 | Soil dil. / " | " |
| IIE6001 | 4.9.87 | Soil dil. / VP3HB | Soil |
| IIE7001 | 11.9.87 | " | " |
| IIE7002 | " | " | " |
| IIE7003 | " | " | " |
| IIE7004 | " | *Cit* . root/ " | Root |
| IIE8001 | 25.9.87 | Soil dil. / " | soil |
| IIE8004 | " | " | " |
| IIE8005 | " | " | " |
| IIE8007 | " | " | " |
| IIE8008 | " | " | " |
| IIE8009 | " | " | " |
| IIE80011 | " | " | " |
| IIE80013 | " | " | " |
| IIE80014 | 25.9.87 | *Cit* . bait/ VP3HB | Soil |
| IIE80015 | " | Soil dil. / " | " |
| IIE9001 | 9.10.87 | *Cit* . bait/ " | " |
| IIE9003 | " | " | " |
| IIE100010 | 26.10.87 | " | " |
| IIE11003 | 13.11.87 | Soil dil. / " | " |
| IIE11004 | " | " | " |
| IIE11008 | " | *Cit* . bait/ " | " |
| IIE12001 | 27.11.87 | Soil dil. / " | " |
| IIE12003 | " | " | " |
| IIE12004 | " | " | " |
| IIE12006 | " | " | " |
| IIE12007 | " | " | " |
| IIE14001 | 31.12.87 | " | " |
| IIE14002 | " | Lupin bait/ " | " |
| IIE14003 | " | Soil dil. / " | " |
| IIE14004 | " | " | " |
| IIE14005 | " | *Cit* . bait / " | " |

| Isolate number | Date | Isolation method | Source |
|---|---|---|---|
| **The Other Sites** | | | |
| FAR001 | 13.10.87 | *Cit* . bait/ VP3HB | Soil |
| FAR002 | " | " | " |
| FAR003 | " | " | " |
| TMD001 | 11.12.87 | " | " |
| TMD002 | 11.12.87 | " | " |
| TMD003 | " | " | " |
| TMD004 | " | " | " |
| TMD005 | " | " | " |
| TMD006 | " | " | " |
| JRNP001 | 7.1.88 | " | " |
| JRNP004 | " | " | " |
| JRNP005 | 7.1.88 | *Cit* . bait/ VP3HB | Soil |
| JRBD003 | 7.1.88 | " | " |
| JRBD004 | " | " | " |
| JRBD006 | " | " | " |
| ***Ph.nicotianae*** | | | |
| **Site I** | | | |
| **Subsite A** | | | |
| IA3001 | 29.6.87 | Soil dil. / VP3HB | soil |
| IA7002 | 29.8.87 | " | " |
| IA7003 | " | " | " |
| IA7005 | " | " | " |
| IA7006 | " | " | " |
| IA7007 | " | " | " |
| IA7008 | " | " | " |
| IA70016 | " | " | " |
| IA70017 | " | " | " |
| **Subsite B** | | | |
| IB4008 | 21.11.87 | " | " |

| Isolate number | Date | Isolation method | Source |
|---|---|---|---|
| **Subsite C** | | | |
| IC40010 | 16.7.87 | Soil dil. / VP3HB | soil |
| IC8001 | 10.9.87 | *Cit* . root / " | Root |
| IC14001 | 9.12.87 | *Cit* . bait / " | soil |
| ID7001 | 29.8.87 | Soil dil. / VP3HB | soil |
| ID7002 | " | " | " |
| ID7003 | " | " | " |
| ID11002 | 18.10.87 | " | " |
| ID11003 | " | *Cit* . bait / " | " |
| ID11004 | " | " | " |
| ID12006 | 5.11.87 | " | " |
| **Site II** | | | |
| **Subsite A** | | | |
| IIA30012 | 23.7.87 | Soil dil. / VP3HB | soil |
| IIA30022 | " | " | " |
| IIA4001 | 3.8.87 | " | " |
| IIA9001 | 9.10.87 | *Cit* . bait / " | " |
| IIA9002 | " | " | " |
| IIA10002 | 26.10.87 | " | " |
| IIA10004 | " | " | " |
| IIA10005 | " | " | " |
| IIA10006 | " | " | " |
| IIA10008 | " | " | " |
| IIA10009 | " | " | " |
| IIA12001 | 27.11.87 | *Cit* . root / " | Root |
| **Subsite B** | | | |
| IIB4002 | 3.6.87 | Soil dil. / VP3HB | soil |
| IIB7005 | 11.9.87 | *Cit* . root / " | Root |
| IIB7006 | " | " | " |
| IIB10006(B) | 26.10.87 | *Cit* . bait / " | soil |
| IIB100011 | 26.10.87 | *Cit* . bait / VP3HB | soil |

| Isolate number | Date | Isolation method | Source |
|---|---|---|---|
| IIB110012 | 13.11.87 | Soil dil / VP3HB | soil |
| IIB13001 | 11.12.87 | Soil dil / " | " |
| IIB130015 | " | " | " |
| **Subsite C** | | | |
| IIC2001 | 6.7.87 | " | " |
| IIC4001 | 3.8.87 | Soil dil / VP3HB | soil |
| IIC4004 | " | " | " |
| IIC4007 | " | " | " |
| IIC12003 | 27.11.87 | *Cit* . root / " | Root |
| IIC12004 | " | " | " |
| IIC12005 | " | " | " |
| IIC13005 | 11.12.87 | Soil dil / " | Soil |
| IIC13008 | " | " | " |
| IIC130010 | " | " | " |
| IIC130012(B) | " | " | " |
| IIC130025(B) | " | *Cit* . root / " | Root |
| IIC130026 | " | " | " |
| IIC130027 | " | Soil dil / " | Soil |
| **Subsite D** | | | |
| IID3001 | 23.7.87 | Soil dil / " | Soil |
| IID3006 | " | " | " |
| IID3007 | " | " | " |
| IID7003 | 11.9.87 | *Cit* . root / " | Root |
| IID80012 | 25.9.87 | Soil dil / " | Soil |
| IID120014 | 27.11.87 | *Cit* . root / " | Root |
| **Subsite E** | | | |
| IIE4004 | 3.8.87 | Soil dil. / VP3HB | soil |
| IIE4006 | " | " | " |
| IIE8003 | 25.9.87 | " | " |
| IIE8006 | " | " | " |
| IIE80010 | " | " | " |
| IIE80012 | " | " | " |
| **The Other Sites** | | | |
| TNS001 | 21.10.87 | *Cit* . root / " | Root |
| TNS002 | " | " | " |

## Appendix Two

### Culture media

All media were made up to 1 litre in distilled water, autoclaved at 15 psi for 15 min. 10 mg β- sitosterol was dissolved 30$cm^{-3}$ diethyl ether, added to the hot media (where required) with caution . Thiamine HCl was sterilized by ultrafiltration(0.45 μm pore size, Whatman) then added to the cooled autoclaved media.

1. **CA** (modified from Brasier, 1969)

| | |
|---|---|
| supernatent from blended frozen peas | 200 g |
| agar (Oxoid) | 10 g |
| β-sitosterol | 10 mg |
| thiamine HCl | 1 mg |

2. **CMA** ( after Benhams, 1931)

| | |
|---|---|
| extracted corn decoction | 40 g |
| agar | 15 g |
| or { corn meal agar (Oxoid) | 17 g} |
| β-sitosterol | 10 mg |
| Thiamine HCl | 1 mg |

3.**CV8A** (after Zentmyer *et al* ., 1976)

| | |
|---|---|
| cleared V8 juice | 200 $cm^3$ |
| agar (Oxoid) | 10 g |

| | |
|---|---|
| β-sitosterol | 10 mg |
| thiamine HCl | 1 mg |

Cleared V8 juice was prepared by adding 2.0 g $CaCO_3$ to 200 $cm^3$ of Campbells V8 juice and centrifuged at 3,500 rpm for 20 min. The clear supernatent was used.

4. **CV8CMOA**

| | |
|---|---|
| cleared V8 juice | 100 $cm^3$ |
| supernatent from blended frozen peas | 100 g |
| cleared blend of whole orange | 100 $cm^3$ |
| corn meal agar | 17 g |
| β-sitosterol | 10 mg |
| thiamine HCl | 1 mg |

Cleared blend of whole orange was prepared by adding 2.0 g $CaCO_3$ to 200 g blended orange, centrifuge at 3,500 rpm for 20 min then using the clear supernatent.

5. **DR** (modified from Ribiero *et al* ., 1975)

| | |
|---|---|
| glucose | 9 g |
| L-aspargine | 200 mg |
| $KNO_3$ | 300 mg |
| $KH_2PO_4$ | 300 mg |
| $MgSO_4.7H_2O$ | 1 g |
| $CaCl_2$ | 200 mg |
| β-sitosterol | 20 mg |
| thiamine HCl | 2 mg |

6. **OMA** (modified from Khaki & Shaw, 1975)

| | |
|---|---|
| oat meal | 10 g |
| agar (Oxoid) | 10 g |

Boiled and autoclaved as above, without filtration.

7. **PA**

| | |
|---|---|
| Supernatent from blended frozen peas | 100 g |
| agar (Oxoid) | 10 g |
| β-sitosterol | 10 mg |
| thiamine HCl | 1 mg |

8. **PDA**

| | |
|---|---|
| potato dextrose agar(Oxoid) | 39 g |
| β-sitosterol | 10 mg |
| thiamine HCl | 1 mg |

9. **RSM** (modified from Ribiero *et al* ., 1975)

| | |
|---|---|
| glucose | 4.5 g |
| agar (Oxoid) | 10 g |
| L-aspargine | 100 mg |
| $KNO_3$ | 150 mg |
| $KH_2PO_4$ | 150 mg |
| $MgSO_4.7H_2O$ | 500 mg |
| $CaCl_2$ | 100 mg |
| β-sitosterol | 10 mg |

| | |
|---|---|
| thiamine HCl | 1 mg |

**10.V8** ( modified from Mircetich *et al* ., 1968)

| | |
|---|---|
| cleared V8 juice | 200 $cm^3$ |
| β-sitosterol | 10 mg |
| thiamine HCl | 1 mg |

**11. SSE** [ modified from non sterile SSE (Chee and Newhook, 1966)]

| | |
|---|---|
| soil | 100 g |
| Dw | 2000 $cm^3$ |

Add soil to water and shake well, allow to settle for 48 h , filter through a Whatman No.1 filter, then autoclave as mentioned above for media.

Appendix Three

C.A.B. INTERNATIONAL
Mycological Institute

FERRY LANE
KEW
SURREY TW9 3AF
ENGLAND
Tel. 01-940 4086

# Identification Services

REPORT N° H580/88/YE1 19 August 1988

YOUR REF: Dr. Meriel G. Jones
The University of Liverpool
Department of Botany
P.O. Box 147
LIVERPOOL
L69 3BX

| Specimen Number | Herb. IMI Number | IDENTIFICATION |
|---|---|---|
| | | Report from Dr G. Hall |
| IA-14002(A) | 322373) | |
| IC-13008(A) | 322374) | |
| IIC-130012(A) | 322375)* | Phytophthora citrophthora (Smith & Smith) Leonian |
| IIE-12003(A) | 322376) | |
| ID-14001(A) | 322377)* | |
| IIB-10005(A) | 322378 | Phytophthora species. |
| * Deposited | | Pond water cultures IMI 322373,6,7,8 had sporangia of P. citrophthora, IMI 322375 had a few immature sporangia, but, none were found in IMI 322378. I have repeated the distilled water culture (reported 9/8/1988) and failed to obtain sporangia, confirming my original report. It is difficult to find a source of pond water locally that is not polluted, but one has been traced on Ham Common, Surrey. Incubation of IMI 322373-7 in this pond water produced sporangia within 4-7 days at room temperature, but none in IMI 322378, which I cannot confirm as P. citrophthora. |
| IA 7007 (B) | 322379) | |
| IIB 7005(B) | 322380) | |
| IIC 132225(B) | 322381) | Phytophthora nicotianae van Breda de Haan |
| ID 7002(B) | 322382) | |
| IID 3007(B) | 322373) | |
| IIE 4006(B) | 322384) | |
| All previously deposited. | | These isolates readily formed sporangia in pond water, 5% of which were caducous. No chlamydospores were formed after 14 days incubation, but, most |

| Specimen number | Herb. IMI number | Identification |
|---|---|---|
| | | isolates had stellate hyphal swellings in water. Therefore, I cannot confirm these isolates as var. parasitica, since they have features of both vars parasitica and nicotianae. We receive many isolates like this each year at CMI, and I am doubtful of the validity of the varietal names. The taxonomy of these organisms needs much closer investigation, and I hope to start a research project on them later in the year. Recently, I received a paper on a similar isolate, which was closest to P. nicotianae var. parasitica by electrophoretic studies. P. nicotianae var. parasitica is commonly isolated from diseased citrus seedlings - see CMI description sheets of plant pathogenic fungi nos 34 & 35.<br><br>Thank you for drawing this occurrence to my attention. I am currently revising and improving methods used at CMI for the identification of Oomycetes to ensure a better service to customers. Consequently feed-back like this is very valuable in this process. Pond water is now routinely used at CMI. If you would like to send any more cultures to CMI for identification, I would be pleased to examine them. Please note that there is no charge for identification services for CAB member countries, of which the U.K. is one. |



B

## Appendix Four

Radial extension rates of 4 randomly chosen isolates of *Ph.citrophthora* and *Ph.nicotianae* at different temperatures on Ribiero synthetic agar. Data are expressed as radial extension rate mm 24 $h^{-1}$.

**Table 4.a.** RER at 25°C

| Isolates | Carbon sources | | | | | |
|---|---|---|---|---|---|---|
| | Sorbitol | Glucose | Fructose | Sucrose | Galactose | Lactose |
| *Ph.citrophthora* | | | | | | |
| IC13008 | 5.9 | 4.9 | 5.2 | 5.0 | 3.6 | 4.6 |
| IA14002 | 5.2 | 4.9 | 4.6 | 4.9 | 3.3 | 4.4 |
| IIC130012 | 6.1 | 3.8 | 3.8 | 4.7 | 3.5 | 4.1 |
| IIE12003 | 5.3 | 5.3 | 5.4 | 5.5 | 3.9 | 4.9 |
| *Ph.nicotianae* | | | | | | |
| ID7002 | 6.2 | 5.5 | 4.9 | 5.0 | 4.9 | 4.1 |
| IA7007 | 5.8 | 5.3 | 4.7 | 4.5 | 4.6 | 4.0 |
| IIC130025 | 5.5 | 5.7 | 5.2 | 5.3 | 4.7 | 4.3 |
| IIB7005 | 5.2 | 4.9 | 4.9 | 4.8 | 4.7 | 3.9 |

**Table 4.b.** RER at 30°C

| Isolates | Carbon sources | | | | | |
|---|---|---|---|---|---|---|
| | Sorbitol | Glucose | Fructose | Sucrose | Galactose | Lactose |
| *Ph.citrophthora* | | | | | | |
| IC13008 | 4.3 | 3.0 | 2.8 | 3.3 | 2.6 | 2.6 |
| IA14002 | 4.1 | 3.2 | 2.9 | 3.1 | 2.7 | 3.3 |
| IIC130012 | 4.8 | 3.2 | 3.0 | 3.0 | 3.0 | 3.5 |
| IIE12003 | 4.0 | 2.0 | 3.4 | 3.7 | 3.2 | 3.0 |
| *Ph.nicotianae* | | | | | | |
| ID7002 | 7.5 | 5.9 | 5.6 | 5.6 | 6.8 | 6.1 |
| IA7007 | 6.9 | 5.9 | 5.9 | 5.7 | 6.0 | 6.7 |
| IIC130025 | 7.8 | 5.9 | 5.5 | 5.9 | 6.6 | 6.3 |
| IIB7005 | 7.5 | 6.0 | 5.6 | 6.0 | 6.9 | 6.6 |

**Table 4.c.** RER at 32°C

| Isolates | Carbon sources | | | | | |
|---|---|---|---|---|---|---|
| | Sorbitol | Glucose | Fructose | Sucrose | Galactose | Lactose |
| *Ph.citrophthora* | | | | | | |
| IC13008 | 0.7 | 0.3 | 0.3 | 0.4 | 0.6 | 0.1 |
| IA14002 | 0.5 | 0.7 | 1.0 | 0.6 | 1.2 | 0.7 |
| IIC130012 | 0.5 | 0.9 | 0.6 | 1.0 | 0.7 | 0.5 |
| IIE12003 | 0.1 | 0.9 | 0.3 | 0.6 | 0.9 | 0.5 |
| *Ph.nicotianae* | | | | | | |
| ID7002 | 7.3 | 4.8 | 4.8 | 5.1 | 5.8 | 6.0 |
| IA7007 | 6.7 | 4.3 | 4.5 | 5.0 | 5.9 | 5.0 |
| IIC130025 | 5.9 | 3.8 | 4.0 | 4.9 | 5.8 | 5.3 |
| IIB7005 | 5.8 | 4.1 | 3.6 | 4.6 | 6.1 | 4.1 |

**Table 4.d.** RER at 35°C

| Isolates | Carbon sources | | | | | |
|---|---|---|---|---|---|---|
| | Sorbitol | Glucose | Fructose | Sucrose | Galactose | Lactose |
| *Ph.citrophthora* | | | | | | |
| IC13008 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| IA14002 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| IIC130012 | 0.0 | 0.1 | 0.0 | 0.0 | 0.1 | 0.1 |
| IIE12003 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.1 |
| *Ph.nicotianae* | | | | | | |
| ID7002 | 1.9 | 1.5 | 1.0 | 1.3 | 1.7 | 1.0 |
| IA7007 | 1.5 | 1.5 | 0.8 | 1.3 | 1.7 | 1.3 |
| IIC130025 | 1.1 | 1.2 | 1.2 | 1.6 | 1.6 | 1.2 |
| IIB7005 | 1.3 | 0.9 | 1.2 | 1.2 | 1.8 | 1.0 |

**Table 4.e.** RER at 37°C

| Isolates | Carbon sources | | | | | |
|---|---|---|---|---|---|---|
| | Sorbitol | Glucose | Fructose | Sucrose | Galactose | Lactose |
| *Ph.citrophthora* | | | | | | |
| IC13008 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| IA14002 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| IIC130012 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| IIE12003 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| *Ph.nicotianae* | | | | | | |
| ID7002 | 0.8 | 0.6 | 0.2 | 0.6 | 0.8 | 0.3 |
| IA7007 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| IIC130025 | 0.2 | 0.2 | 0.3 | 0.5 | 0.3 | 0.2 |
| IIB7005 | 0.0 | 0.5 | 0.2 | 0.5 | 0.5 | 0.1 |

## Appendix Five

Results of mycelium wet weight, residual glucose and protein concentration obtained from 63 *Ph.citrophthora* isolates after 5 days incubation using methods mentioned in Sections 6.2.1 and 6.2.3.2.

| Isolate | Mycelium wet weight g | Residual glucose $mg.cm^{-3}$ | Protein $mg.cm^{-3}$ |
|---|---|---|---|
| IB2001 | 2.4 | 0.3 | 0.54 |
| IB4001 | 2.8 | 0.8 | 0.63 |
| IB4002 | 2.7 | 0.6 | 0.57 |
| IB6001 | 3.1 | 0.4 | 0.65 |
| IB7001 | 3.1 | 0.6 | 0.49 |
| IB7002 | 2.1 | 1.0 | 0.58 |
| IB7003 | 3.3 | 1.0 | 0.54 |
| IB7004 | 3.0 | 0.2 | 0.58 |
| IB7005 | 3.7 | 0.6 | 0.64 |
| IB8001 | 4.0 | 0.5 | 0.52 |
| IB8002 | 2.6 | 0.1 | 0.72 |
| IB11004 | 3.4 | 0.1 | 0.65 |
| IB12002 | 2.8 | 0.3 | 0.40 |
| IB12004 | 2.1 | 0.2 | 0.67 |
| IB12005 | 2.0 | 0.4 | 0.43 |
| IB12006 | 2.5 | 4.9 | 0.54 |

| Isolate | Mycelium wet weight g | Residual glucose $mg.cm^{-3}$ | Protein $mg.cm^{-3}$ |
|---|---|---|---|
| IB12007 | 3.7 | 2.3 | 0.38 |
| IB120010 | 3.2 | 4.2 | 0.54 |
| IB120012 | 2.6 | 0.8 | 0.43 |
| IB13001 | 3.8 | 0.4 | 0.35 |
| IB13002 | 2.2 | 2.0 | 0.50 |
| IB13005 | 2.8 | 0.6 | 0.43 |
| IB13006 | 2.8 | 0.3 | 0.57 |
| IB13007 | 2.7 | 0.4 | 0.54 |
| IB13008 | 3.0 | 0.5 | 0.54 |
| IIE3003 | 2.6 | 0.2 | 0.46 |
| IIE5001 | 2.6 | 0.3 | 0.53 |
| IIE7001 | 2.4 | 0.2 | 0.59 |
| IIE7002 | 2.9 | 0.2 | 0.47 |
| IIE7003 | 2.3 | 2.8 | 0.67 |
| IIE7004 | 3.7 | 0.2 | 0.57 |
| IIE8001 | 2.7 | 5.7 | 0.61 |
| IIE8004 | 3.9 | 2.7 | 0.43 |
| IIE8005 | 3.2 | 3.8 | 0.59 |
| IIE8008 | 3.8 | 2.5 | 0.38 |
| IIE8009 | 1.3 | 4.8 | 0.58 |
| IIE80011 | 1.8 | 1.8 | 0.91 |
| IIE80014 | 3.3 | 2.3 | 0.53 |
| IIE80015 | 4.3 | 0.8 | 0.66 |
| IIE9001 | 3.6 | 5.7 | 0.65 |

| Isolate | Mycelium wet weight g | Residual glucose $mg.cm^{-3}$ | Protein $mg.cm^{-3}$ |
|---|---|---|---|
| IIE9003 | 5.6 | 0.1 | 0.45 |
| IIE11003 | 2.5 | 1.1 | 0.87 |
| IIE11004 | 2.7 | 0.5 | 0.67 |
| IIE112001 | 2.3 | 0.5 | 0.64 |
| IIE12003 | 5.0 | 0.7 | 0.58 |
| IIE14001 | 3.3 | 0.2 | 0.34 |
| IIE14002 | 3.6 | 1.7 | 0.35 |
| IIE14003 | 3.0 | 1.1 | 0.50 |
| IIE14005 | 3.7 | 1.5 | 0.41 |
| FAR001 | 4.2 | 2.7 | .38 |
| FAR003 | 4.6 | 1.6 | 0.35 |
| TMD001 | 1.8 | 1.7 | 0.8 |
| TMD002 | 3.2 | 1.6 | 0.4 |
| TMD003 | 2.6 | 3.8 | 0.47 |
| TMD004 | 2.6 | 2.3 | 0.51 |
| TMD005 | 3.9 | 0.2 | 0.38 |
| TMD006 | 4.0 | 0.2 | 0.60 |
| JRNP001 | 2.6 | 2.0 | 0.68 |
| JRNP004 | 2.2 | 1.7 | 0.75 |
| JRNP005 | 2.7 | 1.5 | 0.65 |
| JRBD003 | 2.7 | 1.2 | 0.76 |
| JRBD004 | 2.7 | 1.5 | 0.65 |
| JRBD006 | 3.3 | 1.6 | 0.46 |

## Appendix Six

The grouping of bands for the 63 *Ph.citrophthora* isolates assessed for protein profiles. All isolates (excluding 1,2 & 5) were used in the principal component analysis.

| Isolate | Marker bands | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| 1. IB2001 | 3 | 2 | | 7 | 1 | 5 | 3 | 2 | 1 | 2 |
| 2. IB4001 | 3 | 2 | | 7 | 1 | 5 | 2 | 2 | 1 | 2 |
| 3. IB4002 | 1 | 1 | 2 | 3 | 1 | 4 | 2 | 2 | 1 | 2 |
| 4. IB6001 | 1 | 1 | 2 | 3 | 1 | 4 | 2 | 2 | 1 | 2 |
| 5. IB7001 | 5 | 2 | | 7 | 1 | 4 | 2 | 2 | 1 | 2 |
| 6. IB7002 | 1 | 1 | 2 | 3 | 1 | 4 | 2 | 2 | 1 | 2 |
| 7. IB7003 | 6 | 3 | 3 | 5 | 2 | 4 | 3 | 2 | 1 | 2 |
| 8. IB7004 | 1 | 1 | 2 | 3 | 1 | 6 | 2 | 2 | 1 | 2 |
| 9. IB7005 | 1 | 1 | 4 | 3 | 1 | 6 | 2 | 2 | 1 | 2 |
| 10.IB8001 | 2 | 1 | 2 | 3 | 1 | 6 | 2 | 2 | 1 | 2 |
| 11.IB8002 | 1 | 1 | 2 | 3 | 1 | 6 | 2 | 2 | 1 | 2 |
| 12.IB11004 | 2 | 1 | 2 | 3 | 1 | 4 | 2 | 2 | 1 | 2 |
| 13.IB12002 | 2 | 1 | 2 | 3 | 1 | 6 | 2 | 2 | 1 | 2 |
| 14.IB12004 | 3 | 1 | 4 | 3 | 1 | 4 | 2 | 2 | 1 | 2 |
| 15.IB12005 | 3 | 1 | 3 | 3 | 1 | 6 | 2 | 2 | 1 | 2 |
| 16.IB12006 | 3 | 1 | 2 | 4 | 1 | 6 | 2 | 2 | 1 | 2 |
| 17.IB12007 | 4 | 1 | 2 | 3 | 1 | 5 | 2 | 2 | 1 | 2 |
| 18.IB120010 | 4 | 2 | 2 | 3 | 2 | 5 | 2 | 2 | 1 | 2 |
| 19.IB120012 | 4 | 1 | 2 | 3 | 1 | 4 | 2 | 2 | 1 | 2 |

| Isolate | Marker bands | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| 20.IB13001 | 2 | 4 | 4 | 5 | 1 | 6 | 2 | 2 | 1 | 2 |
| 21.IB13002 | 4 | 4 | 4 | 3 | 1 | 5 | 3 | 2 | 1 | 2 |
| 22.IB13005 | 4 | 4 | 4 | 3 | 1 | 5 | 3 | 2 | 1 | 2 |
| 23.IB13006 | 4 | 4 | 4 | 4 | 2 | 4 | 2 | 2 | 1 | 2 |
| 24.IB13007 | 4 | 4 | 4 | 5 | 1 | 6 | 2 | 2 | 1 | 2 |
| 25.IB13008 | 4 | 4 | 4 | 3 | 1 | 5 | 2 | 2 | 2 | 2 |
| 26.IIE3003 | 2 | 4 | 4 | 3 | 1 | 5 | 2 | 2 | 1 | 2 |
| 27.IIE5001 | 2 | 4 | 3 | 4 | 1 | 5 | 2 | 2 | 1 | 2 |
| 28.IIE7001 | 2 | 4 | 3 | 4 | 1 | 5 | 3 | 2 | 1 | 2 |
| 29.IIE7002 | 4 | 3 | 4 | 3 | 1 | 5 | 2 | 2 | 1 | 2 |
| 30.IIE7003 | 4 | 3 | 2 | 3 | 1 | 5 | 2 | 2 | 1 | 2 |
| 31.IIE7004 | 4 | 3 | 2 | 3 | 1 | 5 | 2 | 2 | 1 | 2 |
| 32.IIE8001 | 3 | 3 | 2 | 3 | 2 | 4 | 2 | 2 | 1 | 2 |
| 33.IIE8004 | 3 | 3 | 2 | 3 | 4 | 4 | 2 | 2 | 1 | 2 |
| 34.IIE8005 | 3 | 3 | 2 | 4 | 4 | 4 | 2 | 2 | 1 | 2 |
| 35.IIE8008 | 3 | 3 | 2 | 2 | 4 | 4 | 2 | 2 | 1 | 2 |
| 36.IIE8009 | 3 | 3 | 2 | 3 | 4 | 4 | 2 | 2 | 1 | 2 |
| 37.IIE80011 | 4 | 3 | 2 | 2 | 4 | 4 | 2 | 2 | 1 | 2 |
| 38.IIE80014 | 4 | 3 | 2 | 4 | 2 | 6 | 2 | 2 | 1 | 2 |
| 39.IIE80015 | 1 | 2 | 2 | 4 | 1 | 4 | 2 | 2 | 1 | 2 |
| 40.IIE9001 | 4 | 3 | 2 | 2 | 2 | 6 | 2 | 2 | 1 | 2 |
| 41.IIE9003 | 4 | 3 | 2 | 3 | 2 | 6 | 3 | 2 | 1 | 2 |
| 42.IIE11003 | 4 | 3 | 2 | 1 | 2 | 6 | 3 | 2 | 1 | 2 |
| 43.IIE11004 | 4 | 3 | 2 | 4 | 1 | 5 | 3 | 2 | 1 | 2 |

| Isolate | Marker bands | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| 44.IIE112001 | 5 | 3 | 2 | 1 | 2 | 5 | 2 | 2 | 1 | 2 |
| 45.IIE12003 | 2 | 1 | 2 | 2 | 2 | 5 | 2 | 2 | 1 | 2 |
| 46.IIE14001 | 2 | 1 | 2 | 2 | 2 | 5 | 2 | 2 | 1 | 2 |
| 47.IIE14002 | 5 | 3 | 4 | 5 | 2 | 5 | 2 | 2 | 1 | 2 |
| 48.IIE14003 | 2 | 3 | 4 | 5 | 2 | 6 | 2 | 2 | 1 | 2 |
| 49.IIE14005 | 5 | 3 | 3 | 4 | 2 | 4 | 2 | 2 | 1 | 2 |
| 50.FAR001 | 5 | 3 | 3 | 4 | 2 | 3 | 2 | 2 | 1 | 2 |
| 51.FAR003 | 6 | 3 | 3 | 3 | 4 | 5 | 3 | 2 | 1 | 2 |
| 52.TMD001 | 5 | 3 | 2 | 3 | 4 | 5 | 2 | 2 | 1 | 2 |
| 53.TMD002 | 5 | 3 | 2 | 3 | 4 | 3 | 2 | 2 | 1 | 2 |
| 54.TMD003 | 5 | 3 | 2 | 3 | 3 | 3 | 2 | 2 | 1 | 2 |
| 55.TMD004 | 2 | 3 | 4 | 4 | 2 | 3 | 2 | 2 | 1 | 2 |
| 56.TMD005 | 2 | 3 | 4 | 4 | 2 | 4 | 2 | 2 | 1 | 2 |
| 57.TMD006 | 2 | 3 | 4 | 3 | 2 | 5 | 2 | 2 | 1 | 2 |
| 58.JRNP001 | 4 | 3 | 2 | 3 | 1 | 5 | 2 | 2 | 1 | 2 |
| 59.JRNP004 | 4 | 3 | 4 | 3 | 2 | 4 | 2 | 2 | 1 | 2 |
| 60.JRNP005 | 6 | 1 | 4 | 3 | 2 | 4 | 3 | 2 | 1 | 2 |
| 61.JRBD003 | 3 | 2 | 3 | 3 | 2 | 5 | 2 | 2 | 1 | 2 |
| 62.JRBD004 | 6 | 3 | 4 | 3 | 2 | 6 | 3 | 2 | 1 | 2 |
| 63.JRBD006 | 3 | 3 | 4 | 3 | 1 | 4 | 2 | 2 | 1 | 2 |