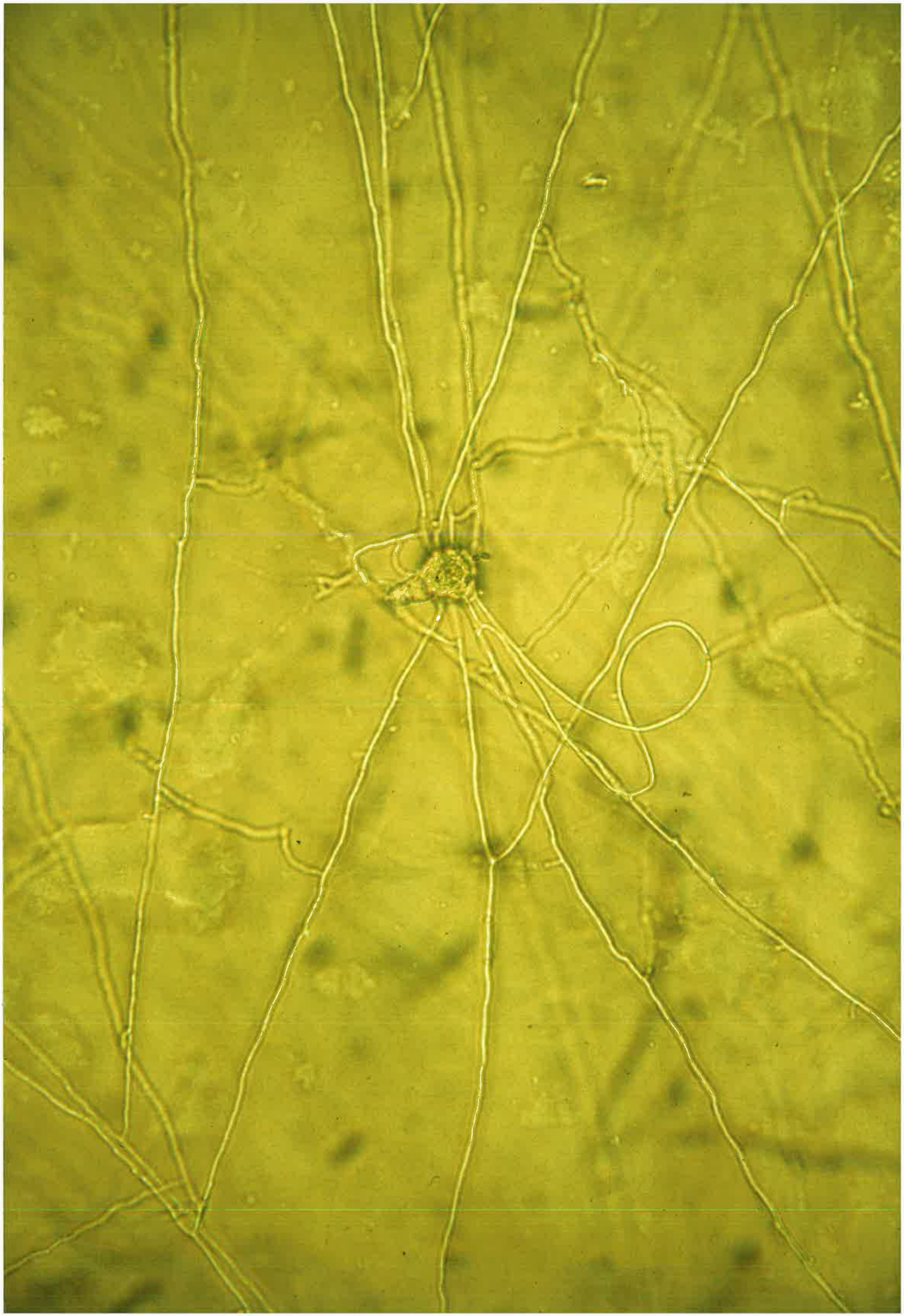

And yet relation appears,

A small relation expanding like the shade

Of a cloud on sand, a shape on the side of a hill.

-WALLACE STEVENS

"Connoisseur of Chaos"

Opposite Plate: A germinating oospore of *Phytophthora citrophthora*.

I dedicate this thesis to the memory of my grandmother

Anna Ciornohus

1910 – 1989

whose courage and integrity gave support to my own "vision"

THE USE OF TISSUE CULTURE AND MOLECULAR TECHNIQUES TO ASSESS VARIATION WITHIN *PHYTOPHTHORA CITROPHTHORA* AND SCREEN FOR DISEASE RESISTANCE IN ITS CITRUS HOST.

by

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ABBREVIATIONS

A	absorbance
BSA	bovine serum albumin
cm	centimetres
DMSO	dimethyl sulfoxide
DTT	dithiothritol
DW	dry weight
EDTA	ethylenediaminetetra acetic acid
FW	fresh weight
g	grams
hr	hours
kb	kilobase
l	litre
mA	milliAMPS
Mbp	mega base pairs
min	minutes
ml	millilitre
mm	millimetres
MW	molecular weight
nm	nanometers
O.D.	optical density
o.p.m.	orbits per minute
r.p.m.	revolutions per minute
RNA	ribonucleic acid
RO	reverse-osmosis

s.e.d.	standard error of difference
SDS	sodium dodecyl sulphate
SDW	sterile distilled water
sec	second
spp.	species
Tris	tris(hydroxymethyp)aminimethane
UV	ultraviolet
V	volts
v	volume
μg	micrograms
μl	microlitre

SUMMARY

In the Citrus industry, control strategies for *Phytophthora* root rot have focused on selecting resistant rootstocks by means of long–term breeding programmes. These programmes, however, are inefficient because: 1) the production of a hybrid Citrus rootstock depends on the striking of cuttings and/or the raising of nucellar seedlings and consequently there is a considerable time interval before hybrids can be evaluated for resistance to disease; and 2) there is little information on the variation, particularly with respect to pathogenicity, within *P. citrophthora* populations.

Rapid techniques for screening for disease resistance, both *in vivo* and *in vitro*, were developed which could distinguish between cultivars with known resistance and susceptibility to *P. citrophthora* in the field. In growth cabinet experiments, 2 to 3 month–old seedlings of cultivars commonly used as rootstocks in South Australia (Carrizo Citrange, McKillops Rough Lemon and Symons Sweet Orange) were screened using root and collar inoculation techniques. In the former, seedlings were inoculated by immersing roots in a zoospore suspension (10^4 zoospores per ml) for 24 hr before being planted in 1:1 (v/v) steamed recycled soil/ peat medium and flooded for a further 24 hr. After 7 days, a correlation was observed between reduction of infection in taproots and increased plant growth in resistant seedlings (Carrizo Citrange); this response differed significantly from that of susceptible seedlings (McKillops Rough Lemon and Symons Sweet Orange). In collar inoculation trials, mycelial discs were applied to wounds in the collar region of the stem and lesion length was assessed 14 days later. Lesion length was significantly greater in susceptible seedlings

(McKillops Rough Lemon and Symons Sweet Orange) than in resistant seedlings (Carrizo Citrange). Results obtained from seedling inoculations were then used as a "standard" against which to compare the responses of cultivars in tissue culture experiments.

The screening technique used in tissue culture experiments was a modification of an excised twig assay, analogous to collar inoculation of seedlings. After a multiplication cycle which took 6 – 8 weeks, excised shoots of both susceptible (McKillops Rough Lemon, Symons Sweet Orange) and moderately resistant cultivars (Carrizo Citrange, Cleopatra x English Poncirus Trifoliata, Cleopatra x Swingle Poncirus Trifoliata) were placed upright in agar medium colonised with the fungus and development of necrosis was measured over 5 – 6 days. Necrosis developed more rapidly on shoots of susceptible cultivars than on those of resistant cultivars. Both the *in vivo* and *in vitro* methods developed here permit a rapid and efficient primary screening of Citrus rootstocks for resistance to *Phytophthora* infection and would, therefore, be a useful adjunct to Citrus breeding programmes.

The extent of variation within *P. citrophthora* was investigated by examining isolates obtained from Citrus hosts from a wide geographic distribution. Parent isolates and progeny derived from single uninucleate zoospores were compared with respect to colony and sporangium morphology, growth rate and pathogenicity. There were significant differences between parent isolates in all parameters measured, including aggressiveness on excised shoots using the tissue culture screening technique. The majority of single zoospore–derived progeny were similar to their respective parents.

Improved techniques for obtaining oospores *in vitro* were developed in this study. Oospores were produced on carrot agar and in tissue culture on shoots and rooted shoots of Symons Sweet Orange. Using the techniques described, isolates could be classified, with confidence, as A1 or A2 mating types. There were significant differences in oospore morphology between mating combinations. Oospore germination ranged from 0 to 13% depending on the particular mating combination. This difference was attributed to a 'specific mating ability', where different mating combinations were found to have different optimal requirements for both mating vigour and oospore germination.

Restriction Fragment Length Polymorphisms (RFLPs) were used to examine genetic variation among isolates of *P. citrophthora*. Sixteen DNA probes derived from a *P. citrophthora* genomic library were used to identify RFLPs between isolates using the restriction endonucleases, *Bam* HI and *Dra* I. Polymerase Chain Reaction (PCR) using several random primers synthesised from plant sequences was also examined as an alternative technique for identifying intraspecific variation within *P. citrophthora*. Both RFLP and PCR analyses revealed two groupings within the *P. citrophthora* isolates which were correlated with the mating type of the isolate (A1 or A2).

In addition, Contour-clamped Homogenous Electric Field Electrophoresis (CHEF) was used to determine the number and size of chromosomes in *P. citrophthora*. Several techniques for preparing DNA for electrophoresis were tested and the most successful involved embedding ground mycelium in agarose plugs and then treating with proteinase K in the presence of EDTA and SDS. The migration of DNA from *P. citrophthora* (isolate C23) was compared to that of DNA from *Schizosaccharomyces pombe* and

Saccharomyces cerevisiae and five bands ranging in size from 2.5 to 5.7 megabases, were detected, suggesting that at least five chromosomes are present in *P. citrophthora*. This technique requires refinement before it can be used to determine variability between isolates.

Although genetic analysis revealed that isolates of *P. citrophthora* could be clearly divided into two groups (A1 or A2), these two groups were morphologically indistinguishable. One hypothesis proposed to explain this result is that the A1 and A2 groups may represent distinct but closely related species. Alternatively, it may be that the probes hybridised to a mating-type locus located on a particular chromosome.

DECLARATION

I HEREBY DECLARE that the work presented in this thesis contains no material which has been accepted for the award of any other degree or diploma in any university. To the best of my knowledge and belief, no material herein has been previously published or written by another person, except where due reference is made in the text. I give consent to this copy of my thesis being available for photocopy and loan if it is accepted for the award of the degree.

Christina Son

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1. INTRODUCTION

1.1 THE IMPORTANCE OF *PHYTOPHTHORA CITROPHTHORA* TO THE CITRUS INDUSTRY

Australia is an important contributor to world Citrus production. In 1986 the Australian Citrus industry produced 704,000 tonnes of fruit (representing approximately 1.6% of global production) from 8.3 million trees of which 74% bore fruit (Gallasch, 1988). More than half of Australia's exports are from South Australia, the major markets being New Zealand, Singapore and Hong Kong (G. Walker, pers. comm.). Over the past 100 years, *Phytophthora* root rot has been very costly to the Citrus industry; for example, a severe outbreak occurred in 1942 when nearly 50% of the Citrus plantings in the Murrumbidgee Irrigation Area were forced out of commercial production (Broadbent, 1977).

There are two principal *Phytophthora* species that cause root and collar rot of Citrus, *Phytophthora citrophthora* (R. E. Smith and E. H. Smith) Leonian and *P. parasitica* Dastur. Their relative importance is determined by temperature, *P. citrophthora* is the prevalent species in temperate and sub–tropical regions, whereas *P. parasitica* is found in subtropical and tropical regions. There are other less important pathogens of Citrus found amongst *Phytophthora* species: *P. palmivora* Butler and *P. citricola* Sawada have been reported to attack Citrus in some tropical areas. *P. hibernalis* Carne and *P. syringae* (Klebahn) Klebahn also occur in limited areas and are usually associated with brown rot attacks in localities with cool, moist winters (Broadbent 1977).

In recent years, losses due to *Phytophthora* have been reduced by using *Phytophthora*–resistant rootstocks in replant situations. However, propagation of hybrid Citrus seedlings from breeding programmes aimed at developing improved rootstock characters (e.g. disease resistance, salt tolerance), depends on vegetative propagation or utilisation of polyembryony to raise nucellar seedlings. Both of these methods can take many years to provide enough clonal seedlings for screening for commercially desirable traits.

Tissue culture has been employed in the Citrus industry principally to select for new phenotypes from large cell populations (callus) cultured under defined conditions. Recently, the possibility of using tissue culture techniques to identify and select disease–resistant plants *in vitro* for rootstock breeding programmes of woody horticultural plants, has been investigated (Daub, 1986; Scott *et al.*, 1992; Sharma and Skidmore, 1988). For this purpose, plants generally have been propagated by means of shoot multiplication because callus is considered to be clonally unstable (Barlass and Skene, 1986). It has been suggested that incorporation of tissue culture techniques could both complement and shorten screening and selection processes because, instead of whole plants, excised tissues are used to screen for desirable traits such as disease resistance (Kester *et al.*, 1986).

A prerequisite for the development of any screening technique (including tissue culture) to select for disease resistance is a thorough understanding of the pathogen, in this case *P. citrophthora* and its association with the host, as the success or failure of host

resistance often depends on the capacity for variation in the pathogen population. Unlike other *Phytophthora* spp., *P. citrophthora* has not been extensively researched. Thus, much of our current knowledge about *P. citrophthora* is based on information extrapolated from other *Phytophthora* species.

In this important group of pathogens, variability with regard to pathogenicity, morphology of vegetative and reproductive structures and other features, is still poorly understood in spite of the fact that the literature on variation within *Phytophthora* spp. has increased dramatically since a review by Erwin *et al.* in 1963. Of particular interest are the mechanisms by which variation occurs. *P. citrophthora* is considered a difficult species to study because the sexual stage has rarely been reported and sexual recombination in most other species of fungi provides the usual means of variation and generation of new physiological races. However, recent work, brought about by the development of molecular techniques, has provided evidence of somatic recombination in the Oomycetes, suggesting that asexual mechanisms may be important in species such as *P. citrophthora* (Michelmore and Hulbert, 1987).

Thus, it is clearly important to examine further the interaction between *P. citrophthora* and its Citrus host as well as to develop techniques which could be used by the Citrus industry to improve their current breeding programmes aimed at producing disease resistant rootstocks. The present study was undertaken for these reasons; the aims are outlined below.

1.2 AIMS OF THIS PROJECT

(i) to develop rapid screening techniques (including the application of tissue culture) to distinguish between Citrus cultivars known to be resistant and susceptible to infection by *P. citrophthora*.

(ii) to extend the use of tissue culture techniques to examine host/pathogen interactions, in particular the hypothesis that chemotropism may play an important role in the susceptibility of cultivars to *P. citrophthora*.

(iii) to examine the extent of variation, morphological, genetical, and pathological, between isolates of *P. citrophthora*. The isolates examined were restricted to those taken from Citrus as it has been shown that isolates that have been derived from other hosts may actually be a different *Phytophthora* species (Förster *et al.*, 1990a). Isolates were also chosen to represent a geographically diverse cross-section of the population of *P. citrophthora*.

(iv) to produce and germinate oospores (the sexual stage) in culture.

(v) to determine chromosome number and size for *P. citrophthora* and thus gain some appreciation of its genetic complexity.

2. LITERATURE REVIEW

2.1 INTRODUCTION

The current study was initiated in 1989 and this chapter provides a review of the relevant literature up to that date. The review has been divided into different sections, providing background information on several relevant areas. The first section examines the pathogen, *P. citrophthora*; the life cycle of the fungus; root and collar rot of Citrus; variation in taxonomic characters and pathogenicity; mechanisms for generating variation; and the possibility of using molecular markers to examine genetic variability. The second section examines the Citrus host and particularly the importance of the various rootstocks in screening for resistance to root and collar rot diseases. The last section examines the interaction between *P. citrophthora* and Citrus with regard to the use of tissue culture techniques in screening for disease resistance and how this compares with screening in the field.

2.2 THE PATHOGEN – *PHYTOPHTHORA CITROPHTHORA*

Phytophthora citrophthora (R. E. Smith and E. H. Smith) Leonian was originally described in 1906 as *Pythiacystis citrophthora* and in 1925 the species was designated as *Phytophthora citrophthora* (Leonian, 1925).

There have been many assumptions made for *P. citrophthora* based on other *Phytophthora* spp. as knowledge of this species is very limited. The validity of these

assumptions may be questionable as more information on *P. citrophthora* is acquired. However, where information is limited, the literature review presents information on other *Phytophthora* spp. or Oomycete fungi.

Oomycetes are clearly different from other fungi with respect to the following: a) they do not translocate trehalose which is one of the main forms in which carbohydrates are mobilised and accumulated in fungi, and they synthesise lysine by the diaminopimelic acid (DAP) pathway and not by the α–amino–adipic acid (AAA) pathway (the latter pathway is common to fungi but not found in other organisms) (Vogel *et al.*, 1970) ; b) they are diploid for most of their life cycle; c) they have cellulose in their walls ; d) they cannot synthesise sterols; and e) they have well–defined golgi bodies (Deacon, 1984). Work by Förster *et al.* (1990b) supports the suggestion that the Oomycetes may have had a different evolutionary lineage from other fungi. The fungi they considered could be divided into two distinct evolutionary lines based on differences in their 16s rRNA sequences. The first group, the Oomycetes, appeared to be most closely related to heterokont photosynthetic algae whereas the second group contained the Ascomycetes and the Chytridiomycetes.

2.2.1 The life cycle of *P. citrophthora*

It is now widely accepted that *Phytophthora* spp. are asexually reproducing diploid organisms, except during the production of gametes within the oogonia and antheridia prior to fertilisation. This was first demonstrated by Sansome with her work on *Pythium*, *Sclerospora* and *Phytophthora* (Sansome 1961; 1963; 1965), and has since

been confirmed by several other authors (Erselius and Boccas, 1978; Galindo and Zentmyer, 1967; Maia *et al.*,1976). Tetraploidy has been noted in some *Phytophthora* spp. (Sansome and Brasier, 1973; 1974). With few exceptions (Tooley and Therrien, 1987), relative DNA contents of isolates for *Phytophthora* spp. have not yet been determined.

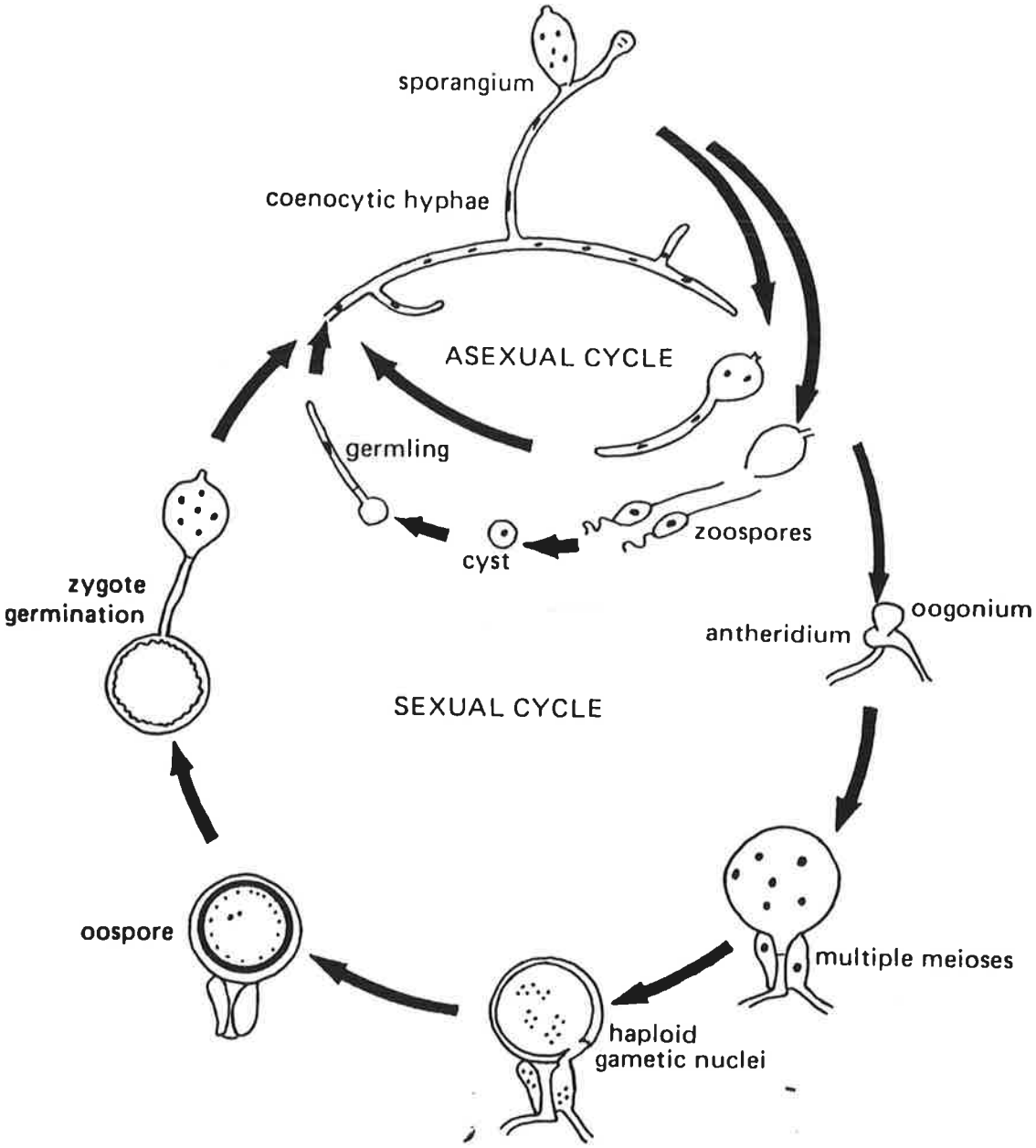
The life cycle of *Phytophthora* is presented in Figure 2.1. The aseptate, branched hyphae of the vegetative mycelium give rise to sporangiophores which bear multinucleate sporangia terminally. Sporangiophore growth is indeterminate; proliferation may occur from the sporangiophore at the base of the sporangium or from within empty sporangia. Mature sporangia of species causing foliar or fruit diseases are usually considered to be deciduous (caducous), but *P. citrophthora* isolates with non–caducous sporangia have been noted (e.g. Ferguson, 1976). Sporangia germinate either by formation of a germ tube(s) that eventually grows to form a mycelium (direct germination) or by differentiation of the cytoplasm within the sporangium into discrete zoospores that are then released through an exit pore (indirect germination).

Sexual reproduction occurs when specialised gametangial hyphae interact to allow plasmogamy and eventually syngamy. The female gametangium (the oogonium) and the associated male gametangium (the antheridium) are both multinucleate cells within which multiple meiosis take place. A single oosphere containing many haploid gametic nuclei differentiates within the oogonium and is fertilised when a

Figure 2.1 The life cycle of *Phytophthora* (Shaw, 1988).

In the asexual stage, the aseptate branched hyphae of the vegetative mycelium give rise to sporangiophores which bear terminal multinucleate sporangia. Sporangia germinate either by formation of a germ tube(s) that eventually grows to form a mycelium (direct germination) or by differentiation of the cytoplasm within the sporangium into discrete zoospores that are then released through an exit pore (indirect germination).

In the sexual stage, multiple meiosis take place in the multinucleate cells of the female gametangium (the oogonium) and the associated male gametangium (the antheridium). A single oosphere containing many haploid gametic nuclei differentiates within the oogonium and is fertilised when a number of male gametic nuclei enter through the fertilisation tube and fuse with single female gametic nuclei. At germination, single diploid zygotic nuclei divide mitotically many times and one or several germ tubes appear and eventually branch into an extensive mycelium. A germ tube may also end in a sporangium, from which zoospores are released.


sporangium
coenocytic hyphae
ASEXUAL CYCLE
germling
cyst
zoospores
zygote
germination
antheridium
oogonium
SEXUAL CYCLE
oospore
multiple meioses
haploid
gametic nuclei

number of male gametic nuclei enter through the fertilisation tube and fuse with single female gametic nuclei (Brasier and Brasier, 1978). At germination, single diploid zygotic nuclei divide mitotically many times and one or several germ tubes appear and eventually branch into an extensive mycelium. A germ tube may also have a terminal sporangium, from which zoospores are released.

Information on the size and number of chromosomes in *Phytophthora* spp. is scarce. In general, the chromosome number is estimated to be 9 – 10 (Erwin *et al.*, 1983) although numbers as high as 22 – 27 (Sansome and Brasier, 1974) and as low as 5 have been reported for *P. megasperma* var. *megasperma* and for *P. megakarya* respectively (Sansome, 1980). The chromosome number of *P. citrophthora* has not been estimated. This partially stems from the difficulty of inducing the sexual stage in culture. However, with the new technique of pulsed–field electrophoresis, the karyotypes of more *Phytophthora* spp. can now be studied without reliance on the production of oospores. For example, Howlett (1989) separated 13–14 bands in the megabase range for *P. megasperma* which agreed with the cytological karyotyping by Hansen *et al.* (1986).

2.2.2 The role of *P. citrophthora* in Citrus Root and Collar Rot

Infection of Citrus by *P. citrophthora* may occur above ground via wounds or natural cracks in the bark (collar rot) or below ground, via tap and feeder roots (root rot). Zoospores are the principal agents of dispersal and infection, especially under moist

conditions. Zoospores are biflagellate and swim or are transported in water, i.e. in surface drainage water or via water–filled pores in wet soils. Zoospores of *Phytophthora* are attracted towards nutrient sources, which include root exudates in soil–borne species, usually in the region of elongation or, in older roots, where a wound has occurred. This effect, known as chemotaxis, has also been observed in initiation of collar rot infections (Whiteside, 1971).

Because of difficulties in methodology, little is known about the development of collar rot infection within the host, particularly in terms of the mode of penetration through the stem region. Techniques have been developed for infection of fibrous roots using zoospores, but for collar infection, techniques have been largely limited to placing mycelium, with the agar in which it was grown, in contact with the cambium after removing a disc of bark. This procedure is not analogous to natural infection. Once in the stem, the fungus grows into the cambium producing a necrotic reaction, which is commonly accompanied by abundant gum exudation (Plate 2.1). On susceptible rootstocks, under favourable conditions for disease development, lesions may extend downward into the crown roots as well as upward on the trunk. Infected nursery trees and young orchard trees develop pale green leaves with yellow veins, typical of a girdling effect and may eventually be killed. Large trees may also die, but are usually only partially girdled, and the injury causes a decline of the canopy with defoliation, twig dieback and short growth flushes (refer to Plates 2.2 and 2.3). It is generally agreed that conditions predisposing the tree to collar rot include high

soil moisture content, soil build-up against the trunk, deep planting and cultivation injury (Whiteside, 1971).

Root infection is thought to occur as follows: germ tubes from several zoospore cysts penetrate the host, growing between the cells of the epidermis. Hyphae grow inter- and intra-cellularly within the root cortex. This results in necrosis of the piliferous layer and cortex. *P. cinnamomi* hyphae then enter the xylem and phloem and grow throughout the stellar parenchyma where they reproduce asexually (Weste and Cahill, 1982). On roots, symptoms of infection occur below the soil line and canopy symptoms may develop without obvious damage to the above-ground portion of the trunk. In this case, *P. citrophthora* can cause a decay of feeder roots, which slough their cortex leaving only the stele, giving the root system a stringy appearance. Feeder root rot of highly susceptible rootstocks causes tree decline and yield losses.

Phytophthora spp. are considered to have a low saprophytic ability, but a study by Sneh and Katz (1988) has shown that *P. citrophthora* can colonise leaf pieces and fruits as well as root and stem tissue in the soil. Survival of *P. citrophthora* in the soil is thought to be in the form of oospores and chlamydospores, although this has not been demonstrated.

Plate 2.1 ***P. citrophthora*** **infection on a Citrus tree.**

A. In the stem, the fungus grows into the cambium producing a necrotic reaction, which is commonly accompanied by abundant gum exudation indicated by an arrow at the base of the trunk.

B. Branches of an infected tree showing the distinctive symptom of twig dieback, indicated by an arrow. Next to this tree, the healthy branches of an uninfected tree serve as a comparison.

C. A dead tree in a Citrus orchard with known *P. citrophthora* infection. Pear–baiting techniques isolated *P. citrophthora* from the soil around the roots of this tree.

This orchard is typical of many of the earlier orchards established when the benefits of using resistant rootstocks were relatively unknown. Susceptible plants (e.g. Symons Sweet Orange) are interspersed amongst the more tolerant cultivars (e.g. Carrizo Citrange) which results in the patchy distribution of infection observed in the above photographs.

A

B

C

2.3 VARIABILITY WITHIN *P. CITROPHTHORA*

Variability is examined with respect to two criteria: taxonomic characters and pathogenicity. Concepts of variability and how it occurs in *Phytophthora* spp., especially in the development of new races, cannot be inferred from other fungi because, as noted previously, most of these are haploid during the vegetative part of their lifecycle. Therefore, there is a need to develop concepts more appropriate to organisms with a diploid somatic phase. The development of such concepts has obvious taxonomic implications as well as other consequences in, for example, host–pathogen interactions (dealt with in Section 2.6).

2.3.1 Taxonomic Variability

Recognition of the genus *Phytophthora* ("plant destroyer") began with the work of Anton de Bary in 1876, when he described the fungus causing late blight of potato, *Phytophthora infestans*, as the type species. This was followed by the description of other *Phytophthora* spp. including *P. cactorum* in 1886, *P. phaseoli* in 1889, and *P. nicotianae* in 1896 (Zentmyer, 1983). Today, 43 species of *Phytophthora* (Waterhouse *et al.*, 1983) are known to cause a wide range of diseases on a variety of hosts including food crops, forest trees and ornamental plants.

The taxonomy of the genus *Phytophthora* is based largely upon morphological and cultural criteria and generally requires determination of a large number of characters. There are several taxonomic keys in existence (Leonian, 1934; Rosenbau, 1917;

Tucker, 1931). Waterhouse (1956; 1963; 1970) and her associates (Newhook *et al.*, 1978; Stamps *et al.*, 1990) provide the keys currently in general use, although recently Ho (1981) developed a synoptic key for identification of *Phytophthora* species.

Details of sexual structures which are of value for identification purposes include morphological variations in antheridia, oogonia and oospores (Newhook *et al.*, 1978). Included are the type of antheridium, whether paragynous or amphigynous and whether or not antheridia are subterminal, have tangled hyphae, are elongated and contain residual oil globules. Characteristics of the oogonia include variations in size, ornamentation, tapering of bases, thickness of walls, clustering, eccentricity of stalks, or becoming rough with age. Oospore diameter is used and seems to vary with the size of oogonium, except when the oogonium is markedly aplerotic.

Characters of the asexual structures may be more important in the classification of certain species of *Phytophthora*, such as *P. citrophthora*, where the sexual stage is difficult to produce in culture. Waterhouse (1983) has included characteristics of the sporangia, such as the type of papillation, nature of the exit pore, shape, length to breadth ratio, whether or not they are caducous, and length of the pedicel attached to sporangia of caducous species, as well as the presence or absence, and characteristics, of other asexual bodies such as chlamydospores and hyphal swellings.

Environmental factors, such as age, temperature and medium, have been shown to affect certain morphological characters, such as caducous sporangia. Other characters, for example, the pedicel, were more "stable" because they were apparently not affected by environmental factors (Al–Hedaithy and Tsao, 1979) and may have arisen from selection pressures applied by the different natural habitats of the species (Gallegly, 1983). This may explain the inconsistent observations found for sporangia of *P. citrophthora* which have been described as caducous (Waterhouse, 1970) or noncaducous (Ferguson, 1976). Similarly for the sexual structures, oospores produced on synthetic media or on media containing Citrus root extract were consistently larger than those produced on carrot agar, cornmeal agar, clear V–8 juice, or clear V–8 broth (Ribeiro *et al.*, 1975). In sexual matings, conditions are difficult to standardise because the species used in pairings may have different requirements. Therefore the conditions under which asexual and sexual structures are assessed must be specified clearly; this may explain inconsistencies observed in morphological characters not accounted for in current taxonomic keys.

Morphological characters are the chief criteria used for interspecific distinctions. In addition, other characters are also used in the keys, for example, growth rates at cardinal temperatures and colony pattern on agar media. All species of *Phytophthora* are plant pathogens but a few species have a restricted host range and therefore pathogenicity has also been used as a taxonomic criterion in some keys (Tucker, 1931). For example, the pathogen causing red stele of strawberry is classified as *P. fragariae* and that causing downy mildew of lima bean as *P. phaseoli*. However, the

root and crown rots caused by soil–borne *Phytophthora* spp. may be one of several species.

The principal limitation to the use of the keys currently available is that little allowance has been made for variation within isolates from the same species (intraspecific variation) as well as overlap between species (interspecific variation) for particular morphological characters. Earlier keys are more restrictive, in terms of size specifications for morphological characters, than are later keys (e.g. Waterhouse, 1963 compared to Ribeiro, 1978 or Newhook *et al.*, 1978). Many examples of intraspecific variation have been recorded. For example, Leonian (1934) observed extreme variation in sporangium size within a single zoospore–derived isolate of *P. parasitica* var. *rhei*. In *P. citrophthora*, Ferguson (1976) found differences in the size and shape of sporangia for 11 isolates studied. Thus a single "type" culture cannot conceivably represent the intraspecific variability of an entire population or the "mean" of this variability. More knowledge of the normal range of variation of a species is crucial and to this end other methods of examining taxonomic variability have been developed (e.g. biochemical and DNA analyses).

Over the past two decades, a number of attempts have been made to use serological techniques to identify pythiaceous fungi. In the earlier studies, the antisera raised were genus–specific or reacted with related genera, or with groups of species of the same genus (Banowitz *et al.*, 1984; Krywienczyk and Dorworth, 1980; MacDonald and Duniway, 1979; Malajczuk *et al.*, 1975; Merz *et al.*, 1969). Species–specific

antisera were also obtained (Burrell *et al.*, 1966; Halsall, 1976; White, 1976). These were usually prepared by cross-absorption of the antiserum with antigens from another species followed by immunofluorescence and immunodiffusion techniques to determine the resultant specificity. Unfortunately with respect to distinguishing between species, the results were equivocal. No evidence could be found of antigens specific for mating type, isolate, or physiologic race (Burrell *et al.*, 1966; Buxton *et al.*, 1961; Halsall, 1976; Holland and Choo, 1970; White, 1976).

Recently, Hardham *et al.* (1986) used serological techniques to examine variability in *Phytophthora* species. Monoclonal antibodies raised to components on the surface of glutaraldehyde-fixed zoospores and cysts of *P. cinnamomi* were tested against six isolates of *P. cinnamomi*, six other species of *Phytophthora* and three species of *Pythium*. The results yielded the first evidence of isolate-specific as well as species-specific and genus-specific markers. Further development of such highly specific probes would be valuable to monitor the occurrence and spread of individual isolates, their response to changing conditions and their effects on different host plants. Polyclonal and monoclonal antibodies have been used in this way by Pepin and Prager (1989) to detect *Phytophthora* infection in plant tissue.

Determination of total protein patterns by gel electrophoresis has also been used to identify species of *Phytophthora*. The first evidence that total protein patterns may be useful in identification of pythiaceous fungi was provided by Clare (1963) who used starch gel electrophoresis to produce distinct patterns for each of six species of

Pythium. Using the same method, Clare and Zentmyer (1966) found that the protein patterns of *P. cinnamomi*, *P. citrophthora* and *P. palmivora* were distinctly different and that the patterns of different isolates of the same species were identical, regardless of geographic or host origin. Using polyacrylamide gel electrophoresis of total protein Hall *et al.* (1969) obtained similar results except that the *P. palmivora* isolates showed some intraspecific variation which was attributed to the different morphological forms of the species (Gallegly, 1983). Later, Gill and Zentmyer (1978) found that the protein patterns obtained by polyacrylamide gel (disc) electrophoresis of 30 isolates of *P. cinnamomi* from 17 different hosts and widely separated geographic areas were identical except for a minor variation in one isolate. They also found that neither the length of time an isolate had been in culture nor the compatibility type (A1 or A2) affected the protein patterns. This finding was also found by Ferguson (1976) who found the banding pattern for *P. citrophthora* isolates to be similar and independent of the two media upon which the isolates were grown. This was in contrast to the morphology of the isolates which showed a great deal of variation directly attributed to differences in the growth media . In general, however, it is difficult to distinguish intraspecific variation using protein analysis as minor bands are difficult to characterise and it is principally the major bands which are analysed.

For taxonomic purposes, isozyme analysis offers several advantages over techniques for visualisation of total protein banding patterns. Isozyme patterns are less complex and are easier to differentiate and interpret. Isozyme analysis also provides

information about protein function, whereas other electrophoretic techniques only separate proteins on the basis of their physical properties. Isozyme analysis has shown that isolates of *P. citrophthora* fall into two major groups, one from *Theobroma Cacao* L. (Cocoa) and the other from Citrus (Förster *et al.*, 1990a). This finding has been confirmed by RFLP analysis (see below). Most work on isozyme analysis has distinguished clear interspecific variation between species (e.g. Cacciola *et al.*, 1990). In contrast, one study found that in three *Phytophthora* spp. isolated from Citrus (*P. parasitica*, *P. citrophthora* and *P. citricola*) there was some overlap suggesting a continuum between the three species (Vallavieille and Erselius, 1984).

One relatively new approach is to use DNA analyses to examine genetic variation. Here, the relationships, particularly within species, can be defined more accurately than using protein analyses.

Förster *et al.* (1987) have analysed restriction sites in mitochondrial DNA (mt DNA) to examine diversity within several species of *Phytophthora* including *P. citrophthora*. Among isolates of *P. citrophthora*, seven Citrus isolates from Australia, California, and South Africa had identical mtDNA patterns; the same was the case for five Cocoa isolates from Brazil. The mtDNAs from the Cocoa and the Citrus isolates were not closely related to each other and formed two distinct subgroups. Thus, whilst variability was dependent on the host from which the isolates were derived, there was uniformity within the subgroups themselves, which confirms the distinction found by isozyme analysis.

Goodwin *et al.* (1990) also found this "host-specialised" subgrouping of *P. citrophthora* using RFLPs and isozyme analysis, but argued that although there was variation among the isolates derived from different hosts, isozyme patterns of all *P. citrophthora* isolates from Cocoa, Citrus, *Actinidia chinensis* (Kiwi) and *Juglans regia* L (Walnut) clustered separately from those of other *Phytophthora* species. They also argued that the isolates examined were more closely related on the basis of geographical proximity than host specificity, as the RFLP pattern of one isolate from Australia had low similarity coefficients compared with other Citrus isolates taken from southern California.

Recently there has been some interest in DNA polymorphisms based on the Polymerase Chain Reaction (PCR) using random primers. Using this technique, polymorphisms are sought in the distance between two short target sequences rather than the presence or absence of restriction endonuclease sites as in RFLP analysis. Lee *et al.* (1991) found that this technique could be used to separate different species of *Phytophthora* as well as *Phytophthora* from other Oomycete genera. The advantage of PCR lies in its sensitivity and specificity, particularly where DNA samples are limited.

Certainly, while many species are apparently separated by morphological characters, techniques such as molecular analysis may prove valuable to distinguish or delimit isolates such as those of *P. citrophthora* whose variability on the basis of morphological characters results in an overlap of types or are not easily separated by

morphological criteria. However, defining variability based totally on a molecular system may not accurately represent the true biological species and should be used in conjunction with other classification systems.

2.3.2 Variability in Pathogenicity

In the literature the terms "pathogenicity", "aggressiveness" and "virulence" are used in different ways, and it is therefore necessary to define these terms in this thesis. Pathogenicity is used in a broad sense to describe the ability of the species to cause disease on a particular host cultivar. Virulence is used to describe how individual isolates within that species vary in their ability to cause disease on a particular host cultivar (Miles, 1955). This distinction may be best illustrated by an example of a non-virulent strain of a pathogenic species. Aggressiveness, on the other hand, is used to describe differences in disease severity caused by different isolates, but is distinct from virulence in that it is independent of differences between behaviour on host cultivars (Vanderplank, 1978).

Variability in virulence among isolates within a species has long been recognized in *Phytophthora* (review by Erwin *et al.*, 1983). Although Citrus is the main host of *P. citrophthora*, this fungus has been shown to attack hosts in 15 families (Broadbent, 1977) but no work has been done on its pathogenicity with respect to these other hosts. No intraspecific variation in aggressiveness in *P. citrophthora* has been reported (Broadbent, 1971).

There are many examples where single zoospore-derived cultures of *Phytophthora* species have been shown to differ in pathogenicity from the parental cultures and from one another (Hilty and Schmitthenner, 1962; Kennedy *et al.*, 1986). No relationship, however, has been found between pathogenicity of single zoospore-derived cultures and their cultural characteristics (Erwin, 1983).

Loss of pathogenicity with continued culturing has been recorded repeatedly (Caten, 1971; Erwin, 1966; Jeffrey *et al.*, 1962). This type of variation is of great significance to both plant breeders and pathologists. Problems of culturing *Phytophthora* species on defined media, of establishing colonies from single spores and of germinating oospores have prevented detailed studies of the cellular basis for these changes.

Inoculum concentration can be an important determinant of pathogenicity. Eye *et al.* (1978) reported that inoculation of soybean seedlings with 500 zoospores per seedling of *P. megasperma* f. sp. *calen* did not induce disease, inoculation with 10^4 zoospores induced disease on susceptible but not resistant plants, and inoculation with 10^5 zoospores induced disease on some resistant, as well as susceptible, plants.

2.3.3 Summary

Our knowledge of the full range of variability, both taxonomic and pathogenic, within *Phytophthora* and, in particular, *P. citrophthora*, is still limited. The taxonomy of the genus is based largely on morphological characters (e.g. Waterhouse, 1963). These

characters are highly variable not only between species, thereby making classification difficult, but also within species. In addition to morphology, serological techniques, soluble protein patterns, isozyme and DNA analyses have also been used to study both intra- and inter-species variation in *Phytophthora*.

From our knowledge of other *Phytophthora* spp., new pathogenic types are constantly being generated in the field. However, pathogenicity studies have been largely neglected in *P. citrophthora*.

2.4 MECHANISMS OF VARIABILITY IN *PHYTOPHTHORA* SPECIES

Despite the apparently rare occurrence of sexual reproduction (see Section 2.2.1.), *Phytophthora* species appear to be highly variable both in terms of taxonomic characters and in pathogenicity. *P. infestans* has been used to examine the inheritance of virulence phenotypes. Much work points to the existence of a gene-for-gene system (Shaw, 1990). However, data from Spielman *et al.* (1989) indicate that in some isolates avirulence is recessive. Work with another Oomycete, *Bremia lactucae*, suggests that asexual mechanisms of recombination may be also be important in generating new pathotypes (Hulbert and Michelmore, 1988).

2.4.1 Uninucleate and Multinucleate Zoospores

In studies on the inheritance of virulence or other traits, it is necessary to use single spores that are uninucleate to obtain genetically pure strains. Researchers have

examined the number of nuclei in zoospores of different *Phytophthora* species by applying various DNA staining techniques and most report that zoospores are predominantly uninucleate (Caten and Jinks, 1968; Converse and Shiroishi, 1962; Shepherd and Pratt, 1974). In contrast, a high number of multinucleate zoospores has been reported occasionally for the same *Phytophthora* spp. (Gallegly and Eichenmuller, 1959; Clayson, 1958). Caten and Jinks (1968) attributed these contrasting findings to differences between isolates and the conditions used to liberate the zoospores.

There is still some uncertainty as to the incidence of variability between isolates with regard to the ratio of uninucleate to multinucleate spores. For example, little variation has been found between isolates of *P. cinnamomi* in contrast to the greater variability found within *P. fragariae* (Shephard and Pratt 1974, and Converse and Shiroishi 1962, respectively). However, there is strong evidence that the number of nuclei per spore is influenced by cultural conditions. For example, conditions unfavourable for zoospore formation in *Phytophthora* spp. (i.e. temperatures above the optimum and poor aeration) resulted in incomplete cleavage of the protoplasm in the sporangium or the formation of giant spores with many nuclei (Cotner, 1930). Furthermore, in both *P. cinnamomi* and *Colletotrichum* spp. the larger spores contained more than one nucleus (Shepherd and Pratt, 1974 and TeBeest *et al.*, 1989, respectively). In addition, TeBeest *et al.* (1989) found that spores of *Colletotrichum* spp. were more variable in nuclear number when grown in liquid culture than when grown on agar.

2.4.2 Asexual Mechanisms of Variation

Attempts to determine the mechanism(s) responsible for the variation arising through asexual processes within *Phytophthora* species are greatly hampered by the lack of information on the genetics of the genus. The uncertainty about the degree of ploidy of the somatic nucleus previously referred to in Section 2.2.1 is one example where basic information about *P. citrophthora* is inadequate.

Any somatic 'recombination' between strains presumably requires some form of fusion between somatic structures from each strain and several workers have observed fusions between hyphae in mixed cultures (Dyakov and Kuzovnikova, 1974; Shattock and Shaw, 1976). Zoospore fusion has been reported for *P. infestans* under laboratory conditions (Hiddema and Kole, 1954). If it occurs in the field, where zoospores of different genotypes would mix readily in water, it would provide a possible means for heterokaryon synthesis. The existence of binucleate zoospores within *Phytophthora* species further supports the possibility of heterokaryosis. However, in most of the above studies one cannot discount the possibility that the new phenotype resulted from mutation of just one parent.

Layton and Kuhn (1988ab) generated new phenotypes from pairs of soybean isolates of *P. megasperma* using a protoplast fusion technique. Protoplasts from lines resistant to metalaxyl or to fluorotryptophan were fused in the presence of polyethylene glycol. Most regenerants, selected on medium containing both

chemicals, gave rise to zoospore progeny which segregated into the two parental phenotypes, suggesting they were heterokaryons.

Hulbert and Michelmore (1988), working with *Bremia lactucae*, have presented the most convincing evidence to date of a natural somatic fusion in the Oomycetes. The authors examined the RFLPs at 35 nuclear loci of twenty–five isolates representing diverse geographic origins and virulence. The European isolates exhibited great diversity which was consistent with the frequent occurrence of the sexual cycle, in contrast to isolates from Japan, California, Australia and Wisconsin where there was an infrequent occurrence of the sexual cycle. One of the Californian isolates (pathotype IV) appeared to be a somatic hybrid between two other Californian isolates (pathotype II and III). The authors suggested that mitotic crossing over during prophase in heterozygous diploid nuclei and improper disjunction during mitotic divisions may be possible mechanisms by which variation within the asexual populations in Australia and California had arisen. Early studies of somatic recombination in heterozygous diploid nuclei of filamentous fungi have shown that the processes of mitotic crossing–over and non–disjunction during mitotic divisions are rare events (Roper, 1966) but the former, in particular, has been proposed as a compensatory mechanism for generating variation in species of *Phytophthora*, such as *P. citrophthora,* where the sexual stage rarely occurs (Khaki and Shaw, 1974).

It is unlikely that mutation of chromosomal genes can provide an explanation for the variation observed within *Phytophthora* spp. because the fungus is diploid for most of its life cycle and therefore any recessive mutations would not be phenotypically detected.

Alternatively, somatic variation may be under the control of nonchromosomal elements (MacIntyre and Elliott, 1974). Spontaneous variation in several quantitative characters, including growth rate, sporulation and pigmentation, has been shown to be under cytoplasmic control in species of *Aspergillus* and *Penicillium* (Croft, 1966; Jinks, 1966). One of the characteristics of cytoplasmic control is that it often leads to segregation in the asexual progeny of an individual derived from a single homokaryotic spore (Jinks, 1966). The persistent segregation in high and low growth rates observed in successive single zoospore-derived generations of *P. infestans* is therefore supportive of cytoplasmic control as a possible asexual mechanism in *Phytophthora* species (Caten and Jinks, 1968). Furthermore, virus-like particles may carry non-chromosomal genes in *Phytophthora* species. Virus-like particles have been found in *P. infestans* (Corbett and Styer, 1976) and *P. drechsleri* (Erwin *et al.*, 1983). In the latter, virus-like particles were found within the nuclei of zoospores. Different rates of virus replication in different nuclei could yield a zoospore population with a wide range of virus concentrations. It is possible that isolates derived from such zoospores would show differing growth rates and other characteristics, related to the frequency of virus particles in the zoospore nucleus.

However, the variability demonstrated particularly morphological, may not be directly related to changes in genotype but rather may arise from the chemical and physical processes of cellular physiology (Harold, 1990). There is certainly a connection between genes and form, but even if we had all the information encoded in the yeast genome we still could not infer the shape of the cell, let alone the succession of shapes during budding. Similarly in *Phytophthora* spp. the diversity of size and shape of sporangial cells observed cannot be explained, for example, in terms of cytoplasmic inheritance alone.

2.4.3 Sexual Mechanisms of Variation

Sexual reproduction in *Phytophthora*, resulting in the production of oospores, provides the means of long–term survival as well as genetic variation via recombination. Both homothallism and heterothallism occur in species of *Phytophthora*. Homothallic species are self–fertile, whereas the heterothallic species need to be grown in the presence of a suitable mating type before oospores will form. *P. citrophthora* is heterothallic, although the sexual stage is considered rare and details are not included in some taxonomic keys.

Genetic work with several homothallic and heterothallic species shows inheritance of traits in a Mendelian manner, typical of a diploid state (Shaw, 1988). This means that recessive mutations, the favourite markers for analysis of haploids, are difficult to select, that F2 and backcross progeny are needed to identify most genotypes and that deleterious recessive mutations can accumulate and result in inbreeding depression

(Shaw, 1983). There are also difficulties with species which are apparently heterothallic because in these species both crossing and selfing of paired strains of opposite mating type (A1 and A2) can occur. It has been demonstrated, that for some heterothallic species, when strains of opposite mating type are paired but separated by a polycarbonate membrane, one or both strains can reproduce by selfing. Ko (1978) postulated that one strain produces a mating type–specific hormone that induces the other to respond by production of sex organs. The outcome of a pairing of an A1 with an A2 of the same species is therefore unpredictable as, in addition to selfs of each parent, two kinds of hybrid may occur. Galindo and Gallegly (1960), working with *P. infestans* showed that some gamentangial unions resulted from interaction of an A1 antheridium with an A2 oogonium, others from interaction of an A1 oogonium with an A2 antheridium. The implications of this lie in the interpretation of genetic data. If two kinds of selfed progeny are produced in addition to hybrid individuals, the proportions of the different phenotypes in the progeny of a cross of marked strains will depend on the relative frequencies of the two selfings and the crosses as well as on the ability of the different kinds of zygote to germinate.

Furthermore, in *Phytophthora* many inter– and intra–specific crosses produce oospores (Savage *et al.*, 1968), but whether these are products of recombination events remains unclear. Where oospores are not formed in matings, the literature refers to "genetic incompatibility" (Vorob'eva and Gridnev, 1981) or "genetic blockage" (Gallegly, 1983). For example, sexual pairing is only possible between isolates of opposite types of compatibility (A1 x A2). However, the formation of

oospores does not necessarily mean that sexual recombination or genetic exchange of information has occurred and the possibility of selfing in many *Phytophthora* species may be greater than originally envisaged. For example, although progeny derived from *P. parasitica* x *P. cinnamomi* oospores showed evidence of recombination for compatibility type, pathogenicity and temperature, they all had protein patterns similar to that of *P. parasitica* indicating that selfing may also have occurred (Boccas and Zentmyer, 1976). Techniques should be used to ensure the establishment of single oospore–derived cultures from true hybrid oospores otherwise difficulties in interpretation of results will arise. However, in spite of experiments which have used single oospore–derived cultures, there has been little evidence of formation of progeny considered to be the true product of hybridization (Boccas, 1981; Romero and Erwin, 1969).

Inconsistency in oospore germination also presents a major problem for studying mechanisms of variation. Although there has been much work on producing and germinating oospores in the laboratory, the mechanism of dormancy of oospores remains unknown. Oospore dormancy is considered to be constitutive (Cochrane, 1974; Schmitthenner, 1968; Zentmyer and Erwin, 1970) and it is now known that if certain external conditions are provided the germination of oospores of many species will take place (Ribeiro, 1983). For example, a light requirement for germination has been reported by several investigators (Banihashemi and Mitchell, 1976; Berg and Gallegly, 1966; Ribeiro *et al.*, 1975; Satour and Butler, 1968) and certain species of *Phytophthora* were stimulated more by the blue than the far–red wavelengths (Ribeiro

et al., 1975). Oospores often require a pretreatment at a higher or lower temperature than ambient for growth, before they will germinate. The temperature optimum for oospore germination varies depending on the species of *Phytophthora*: for example, at 15°C, 13% of oospores from *P. megasperma* f. sp. *calen* germinated; at 24°C, 65% germinated; at 27°C, 78% germinated; and at 30°C only 7% germinated (Erwin and McCormick, 1971). Similarly, germination of oospores of *P. cactorum* at 16, 20, 24, and 28°C was 41, 88, 62, and 0%, respectively (Banihashemi and Mitchell, 1976). Exogenous factors are required for the germination of oospores of most *Phytophthora* species examined. In some cases germination has been shown to be stimulated by different enzymes, particularly helicase and glusulase derived from snails (Gregg, 1957; Salvatore *et al.*, 1973), although the reasons remain unclear. Germination has been shown to be affected by the use of different media (Förster *et al.*, 1983; 1982; Leal and Gomez–Miranda, 1965; Satour and Butler, 1968) but is apparently not influenced by the pH of a given medium (Klisiewicz, 1970;Hugenin and Boccas, 1971).

While a high frequency of oospore germination has been reported for homothallic species induced by the appropriate conditions (Banihashemi and Mitchell, 1976; Duncan, 1985; Erwin and McCormick, 1971; Förster *et al.*, 1983; Harris and Cole, 1982; Ko and Arakawa, 1980; Leal and Gomez–Miranda, 1965), a low and inconsistent rate of germination has been reported for heterothallic species (Chee, 1973; Kaosiri *et al.*, 1980; Ribeiro *et al.*, 1975; Satour and Butler, 1968; Shattock *et*

al, 1986; Timmer *et al.*, 1970), although recently Ann and Ko (1988) reported some success with *P. parasitica*.

2.4.4 The Potential of RFLPs for Analysis of Variability in *P. citrophthora*

Studies on variability in *Phytophthora* are complicated by the fact that the species is diploid for most of its lifecycle and because of the existence of both asexual and sexual mechanisms underlying variability. In general, such studies have been largely dependent upon characterisation of a rare recombination event which could be attributed to either mechanism, because it is difficult to distinguish mitotic changes from other sources of change such as aneuploidy, mutation or even from contamination. Molecular markers such as RFLPs overcome this problem by allowing unambiguous identification of experimental strains.

RFLPs result from specific differences in DNA sequence (single base-pair substitutions, additions or deletions, or gross chromosomal changes such as inversions or translocations) that alter recognition sites and hence the fragment sizes obtained by digestion with a type II restriction endonuclease (see Figure 2.2). The DNA fragments are fractionated according to size by agarose gel electrophoresis; the DNA is then transferred to and immobilised on a filter, hybridised to a labelled DNA probe and visualised by autoradiography (Southern, 1975). The number of bands observed is dependent upon the number of loci in the genome homologous to the probe and the number of restriction sites within these DNA sequences. RFLP analyses detect variation in DNA sequences within, and contiguous to, the regions homologous to the

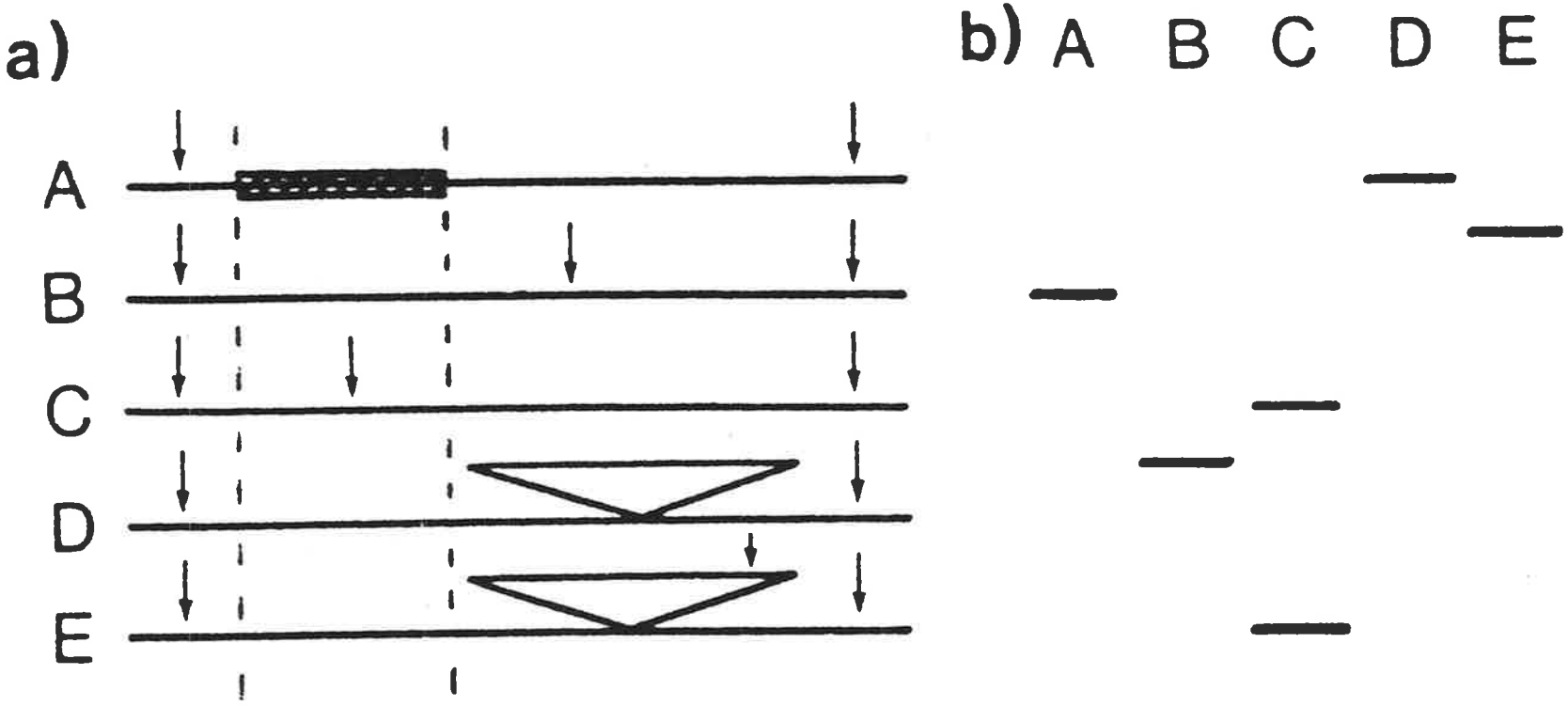
probe. The number of RFLP markers is theoretically unlimited because any low-copy-number DNA segments can be used as probes and several restriction enzymes can be assayed to identify RFLPs with each probe. Intraspecific variation can be readily detected, often between quite closely related individuals (Michelmore and Hulbert, 1987).

Although RFLPs do have some technical disadvantages, for example DNA preparation is both time-consuming and expensive, once isolated the DNA is stable and can be stored for years, so that the RFLPs may be assayed over time. Also, when the DNA is bound to a nylon membrane, each blot may be assayed with a number of different probes. Thus much information can be generated by RFLP analysis.

RFLPs can also be used with other techniques such as Contour-clamped Homogenous Electric Field Electrophoresis (CHEF) to develop genetic maps and elucidate the genetic determinants of virulence and other phenotypic traits. CHEF separates large DNA molecules in straight bands in pulse fields. This technique has been used to karyotype *P. megasperma* (Howlett, 1989). It is potentially useful for organisms such as *P. citrophthora* where the sexual stage is difficult to generate in culture. For example, the position of the RFLPs on the genetic map can be confirmed by probing Southern blots of CHEF gels. As the genetic map approaches saturation, remaining unmapped regions can be then analysed with chromosome-specific probes (Bishop *et al.*, 1983). Chromosome-specific probes are

Figure 2.2 Diagrammatic representation of restriction fragment length polymorphisms.

Part (a) depicts the distribution of restriction endonuclease sites (arrowed) in a short chromosomal fragment in five haploid lines (A to E), containing a region homologous to a cloned probe (shaded). B and C have restriction sites not present in A. D and E have insertions. Part (b) depicts the banding pattern expected from Southern hybridization of the five lines after their genomes have been restricted at the sites shown in a and hybridised with a probe homologous to the shaded region. The gel was run from top to bottom. The largest fragments have the lowest mobilities. (Michelmore and Hulbert 1987).


a)
A
B
C
D
E
b)
A
B
C
D
E

made from DNA eluted from specific bands in the CHEF gels. Similar techniques have been used to demonstrate chromosomal changes associated with antigenic variation in *Trypanosoma brucei* (Johnson and Borst, 1986).

RFLPs can be used in conjunction with dominant selectable markers such as drug resistance. Also, this is particularly useful where diploids or heterokaryons must be tested for recombination resulting from asexual mechanisms of variation (Layton and Kuhn, 1990).

2.4.5 Summary

There are several possible mechanisms responsible for the variability observed in *Phytophthora*. Evidence for somatic recombination in the Oomycetes has been obtained (Hulbert and Michelmore, 1988). However, sexual recombination provides the more common means of generating new pathotypes in nature and there is abundant evidence of intraspecific hybridization in other fungi. In *Phytophthora*, the answer to the question of whether oospores are actual products of sexual recombination remains unclear, particularly in species that are capable of selfing. It is essential to distinguish between hormonal stimulation of the sexual stage and actual fusion of gametangia between two different isolates of the same species or different species. With respect to *P. citrophthora*, the main obstacle is the production and germination of oospores in culture.

RFLP markers have been employed in studies on the genetic mechanisms underlying variability such as determination of recombination events by the unambiguous identification of experimental strains. In addition, RFLP analyses can be used with other selectable markers (such as fungicide–resistant markers) or CHEF where interpretation of results may be difficult or where more information is required.

For genetic analysis, *P. citrophthora* is a difficult organism to work with for several reasons: firstly, *Phytophthora* species are quite distinct from other fungi because, as Oomycetes, they are phylogenetically closer to algae and are diploid for most of their life cycle. Thus, concepts of variability and how it occurs, especially in the development of new strains, is difficult to extrapolate from work done with other fungal species. Secondly, interpretation of genetic events is confounded because *P. citrophthora* is considered to be heterothallic, thus there are the problems of selfing, as well as heterokaryosis to consider for isolates that have multinucleate asexual spores.

2.5 THE HOST – CITRUS

The commonly cultivated Citrus fruit trees belong to three genera, *Citrus*, *Fortunella* and *Poncirus*. They are closely related and are members of the family Rutaceae, subfamily Aurantioideae, subtribe Citrinae. All species of the genus *Citrus* and *Fortunella* are evergreen with persistent 1–foliate or simple leaves, but the related genus *Poncirus* is deciduous, with trifoliate leaves. The genus *Citrus* is subdivided

into two distinct subgenera – *Eucitrus*, (which includes all commonly cultivated species of *Citrus*) and *Papeda.*

2.5.1 Citrus Rootstocks

Originally Citrus trees were grown as seedlings, but today almost all trees in commercial cultivation are propagated on rootstocks. The three genera above have been significant as sources of rootstock or scion cultivars. In fact, from these three genera have come the five rootstocks upon which the world's commercial Citrus industries are based. A number of reviews have been written on this subject (Castle, 1987; Webber, 1948; Wutscher, 1979) and it is clear that rootstocks are important because they affect over 20 Citrus tree characteristics, primarily horticultural and disease response. Among these are: (1) early fruiting and avoidance of juvenility problems; (2) uniform tree size; (3) cropping and fruiting quality control; (4) tolerance to unfavourable soil factors such as salinity, high pH and poor drainage and (5) resistance to *Phytophthora*, parasitic nematodes and viruses.

The Australian Citrus industry requires a range of rootstocks adapted to the different climatic zones, ranging from tropical to arid, and soil conditions. The important Citrus areas are located in the inland river systems such as the Murray and the Murrumbidgee where the mean maximum temperature is 31°C and annual rainfall about 650 mm. However, smaller plantings are found on coastal areas where temperatures are lower and rainfall can exceed 1,200 mm. The soils vary but generally range from loamy clays to loamy sands and sands.

Several rootstocks are commonly used, namely, Rough Lemon (*C. jambhiri*), Sweet Orange (*Citrus sinensis)*, Troyer and Carrizo Citrange (*Poncirus trifoliata* x *C. sinensis*), Cleopatra Mandarin (*C. reticulata*) and Trifoliate Orange (*Poncirus trifoliata*). In the past, Trifoliate Orange has been a significant source of rootstock material, including the Citranges. At present, Rough Lemon is the main rootstock used, but Troyer and Carrizo Citrange have recently become popular, particularly in replant soils, because they have some resistance to *Phytophthora* and to *Citrus* nematode (*Tylenchulus semipenetrans*). (P. Barkley, pers. comm.) Similarly *P. trifoliata* became very popular in New South Wales following tree losses due to *Phytophthora* in the 1940s. However, Trifoliate Orange is not salt tolerant and is better suited to more alkaline soils than are the Citranges. Cleopatra Mandarin is used for shallow soil types.

There are a large number of rootstock varieties that have been bred, selected or introduced into Australia and which still have not been evaluated. Furthermore, the genetic pool for the selection of new and improved rootstocks is very large, as Citrus itself covers a wide range of fruit tree types including uncultivated fruit trees from which many hybrids could be bred. In addition, *Citrus* species have a high frequency of mutations which could be advantageous in selecting for new varieties. The present method of selection, which involves propagation of hybrid Citrus seedlings and the subsequent evaluation of their resistance to disease or stress, is not economically feasible in terms of time and resources.

2.5.2 Micropropagation of Citrus via Tissue Culture

There has perhaps been more published on tissue culture of Citrus than any other fruit tree species. For general reviews on tissue culture and its application to the Citrus industry, refer to Barlass and Skene (1986), Button and Kochba (1977), and Spiegel-Roy and Vardi (1984). Callus and more recently, protoplast culture have been used to generate somaclonal variation in polyembryonic *Citrus* species and cultivars via mutation breeding. Nucellar embryogenesis, meristem culture and shoot-tip grafting *in vitro* have been used to provide virus-free mother stock for budding, which can then be used for international distribution of Citrus material without the risk of spreading disease. Recently, the role of tissue culture for large-scale multiplication of Citrus rootstock or budwood material has been examined and is of specific interest to this study.

Conventional propagation of Citrus seedlings from breeding programmes aimed at improving rootstock characters such as disease resistance or salt tolerance depends on striking cuttings or raising nucellar seedlings. Each of these methods involves a long interval between the production of a hybrid seedling and the evaluation of a particular character, such as disease resistance. Micropropagation via tissue culture offers the opportunity to reduce this interval by enabling hybrids to be clonally multiplied quickly within minimal space. Shoot cultures which regenerate genetically stable plants may be useful in screening for resistance to pathogens such as *Phytophthora*.

Approximately nine different species of *Citrus*, one species of *Poncirus* and an intergeneric cross between *Citrus* and *Poncirus* have been induced to regenerate shoots successfully *in vitro* (Barlass and Skene, 1986). For micropropagation purposes, the method of choice for woody plants, which maintains clonal integrity, is 'axillary shoot proliferation' from pre–existing vegetative meristems. The procedure used for shoot proliferation by tissue culture involves three principal stages: the establishment of an aseptic culture, the multiplication of propagules (i.e. shoots) and the induction of roots.

There are a number of considerations regarding the seedling explant which have been found to influence the regenerative characteristics of the explant in tissue culture and the potential for contamination by microorganisms. These include the age of the plant, the season and the overall quality of the plant from which the explants are obtained and the size of the explant (Murashige, 1974).

Node and internode explants have been the preferred source of multiple shoot cultures for Citrus. Nodal explants with their pre–existing axillary meristems give the best chance for trueness–to–type of the resultant plants. Alternatively, internodes which form adventitious meristems in response to culture provide a potentially useful system for genetic change through mutagenesis (Barlass and Skene, 1982; Broertjes and van Harten, 1978). In general, seedlings have been used as the explant source material. Unlike other heterozygous woody species, the availability of nucellar seedlings in Citrus allows juvenile material to be used for clonal propagation which would

normally be considered unacceptable because the quality of the seedlings would be unknown. In addition, Bouzid (1975) found that the regenerative competence of juvenile material is much higher than that of mature explants. The time of year at which the plant material is collected from the field also has an effect on the number of explants established *in vitro* and the number of cultures which are contaminated with bacteria after sterilisation procedures (Hutchinson, 1984). Material obtained from the glasshouse is less likely to have contamination problems than field–grown material.

The media used for micropropagation of Citrus are principally modifications of the original Murashige and Skoog (1962) medium, depending on the cultivar and age of the mother plant (refer to Barlass and Skene, 1986 for further details). Sucrose concentrations vary from 3 to 5%. Most Citrus tissue cultures have been maintained successfully on media gelled with 0.8 to 1.0% agar, at a pH of 5.7 (Button and Kochba, 1977).

The general concept propounded by Skoog and Miller (1957) that organ differentiation in plants is regulated by an interplay of two groups of hormones, auxins and cytokinins, is generally applicable to Citrus. A high cytokinin–to–auxin ratio promotes shoot formation and a high auxin–to–cytokinin ratio favours root formation. The exogenous requirements for hormones depend on their endogenous levels and this varies with tissue, plant type and phase of growth (Bhojwani and

Razdan, 1983). Therefore, to initiate organ differentiation, the inclusion of only one hormone may be necessary.

The essential plant growth regulator for shoot regeneration in Citrus is the cytokinin, benzyladenine (BA) (Barlass and Skene, 1986). The only report of the successful use of any other cytokinin was with kinetin (Bouzid, 1975), which others have found to be ineffective (Primo Millo and Harada, 1976; Raj Bhansali and Arya, 1978). Root induction, on the other hand, is favoured by the presence of the auxin, α–naphthaleneacetic acid (NAA) (Barlass and Skene, 1986). However, several investigators have found that reducing the macro–nutrient components of the MS medium to half strength and eliminating growth regulators enhances adventitious rooting in some species (Spiegel–Roy and Vardi, 1984). It has been found that for some genotypes, the inclusion of auxins in the medium may be highly inhibitory to shoot production in long–term cultures (Gmitter and Moore, 1986).

Recently, coumarin, a naturally occurring growth–active substance, was used in MS medium to generate whole plants from epicotyl segments of a Swingle Citrumelo Citrus rootstock (Grosser and Chandler, 1986). This offers potential for improving tissue culture techniques in that a single medium can induce both root and shoot formation.

The other major factors in the tissue culture environment are light and temperature, but little systematic research has been done on these topics. In the absence of more

specific data, temperatures in the range of 25 – 30°C appear optimal and are generally used. For shoot proliferation, Button and Kochba (1977) suggest ca 3000 lux (37.5 μE m^{-2} sec^{-1}) for 12 to 16hr per day for Citrus. Other researchers have noted that such light intensities inhibit root development. This adverse effect can be overcome by either wrapping the root portion of tissue culture containers with aluminium foil or by inclusion of charcoal in the nutrient medium (Hu and Wang, 1983).

2.5.3 Summary

Three genera have been important to the Citrus industry as sources of either rootstock or scion material, namely *Citrus*, *Fortunella* and *Poncirus*. Rootstocks are selected on the basis of beneficial horticultural traits and for resistance to various diseases, including *Phytophthora* root and collar rot. In Australia, the Citranges and Rough Lemon are commonly used, but whereas the former is quite tolerant of *Phytophthora*, the latter is highly susceptible (see Section 2.6.1). A large number of rootstocks have yet to be evaluated.

Conventional means of propagation of rootstocks, such as striking cuttings or raising nucellar seedlings, are both time consuming and labour intensive and mean that evaluation of rootstocks for certain characters such as disease resistance may take many years. Therefore micropropagation via tissue culture has been proposed as an alternative means for quick, large-scale multiplication of Citrus plants. There are several different methods for micropropagation of Citrus but internodal and nodal stem segments are the usual sources of explant material for shoot proliferation.

Media and conditions for micropropagation, in general, have been well documented (refer to Barlass and Skene, 1986).

2.6 THE HOST - PATHOGEN INTERACTION

This section will examine how tissue culture techniques have been used to improve our understanding of resistance in host–pathogen interactions and the possibility of using such techniques to screen Citrus material for resistance to *Phytophthora*. It should be noted that the term 'tolerant' is used occasionally instead of 'resistant' (Graham, 1990). As it is difficult to distinguish true tolerance from partial resistance and the term 'resistant' is more common, this term will be used with the understanding that the cultivars used in this study are moderately resistant.

First, however, it is important to examine resistance in the field and the factors which may influence the host or pathogen response, as any response observed *in vitro* should be evaluated in the context of how the host and pathogen behave in natural surroundings.

2.6.1 Traditional Screening Methods

Methods of screening for resistance to *P. citrophthora* in the glasshouse and field have been developed for different Citrus cultivars. The disease caused by *P. citrophthora* is characterised by infection of the roots and of the collar region (see 2.2.2). Therefore several approaches have been used in screening trials, based on either stem inoculations (Broadbent *et al.*, 1971; Fraser, 1942; Klotz and Fawcett,

1930; Rosetti, 1947) and/or root inoculations in aerated water cultures containing the fungus (Broadbent *et al.*, 1971; Klotz *et al.*, 1958) or in soil (Broadbent *et al.*, 1971). Plant parts differ in susceptibility to pathogens and depending on the method of inoculation, a plant may differ in susceptibility to *Phytophthora*. In general, results from both collar and root inoculations indicate that Trifoliate Orange is resistant, its hybrids (e.g. the Citranges) were generally moderately resistant and a range of susceptible responses were shown by the oranges and lemons.

However, factors other than the intrinsic susceptibility of the rootstock to the fungus are involved. The effect of host factors, for example, age and root exudation, the regenerative ability of the roots and the effects of the soil environment, may modify resistance in the field.

2.6.1.1 Effect of Host Factors on Resistance

There are many examples of the influence of host factors such as root exudates, on Citrus host/pathogen interactions. Broadbent (1969) found that zoospores of *P. citrophthora* were attracted equally to roots of both resistant and susceptible *Citrus* species and to the roots of non–hosts tomato and cowpea. Thus it does not appear that the chemotactic response plays a part in the susceptibility or resistance of Citrus to *Phytophthora*.

Phytophthora resistance in Citrus is controlled by multiple genes with complex dominance. The moderate to high levels of resistance in Carrizo Citrange, for

example, have been inherited from Trifoliate Orange, a major germplasm source for resistance to *Phytophthora*. There are different resistant responses, again depending on the method of inoculation. One important resistant response appears to be the ability of roots to regenerate following root infection (Blaker and MacDonald, 1986). Morphological exclusion of the fungus by mature cells does not appear to be an important mechanism contributing to the expression of resistance to root infection. However, the extent of bark and periderm formation may influence the formation of growth cracks in the bark that expose the cambial parenchymous tissue to collar infection in the field (Smith *et al.*, 1991a). Studies have indicated that older plants are less likely to be susceptible to infection as a general consequence of physiological or morphological changes in root and stem during ageing (Graham, 1990; Kim *et al.*, 1989) which may in turn further enhance these resistant responses.

Phytoalexins, including scoparone and sesselin, have been isolated from bark of resistant *Citrus* cultivars inoculated with *P. citrophthora* (Afek *et al.*, 1986, 1989; Sulistyowati and Keane, 1989; Vernenghi, 1987), indicating that biochemical differences may also exist between resistant and susceptible cultivars. Phytoalexins are low molecular weight antimicrobial compounds which have been identified in most dicotyledonous plants. Phytoalexin accumulation is initiated by infection or abiotic stresses and requires specific gene activation and *de novo* synthesis of mRNA and proteins. Resistance to pathogens is then related to the amount accumulated. In resistant reactions the accumulation of phytoalexins is in amounts great enough to

stimulate a hypersensitive response to the fungus and prevent the spread of infection (Rouxel, 1989). Studies have shown that the fungicide fosetyl–Al at particular concentrations may act against *P. citrophthora* by stimulating scoparone production in fungicide–treated susceptible Citrus cultivars (Afek and Sztejnberg, 1989).

2.6.1.2 Effect of Environmental Factors on Resistance

For a root–invading pathogen such as *P. citrophthora*, changes in the soil environment including soil moisture content, salinity and temperature, may have important consequences for both the infection process and the mechanisms of resistance of the host.

Soilborne *Phytophthora* spp. require high soil moisture content and many species require free water for production and dispersal of zoospores. Species of *Phytophthora* vary in water requirements. For example, maximum zoospore formation and release for *P. megasperma* occurs on flooded soils around –0.001 – 0.025 bars but under slightly drier conditions for *P. cinnamomi* at, > – 0.01 bars. However, moisture also affects the environment of the roots and whether they become waterlogged and hence predisposed to infection. Certainly, improving drainage is one of the most effective ways of controlling *Phytophthora* root rot in Citrus (Broadbent, 1977).

The salinity of the soil appears to be an important factor in infection of *Citrus* spp. by *Phytophthora*. For example, it was found that root rot of Citrus caused by *P.*

parasitica increased with increasing soil salinity. The authors attributed this result to the effects of salts which may have increased tissue susceptibility and/or inhibited root growth combined with the ability of *P. parasitica* to tolerate high levels of salinity (Blaker and MacDonald, 1986).

Temperatures below or above the maximum for fungal growth may completely suppress the pathogen. For example, *P. citrophthora* grows best between 24 – 28°C and not below 5°C (minimum) or above 32°C (maximum) (Ferguson, 1976). This may partially explain the pattern of pathogen activity which is observed to increase in winter and decline in the summer months (Greg Walker, pers. comm.). Correspondingly, seasonal changes in susceptibility of Citrus phloem tissue to colonization by both *P. citrophthora* and *P. parasitica* have also been demonstrated (Matheron and Matejka, 1989).

2.6.2 The Use of Tissue Culture Techniques in Screening for Disease Resistance

There have been several areas in which tissue culture techniques have been employed: to examine the mechanisms responsible for disease resistance and more recently, to select for resistant plants at several levels, including cellular via callus cultures and at the whole plant level via micropropagation. For more extensive treatment of the subject, general reviews of the use of tissue culture techniques in plant pathology studies can be found in Daub (1986) and Ingram and Helgeson (1980).

Of significance to this project, however, is the use of tissue culture techniques to select for disease resistant plants. Various *Phytophthora* spp. have been shown to secrete phytotoxic metabolites in culture (Behnke, 1979, 1980; Deaton *et al.*, 1982; Plich and Rudnicki, 1979). Shohet and Strange (1989) used protoplasts from leaves of pigeonpea (*Cajanus cajan*) to detect phytotoxic activity in culture filtrates of *Phytophthora drechsleri* f.sp. *cajani*. They then showed that plant cuttings immersed in desalted culture filtrates showed symptoms similar to those of the disease. In contrast, Vardi *et al.* (1986) found no correlation between sensitivity of Citrus calli to *P. citrophthora* toxin and the field response of the same Citrus cultivars. They concluded that toxins isolated from culture filtrates were of little value in the selection of disease resistant material. Care should be taken when interpreting such results because culture filtrates are rich in substances including secondary metabolites, growth–inhibiting and growth–stimulating substances (Pegg, 1976; Yoder, 1980) and crude or even partially purified extracts may contain a combination of substances which can be phytotoxic but have little to do with disease development.

Callus cultures initiated from Australian native and horticultural species have been used to screen for expression of resistance to *P. cinnamomi*. The extent of hyphal growth on callus was correlated with the susceptibility of the plant from which the callus was derived (McComb *et al.*, 1987). In addition, pine callus tissues infected with hyphae of *P. cinnamomi* have been used to compare susceptible and resistant reactions at the cellular level (Jang and Tainter, 1990; 1991). Resistant reactions showed fewer hyphal penetrations, a greater accumulation of electron dense material

and no morphological changes in the host cell. However, in some other host–pathogen systems it was not possible to draw such a clear distinction between resistant and susceptible responses observed at the cellular level (Cahill *et al.*, 1989). It may be that this type of interaction needs to be elucidated at the molecular level.

Recently, shoots micropropagated by tissue culture techniques have also been used to screen for resistance. Sharma and Skidmore (1988) reported that partial resistance to *P. palmivora* could be identified in shoot cultures of papaya (*Carica papaya* L.) following inoculation of excised shoots with sporangial suspensions of the fungus. Scott *et al.* (1992) reported a modified excised twig assay for screening tissue cultures of almond rootstocks for resistance to *Phytophthora* spp. *in vitro* and found differences in aggressiveness between *P. cambivora* isolates on almond rootstocks which correlated with results from *in vivo* studies.

2.6.3 Advantages and Disadvantages in Using Tissue Culture Techniques to Screen for Resistance

Screening using tissue culture techniques has several advantages over conventional methods as follows: many plants may be screened in a small space where environmental and nutritional factors can be strictly controlled; the material may be inoculated with isolates of the pathogen in the absence of biotic contaminants; plant material *in vitro* is often regenerated quickly and hence preliminary resistance trials may be completed more rapidly than if undertaken in the field; and cloned plant material may be used in replicate inoculations so that the accuracy of the tests is

increased. The last two features have particular significance for screening tree species (Sharma and Skidmore, 1988).

It is important to note that screening using tissue culture techniques also has certain disadvantages. For example, resistance expressed using callus in screening programmes may or may not be related to that expressed by differentiated tissue. Many authors deem the use of crude fungal filtrates to challenge single cells as an inappropriate screening method because many toxins have been poorly characterised and even if shown to be phytotoxic may, in fact, have nothing to do with disease development, particularly on differentiated tissue (Daub, 1986; Vardi *et al.*, 1986). In addition, no callus has been found to completely inhibit fungal growth on its surface (Jang and Tainter, 1990).

The use of differentiated tissue such as zygotic embryos or clonal plantlets via shoot micropropagation techniques has limitations. The main disadvantage is that differentiated tissue takes longer to grow than unorganised cells *in vitro*. In addition, it has been noted that development of necrosis following infection in micropropagated shoots was more rapid, possibly due to the juvenile nature of the tissues which presumably lacked the structural and biochemical barriers to infection operating in woody material (Scott *et al.*, 1992).

For both callus and shoot cultures, authors have made the observation that pathogens may overgrow the culture medium used for the host and physically obscure the

assessment of disease resistance. Such growth would also increase the inoculum potential so that resistant responses which would be sufficient to protect a plant in field conditions may be overcome *in vitro* (Sharma and Skidmore, 1988). To remedy this problem, some authors either repeatedly subcultured the dual host–pathogen cultures to limit the growth of the fungus on the medium (Sharma and Skidmore, 1988) or alternatively, monitored necrosis on a day–to–day basis so that results were obtained before the shoots became overgrown (Scott *et al.*, 1992).

The artificial 'culture' environment presents certain problems. For example, the influence of phytohormones in the growth medium on the expression of disease resistance has been demonstrated in many studies (Haberlach *et al.*, 1978; Miller *et al.*, 1984). It has been suggested that the stimulation of fungal growth by a growth regulator masked resistance in pine callus tissue (Jang and Tainter, 1991).

As noted by several other authors, it is important to standardise environmental conditions (Helgeson, 1983, Scott *et al.*, 1992; Sharma and Skidmore, 1988). It was suggested that slight differences in environmental conditions either during the dual culture phase or in the production of host tissue or fungal inoculum may have been responsible for the accelerated development of necrosis observed (Scott *et al.*, 1992; Sharma and Skidmore, 1988). Temperature has been shown to affect fungal growth and hence the resistant response in culture (McComb *et al.*, 1982).

Thus, pathogens, under the right conditions, can be used to select for disease resistant plants, but when developing tissue culture techniques for screening, many factors must be considered. In addition, any *in vitro* model systems, whether for resistant screening or for pathogenicity studies, will be of value only when the reactions observed are analogous to whole plants at the *in vivo* level. Material showing resistance or partial resistance in culture must, therefore, be further evaluated under field and/or glasshouse conditions.

2.6.4 Summary

Currently there are two main areas where tissue culture techniques have been employed in studies of host/pathogen interactions ; first, to examine the mechanisms responsible for disease resistance and secondly and more recently, to select for resistant plants at several levels including cellular and tissue. It is the latter which is of relevance to this study.

There are advantages in screening in a tissue culture environment that is both nutritionally and environmentally controlled but there are also some disadvantages and these are discussed. Both need to be considered when developing a tissue culture screening technique.

However, the screening of young plants in glasshouses and in the field is, and will continue to be, the primary method of screening for resistance to *Phytophthora* and

must follow preliminary screening using *in vitro* assays based on tissue or cellular reaction to the pathogen. The results from *in vivo* work indicate that Trifoliate Orange is resistant, its hybrids (e.g. the Citranges) are generally tolerant and the oranges and lemons display a range of susceptible responses.

It is also important to compare host/pathogen interactions observed in an artificial environment, such as tissue culture, to those which occur in the natural environment. For example, the effect of host factors including host age and root exudation, the regenerative ability of roots and the effects of the soil environment, have been shown to modify resistance in the field.

3. MATERIALS AND METHODS

3.1 GENERAL TECHNIQUES AND MEDIA USED FOR *P. CITROPHTHORA*

3.1.1 Isolates

Only isolates of *P. citrophthora* obtained from Citrus hosts were examined (refer to Table 3.1) because isolates derived from other plant hosts, e.g. *Theobroma cacao* may actually belong to a different species of *Phytophthora* (Förster *et al.*, 1990a). Isolates originated from geographically diverse locations and continents. The majority were obtained from culture collections, except for C23 and C6, which were isolated using the pear–baiting technique (described in Section 3.1.3). The original identification of these isolates was based on morphology and, in some cases, also by isozyme analysis (Dr. M. Coffey, University of California). An identification code was then allocated to each isolate. Confirmation of the identity of all isolates was made by myself and then by Dr G. Hall (I.M.I.) or Dr J. Walker (B.C.R.I.) using morphological criteria. Some isolates had been previously classified as either A1 or A2 compatibility types.

A major problem associated with the derivation of cultures from other sources was that in some cases the code for a particular isolate may have been changed several times and therefore it was difficult to be certain of the identity of a particular isolate. For example, it was unclear whether two isolates, P3434 and P3367 were originally the same isolate, P717. For this reason the isolates in this study are presented with their original code. Not all isolates were used for all studies as some isolates were obtained at a later date and were used mainly in oospore mating experiments.

Table 3.1 *P. citrophthora* isolates from Citrus hosts used in this study

Isolate	Mating Type	Origin	Date of Isolation	Original Identification	Source	Comments
1. C23	Unknown	Loxton North, South Australia	June 1989	Morphology	Ms C. Son	
2. C6	Unknown	Berri, South Australia	July 1989	Morphology	Ms C. Son	
3. D64660	Unknown	Somersby, New South Wales	March 1989	Morphology	Dr. P. Barkley	
4. P1152	Unknown	Argentina	Unknown	Morphology	Dr. M. Coffey	
5. P1163	Unknown	California	1978	Morphology	Dr. M. Coffey	
6. P3078	Unknown	South Africa	Unknown	Morphology	Dr. M. Coffey	
7. P3382	Unknown	Israel	Unknown	Morphology	Dr. M. Coffey	
8. P6698	Unknown	Taiwan	Unknown	Isozyme analysis	Dr. M. Coffey	
9. P6708	Unknown	Turkey	Unknown	Isozyme analysis	Dr. M. Coffey	
10. P6806	Unknown	Italy	Unknown	Isozyme analysis	Dr. M. Coffey	
11. P6826	Unknown	Greece	Unknown	Isozyme analysis	Dr. M. Coffey	
12. P3693	Unknown	Brazil	Unknown	Isozyme analysis	Dr. M. Coffey	
13. P3582	Unknown	Western Australia	1968	Morphology	Dr. M. Coffey	
14. P3434	A1	New Zealand	1968	Morphology	Dr. M. Coffey	Also coded P717 (Ferguson 1976)
15. P3435	Unknown	New Zealand	1941	Morphology	Dr. M. Coffey	
16. P3418	Unknown	France	Unknown	Morphology	Dr. M. Coffey	
17. P0318	A2	South Australia	Unknown	Morphology	Dr. M. Coffey	Also coded P318 (Ferguson, 1976) (Forster *et al.*, 1990)
18. PAL 2	A1	Portugal	1988	Morphology	Prof. P. Ganhao	
19. PAL 7	A2	Portugal	1988	Morphology	Prof. P. Ganhao	
20. PAL 41	A1	Portugal	1988	Morphology	Prof. P. Ganhao	
21. PAL 45	A2	Portugal	1988	Morphology	Prof. P. Ganhao	
22. PMO 52	A1	Portugal	1988	Morphology	Prof. P. Ganhao	
23. P3367	A1	New Zealand	Unknown	Morphology	Dr. M. Coffey	Also coded P717 (Ferguson, 1976)
24.CBS289	A2	Gomziekte	1935	Morphology	Mrs Snippa	Also coded N222 (Savage *et al.*, 1968)

3.1.2 Culture Media

Approximately 17 ml of media were dispensed into 90mm diameter petri dishes. The pH of the media, prior to autoclaving, was between 6.0 and 6.5, unless otherwise specified. All media were autoclaved at 121°C for 15 min.

1. Corn Meal Agar (CMA)

Corn meal agar (Difco): 17 g per litre distilled water.

2. Lima Bean Agar (LBA)

Lima bean agar (Difco): 23 g per litre distilled water.

3. $P_{10}VP^{+}$ Agar

$P_{10}VP^{+}$ agar was used as a selective medium. 500 ml CMA was autoclaved then cooled to 50°C and the following added: 0.4mg pimaricin (Sigma); 300mg vancomycin hydrochloride (Vancocin, Eli Lily & Co); 33mg pentachloronitrobenzene (PCNB) technical grade (Terrachlor, Agchem); 71.4mg hymexazol (Tachigaren 70% ai, Agchem.) (Tsao and Guy, 1977).

4. Carrot Agar (CA)

Carrot agar was prepared as described in Ribeiro (1978): 200g fresh carrot was blended in 500ml distilled water for 40 seconds at high speed. The supernatant was then filtered

through several layers of cheesecloth and the juice squeezed out from the residue. The volume was adjusted to 1 litre with distilled water and 15g Bitek agar (Difco) was added.

5. Hemp Seed Agar (HSA)

Hemp seed agar was prepared as described by Savage *et al.* (1968): 50g hemp seed was soaked in distilled water overnight. The seed was then autoclaved at 121°C for 15 min in 1 litre of distilled water. The supernatant was strained through cheesecloth, the volume was adjusted to 1 litre and 17g Bitek agar was added.

6. S+L Medium

S+L medium was prepared as described by Ann and Ko (1988). A liquid medium consisting of 1 ml "basal salt solution", 10 mg lecithin (99.8% soy, refined, Natural Products) and 2mg glucose in 100ml distilled water was triturated in an Omni mixer at 6,000 r.p.m. for 1 min and adjusted to pH 7.0 with 1N KOH. The "basal salt solution" contained 100mg $(NH_4)_2SO_4$ (7.6mM); 100 mg $MgSO_4.7H_20$ (4.0mM), 30 mg $CaCl_2.2H_20$ (2.0mM); 3mg $ZnSO_4.7H_20$ (0.1mM); 30mg KH_2PO_4 (2.2mM); and 60mg K_2HPO_4 (3.4mM) in 100ml distilled water. Two grams of Bitek agar were added to 100ml of liquid medium and after autoclaving and cooling to 50°C, antibiotics and fungicides were added aseptically. Antibiotics and fungicides were the same as those used in the selective medium $P_{10}VP^+$ agar above.

7. V8*β* Agar

V8β agar contained 100 ml V–8 juice (Campbell's Soups Pty. Ltd., Aust.), 0.1g $CaCO_3$, 0.02g β–sitosterol (Sigma Chemical Co.) and 17g Bitek agar made up to 1 litre with distilled water.

8. V8*β* Liquid Medium

V8β liquid medium contained 50 ml V–8 juice, 0.1g $CaCO_3$ and 0.02g β–sitosterol made up to 1 litre with distilled water.

3.1.3 Isolation of *P. citrophthora* from Soil

Wicks (1987) found that *Phytophthora* spp. were recovered most frequently at depths between 10 and 25cm. Therefore in this study, four soil samples containing roots were collected at the base of each infected Citrus tree at a depth of 10 – 20cm. The four subsamples from each tree were combined and thoroughly mixed in a sealed plastic bag. Approximately 500g of this combined sample was placed in a plastic container (K16 Food Container, Advanced Products) and mixed with sufficient distilled water to create a layer of water approximately 1cm deep on the surface of the soil. A ripe, green, unblemished Bartlett pear fruit was placed in the centre of each container and pressed 3 – 4cm into the flooded soil. Incubation was at room temperature, approximately 20°C. Controls consisted of pears placed in distilled water without soil. *Phytophthora* infection usually developed within one week. Infected pears displayed firm brown lesions at or below the water line. These pears were swabbed with alcohol and flamed before 0.5 mm pieces were excised from the lesion margin and plated onto CMA and $P_{10}VP^{+}$ agar and

observed for growth of *Phytophthora*. The fungus was then subcultured onto CMA and identified using morphological criteria.

3.1.4 Storage of Isolates

P. citrophthora isolates were first grown on CMA and 0.5mm discs were taken from the actively growing margin. Several discs were placed in 10ml sterile distilled water (SDW) or a single disc was used to inoculate slopes of 10ml CMA + 1% Bitek agar (CMAA) in 1oz MacCartney bottles with the lid loosely tightened to allow exchange of gases (G. Walker, pers. comm.). The SDW cultures were stored in the dark at room temperature. The CMAA cultures were stored in the dark at 4°C. The SDW technique was modified from Castellani (1967). Care was taken to avoid dehydration of stored isolates by checking cultures regularly. After 12 months the CMAA cultures were checked for contamination and subcultured onto fresh CMAA.

3.1.5 Production of Sporangia and Zoospores

1. Small-Scale Method

P. citrophthora isolates were first grown on LBA or CMA for 5 – 7 days at 25°C in the dark. Seven to ten 8 mm discs were cut from the margin of an actively growing colony and placed in a sterile petri dish (90mm in diameter). Discs were flooded with 10 ml SDW and placed under white fluorescent lights for 24 – 48 hr at 25°C. Sporangia had formed by this stage. To induce release of zoospores, plates were placed at 4°C for 20 min, and then at room temperature for 30 min. Zoospore concentration was estimated using a haemocytometer.

2. Large-Scale Method

A method for producing sporangia of *P. cinnamomi* was developed by Chen and Zentmyer (1970) using young cultures washed in a mineral salts solution (as specified below). This technique was modified by Byrt and Grant (1979) for large-scale zoospore production and was further modified in this study for *P. citrophthora* specifically to allow for the production of large quantities of inoculum (between 10^6 and 10^7 zoospores per ml) and to give uniform yields between isolates.

Miracloth mats (Calbiochem R475855, Lot 019591), 60mm diameter, were rinsed with distilled water and autoclaved before being placed on the surface of V8β agar plates. Each miracloth plate was inoculated with seven agar discs (8 mm diameter) taken from 7 – 10 day-old V8β or CMA cultures of *P. citrophthora* and incubated at 25°C for 5 – 7 days in darkness. The miracloth mats were gently lifted off the agar surface and transferred to 250ml Erlenmeyer flasks (two mats per flask) containing 100ml V8b liquid medium. Flasks were shaken on an orbital platform shaker at 160 o.p.m. for 48 hr at 22±1°C. The mats were washed three times for 15 – 30 min with mineral salts solution (10mM $Ca(NO_3)_2.4H_2O$, 5mM KNO_3, 4mM $MgSO_4.7H_2O$, 0.1mM $FeSO_4.7H_2O$ and 0.1mM Na_2EDTA) and then suspended in 80ml of this solution and shaken for a further 48 hr at 160 o.p.m. at 22°C, during which time sporangia formed. The shaking speed is important and it is thought that sufficient aeration during this period is critical for the formation of sporangia (Byrt and Grant, 1979). Cultures were taken off the shaker and

allowed to stand at room temperature for between 15 – 30 min. Zoospores were released spontaneously without a cold shock or any further changes in solution. Zoospore concentrations were determined as before.

3.1.6 Oospore Production

Several methods for producing oospores in culture were tested. These were as follows:

1. CA Sandwich Method (Based on Kellam and Zentmyer, 1986a)

Isolates of known and unknown compatibility types were grown on CA (see 3.1.2) for 5 days. Discs, 8mm in diameter, were cut from colony margins and a stack of three agar discs, each 3mm thick, was made by sandwiching an 8mm–diameter disc of uninoculated CA between two 8mm–diameter discs that contained different isolates to be paired. In controls to test for homothallic oospore formation, each isolate was paired with itself. Four stacks of three discs were placed in a 90 mm–diameter petri dish. Each plate was sealed with parafilm. Plates were placed at 24°C for 24 hr in the dark and then incubated at 21°C in the dark and examined for oospores after 28 and 56 days.

2. HSA Method (Based on Ferguson, 1976)

Discs of HSA (see 3.1.2) 5mm in diameter from colony margins of each of two isolates were placed on the surface of a plate of HSA, 45mm apart. Isolates were also screened for homothallic oospore formation by placing a single 5mm plug in the centre of a HSA plate. Plates were incubated at 20°C in the dark and then observed for the presence of oospores at 28 and 56 days.

3. Tissue Culture Method

Twelve rooted and twelve non-rooted shoots of Symons Sweet Orange were transferred individually to 30ml Polycarbonate Universal Containers (Disposable Products) containing 10 ml of 0.5% water agar. This technique was restricted to examination of one mating combination (C6 x P3382). At the base of the shoots two 5mm carrot agar discs of inoculum were placed. In the single infections, which were to serve as controls for homothallic oospore production, both discs contained either C6 or P3382. In the mixed infections, one disc contained C6 and the other disc contained P3382. Inoculated shoots were placed at 24°C for 24 hr in the dark and then incubated at 21°C in the dark and observed for the presence of oospores at 56 days.

3.1.7 Oospore Germination

This technique was developed by Ann and Ko (1988). An oospore suspension was made by comminuting three CA sandwich blocks (8mm x 9mm, described in Section 3.1.6) containing oospores, with 15ml SDW in an Omni mixer at 4,500 r.p.m. for 1 min. The suspension was filtered successively through 53μm and 10μm nylon mesh (the nylon mesh was disinfected before use by washing in 50% White King domestic bleach {0.4% available sodium hypochlorite) for 20 min followed by three SDW rinses, each of 5 min duration). Oospores retained on the 20μm sieve were washed with SDW.

The oospore suspension was then mixed with an equal volume of aqueous 0.5% $KMnO_4$ to prevent the germination of residual mycelial fragments, chlamydospores and sporangia

present in the oospore suspension. After agitating the mixture for 15 min, the oospores were rinsed with SDW on a 20 μm sieve.

After the final rinse, the oospore suspension (in a total volume of 1 ml SDW) was placed in an Eppendorf tube and the oospore concentration estimated with a haemocytometer. The concentration of oospores was low (< 1 x 10^4 per ml) and therefore samples were not diluted any further. Oospores were plated onto S+L medium (see Section 3.1.2) and incubated at 20 – 21°C under the light regimes specified in the experimental procedure (Chapter 6). Twenty–five 1 cm^2 grid squares placed on the underside of the petri dish were used to assist in locating the oospores. 5μl of an oospore suspension was placed over each grid square.

3.1.8 Staining of Nuclei in Zoospores

Zoospores were obtained using the agar disc method outlined in Section 3.1.5. Zoospores were stained with Dapi (4,6 Diamidino–2–phenylindole, Sigma D1388) at either 0.1 or 0.2μg/ml in TE buffer (10mM Tris–HCl, 1 mM EDTA, pH 8.0). The latter concentration was used for encysted zoospores. Zoospores (10^4 per ml) were first fixed in GF (4% paraformaldehyde in 200 mM Pipes buffer {piperazine–N,N'–bis(2–ethane sulfonic acid) pH 7.2; Sigma}+ 0.4% glutaraldehyde). After 30 min, Dapi stain was added. Stained zoospores were examined under a Zeiss Axioplan fluorescence microscope. The filter set used for Dapi fluorescence was Zeiss 487901 (BP 365, FT 395, LP 397).

3.1.9 Isolation of Single Zoospores

Zoospores were produced according to the method described in Section 3.1.5. The spores were isolated using a technique developed for isolation of resting spores of *Plasmodiosphora brassicae* (Jones *et al.*, 1982, modified by Scott, 1985).

Approximately 10ml of 0.5% gelrite in distilled water was poured into the inverted lids of sterile 90mm petri dishes and allowed to set. Gelrite is a transparent "agar substitute" used mainly in tissue culture media (see Section 3.3.2). A zoospore suspension was filtered through sterile Kimwipe tissues (Kimberley Clark) lining a small plastic funnel inserted into a 10ml sterile plastic Eppendorf tube. The zoospores were collected and the concentration estimated with a haemocytometer and, where necessary, adjusted to 10^3 per ml. 100 μl of zoospore suspension was pipetted directly onto the gelrite and evenly distributed over the surface with a sterile glass spreader. Single, well separated zoospores were located by scanning with a microscope at 200x magnification. A spore punch, consisting of a 10μl capillary tube mounted in a dummy microscope objective (Keyworth, 1959) was used to mark out an agar disc (approx. 1mm diameter) bearing a single zoospore. After checking that the disc carried only one spore, it was removed using a sterilised flattened needle and placed in the centre of a CMA plate. Incubation was at 25°C in the dark. From approximately twenty single zoospore-derived progeny per parent isolate, three were randomly selected for further studies. Abnormally large spores were avoided as these were more likely to be binucleate (Chapter 4).

3.2 GENERAL TECHNIQUES AND CONDITIONS FOR GROWING CITRUS

3.2.1 Citrus Rootstocks

The Citrus rootstock cultivars chosen for this study are commonly found in South Australia because of their suitability to the saline soil conditions (G. Walker, pers comm.). These rootstocks had been assessed in both glasshouse and field trials and their resistance or susceptibility to *P. citrophthora* infection was known. They were: Carrizo Citrange (*C. sinensis* x *P. trifoliata*, moderately resistant to *Phytophthora*; Broadbent and Gollnow, 1992); McKillops Rough Lemon (*C. jambhiri*, highly susceptible; Broadbent and Gollnow, 1992); and Symons Sweet Orange (*C. sinensis*, highly susceptible; Broadbent and Gollnow, 1992). In addition, 28/25 (*C. reticulata* x English *P. trifoliata*, F1; Acc. No. 65–260–35) and 1/31 (*C. reticulata* x Swingle *P. trifoliata* Acc. No. 56–43–13) were tested in tissue culture studies (Chapter 6). Both cultivars had been classified as being moderately resistant to *Phytophthora* (Barkley, pers. comm.; Gallasch and Dalton, 1989). It is important to note that not all Rough Lemons are the same in terms of susceptibility to *Phytophthora*, i.e. McKillops Rough Lemon is more susceptible than Watkins Rough Lemon and similarly not all Trifoliate hybrids have the same resistance (P. Barkley, pers. comm.).

3.2.2 Germination of Citrus Seeds

A simple and quick technique for germination of Citrus seeds, modified from Sykes (1985) which gave uniform germination rates across cultivars (data not shown) was used. Seeds of uniform size were selected, washed to remove any fungicide and soaked in distilled water for at least 2 hr, but not longer than 16 hr. The seeds were put on paper

towelling to remove exudates before being surface sterilised by soaking in 1 or 2% NaOCl for 10 min and rinsed three times with SDW. The outer seed coat was then removed aseptically and 20 seeds were placed in a sterile petri dish (90mm diameter) lined with sterile filter paper (Whatmans 1MM) soaked in SDW. Seeds germinated only when part of the seed coat was removed. Seeds were placed in a 28°C incubator in the dark. After 8 days, germinated seeds were transferred to trays (Rite Gro Kwikpots, Greens Horticulture) and covered with steam–sterilised recycled soil (see Section 3.2.3) at a depth of 2cm. The trays were covered with clear polyethylene film and watered daily with reverse–osmosis (RO) water to keep the soil moist until shoots were produced. Trays were placed in the growth cabinet (Section 3.2.4). Once the shoots had emerged, the polyethylene film was removed and the plants watered as required. When the shoots were 3 – 4cm in height, plants were transferred to black 3" diameter pots containing steam–sterilised recycled soil. Uniform seedlings 10cm in height were used for all experiments. At this stage they were between 2 and 3 months of age.

3.2.3 Potting Media

Preliminary experiments indicated that both resistant and susceptible Citrus cultivars grew best in recycled soil and there was less variability in growth rates between different cultivars compared to other soil types (data not presented). The recipe for recycled soil was as follows: 0.5m^3 of used and composted experimental soil (composted for up to 2 years) was mixed with 0.1m^3 of "Detorf" peatmoss with the following nutrients added: 500g bloodmeal; 200g potassium sulphate; 100g super phosphate; and 200g ground limestone. The soil was sterilised at 75°C for 45 min using aerated steam. In later

experiments the recycled soil was modified by adding 50% peat, because its clay texture resulted in waterlogging problems which confounded results (see Chapter 5). The soil pH was 6.0.

3.2.4 Growing Conditions

Plants were provided with a 16 hr day (65/80 watt white fluorescent lights, Phillips) in a growth cabinet. The temperature was maintained at 18°C (night) and 25°C (day). Plants were watered from the top every second day with RO water and allowed to drain, avoiding problems with waterlogging (Tsao and Garber, 1960). They were supplied weekly with a half-strength Hoaglands solution made-up in RO water, based on the recipe by Hoagland and Arnon (1938) which contained the following in mg per litre: 68.0 KH_2PO_4 (0.5mM); 118.08 $Ca(NO_3)_2.4H_20$ (0.5mM); 50.55 KNO_3 (0.5mM); 123.24 $MgSO_4.7H_20$ (0.5mM); 17.25 FeNaEDTA (47μM); 0.9 $MnCl_2.4H_20$ (4.55μM); 1.43 H_2BO_3 (23.15μM); 0.04 $CuSO_4.5H_20$.(0.16μM); 0.11 $ZnSO_4.7H_20$ (0.39μM); 0.014 $Na_2MoO_4.2H_20$ (0.06μM); and 0.027 $CoSO_4.6H_20$ (0.1μM).

3.3 TISSUE CULTURE TECHNIQUES

3.3.1 Initiation of Citrus Cultures

Young shoots were excised and the leaves and thorns carefully trimmed back before being surface sterilised in 50% domestic bleach (White King, 0.4% available sodium hypochlorite) containing 0.001% of the emulsifier Tween 20^R. The shoots were immersed in this solution for 15 min and gently agitated at intervals before being rinsed three times in SDW. The shoot segments were then cut into single node segments of 1.0

– 1.5cm in length. Initially, shoots were immersed in 80% alcohol for 30 sec before the surface–sterilisation process but this was later discontinued because it was found to damage the explants. Fungal and bacterial contamination could usually be detected within 2 weeks of culture initiation. Contaminated cultures were discarded. Contamination occurred in 10 – 20% of excised shoots surface sterilised using this method.

3.3.2 Multiplication Media for Shoot Production

Nodal segments were placed in 30ml Polycarbonate Universal Containers (Disposable Products) containing 10ml of culture medium. The basal medium contained the mineral salts and vitamins described by Murashige and Skoog (MS medium, 1962, see Table 3.2 for details), 3% sucrose and was adjusted to pH 5.7 with 0.1N NaOH, solidified with 0.6% Bitek agar and autoclaved at 121°C for 15 min. After 2 weeks uncontaminated nodal segments were transferred to MS medium supplemented with $2mgl^{-1}$ benzyladenine (BA). After a period of 1 – 2 weeks, shoots were produced at the nodes. These were excised and five shoots were placed in a 250ml Polycarbonate Specimen Container (Disposable Products) again containing MS medium (50ml) at full strength, supplemented with $2mgl^{-1}$ BA but with a combination of .18% gelrite (Merck) and .08% Bitek agar. This gave a clear medium which permitted detection of contaminants. Bitek agar was added to overcome vitrification problems which arose when using gelrite only.

TABLE 3.2 **MS salts and vitamins (Murashige and Skoog, 1962).**

	Final concentration ($mg L^{-1}$)
MACRONUTRIENTS	
$NH4NO_3$	1650
KNO_3	1900
$CaCl_2.2H_2O$	440
$MgSO_4.7H_2O$	370
KH_2PO_4	170
MICRONUTRIENTS	
$MnSO_4.H_2O$	22.3
$ZnSO_4.7H_2O$	8.6
H_3BO_3	6.2
KI	0.83
$NaMoO_4.2H_2O$	0.25
$CoCl_2.6H_2O$	0.025
$CuSO_4.5H_2O$	0.025
FE SOURCE	
$FeSO_4.7H_2O$	27.8
Na_2.EDTA	37.3
VITAMINS	
Myo–inositol	100
Glycine	2.0
Nicotinic acid	0.5
Pyridoxine–HCl	0.5
Thiamine–HCl	1.0

3.3.3 Rooting Medium

Both Symons Sweet Orange and Carrizo Citrange shoots were excised from shoot clusters on multiplication media and transferred to MS basal medium to allow elongation of the shoots. Shoots were then transferred to MS medium containing $5mgl^{-1}$ α–naphthaleneacetic acid (NAA) for 28 days. Rooted shoots were transferred to basal MS medium.

3.3.4 Tissue Culture Conditions

All cultures were maintained at 25 ± 2°C, under cool white fluorescent lights (approx. 35 μEs^{-1} m^{-2}), 16 hr day.

3.4 TECHNIQUES FOR INOCULATION OF SEEDLINGS

Note that techniques for inoculation of tissue cultures are discussed in the relevant Section.

3.4.1 Root Inoculation Techniques

1. Root–Dipping Method

Plants were unearthed and washed prior to being placed in beakers with roots immersed in mineral salts solution (see Section 3.1.5) containing zoospores adjusted to the appropriate inoculum levels. Control plants were dipped in mineral salt solution without zoospores. Beakers were wrapped in aluminium foil. Roots were left in the different inoculum suspensions for a period of 24 hr at 25°C. On transplanting to soil, plants were flooded in the following manner: pots were placed in water so that the lower two thirds

of the pots were immersed (20cm in depth), for 24 hr. After flooding, plants were removed and allowed to drain freely. It was decided to use this method of flooding as totally submerging the pots can result in the collar region of young seedling becoming waterlogged. To avoid cross-infection during flooding, replicates for one treatment were placed together in the same tray.

2. Root-Drenching Method

The root drenching method was modified from Reifschneider *et al.* (1986). Citrus plants were watered to field capacity prior to inoculation. Roots were inoculated by adding 20ml of the appropriate zoospore suspension in mineral salts solution (see Section 3.1.5) to soil at the base of the stem. Mineral salt solution without zoospores was added to control plants.

3.4.2 Collar Inoculation Techniques

The collar region of the stem (10 – 15mm above the soil surface for the seedlings used in these studies) was wiped with ethanol and the bark cut longitudinally to reveal the cambium. In between the cambium and bark, a 2.5mm mycelium disc of *P. citrophthora* isolate C23, taken from the actively growing margin of a CMA plate was placed with the fungus facing towards the cambium. The area was carefully bound with Parafilm tape taking care to avoid contamination by soil. To avoid splash contamination, the pots were not flooded and plants were watered from the bottom by placing pots on moistened mats in shallow trays. To avoid desiccation of the agar discs, the area was sprayed

gently with water – this was particularly important in the early stages before the fungus had infected the plants. The Parafilm was removed after 2 weeks.

3.4.3 Incubation Conditions

Inoculated plants were maintained in a growth cabinet as described in Section 3.2.4.

3.4.4 Re-isolation of *P. citrophthora* from Inoculated Plant Material

To check that *P. citrophthora* was the causal agent of infection in host plants, 2cm root segments were surface sterilised with 10% White King (0.4% available sodium hypochlorite) for 15 min followed by three rinses with SDW and root segments were plated out on $P_{10}VP^{+}$ selective medium or CMA (see Section 3.1.2) and incubated at 25°C in the dark. The roots were then cleared with 10% KOH, stained with 0.05% trypan blue in lactophenol for 24 hr and examined microscopically for the presence of *P. citrophthora*.

3.5 ISOLATION OF DNA FROM *P. CITROPHTHORA*

3.5.1 Preparation of Fungal Material

Two CMA plugs, 0.5mm in diameter, containing fungal mycelium were used to inoculate 100ml of V8β (see Section 3.1.2) in 250ml Erlenmeyer flasks. After 10 – 14 days standing at 25°C in the dark, the broth cultures were filtered through sterilised miracloth and rinsed three times with SDW. The mycelium was blotted dry with sterilised Whatman No. 3 filter paper.

3.5.2 Large-Scale Isolation of Total DNA

Different methods for extraction of DNA were tested. These included methods described by Murray and Thompson (1980), Panabieres *et al.* (1989) and Guidet *et al.* (1991). The method of Guidet *et al.* (1991) gave the highest yields (145 - 690 μgDNA/g mycelium) and was chosen for subsequent work. The major difference between this and the other methods was the inclusion of an antioxidant in the extraction buffer and the re-extraction of the chloroform/phenol interphase. The method is outlined below.

For large-scale DNA extractions mycelium from up to 10 flasks (approx. 1.7g fresh weight {FW}) was ground to a fine powder in liquid nitrogen and then resuspended in 20 ml extraction buffer (0.1M Tris-HCl, 0.1M NaCl, 0.1M Na_2SO_3, 0.01M EDTA, 1% sarkosyl, pH 8.5) and ground again. This extract then was transferred to 40ml plastic centrifuge tubes and an equal volume of phenol-chloroform-iso-amyl alcohol (25:24:1) was added. The sample was then placed on an end-over-end shaker (20 r.p.m.) for 1 hr at 4°C. After centrifugation for 5 min at 5000g at 4°C (rotor JA10, Beckman) the organic phase was discarded and the aqueous phase was re-extracted as described above with the exception that the samples were shaken for 30 min. After centrifugation, the aqueous phase was transferred to a 250ml plastic centrifuge tube to which 0.1 volume of 3M sodium acetate was added. The sample was mixed well and 2.5 volumes of ethanol (99%) were added and mixed again. The DNA was left to precipitate at room temperature for 30 min and then pelleted by centrifugation at 10,000g for 10 min at 4°C. The DNA pellet was washed twice with 70% ethanol, partially dried in a vacuum centrifuge (Speed Vac Concentrator, Savant) and resuspended in 1ml R40 (40μg/ml

RNAse A {Boehringer Mannheim} in TE buffer {0.01M Tris–HCI pH 8.4, 0.001 EDTA}). The DNA was further purified by centrifugation to equilibrium in caesium chloride–ethidium bromide gradients as described in Section 3.5.4.

3.5.3 Small–Scale Isolation of Total DNA

For single zoospore–derived progeny, where material was limited, small–scale DNA samples were prepared in a manner similar to the above except that only one flask of mycelium (approx FW = 0.35g) was used. Therefore smaller amounts of DNA (approx. 500 – 1000ng) were isolated. The mycelium was blotted dry with Whatman paper No. 3 and placed in 2 ml Eppendorf tubes to which 750μl of extraction buffer (see Section 3.5.2) was added and gently macerated. This was then placed in liquid nitrogen before being macerated again. An equal volume of phenol/chloroform/iso–amyl alcohol (25:24:1) was added and the sample placed on an end–over–end mixer (20 r.p.m.) for 30 min. After centrifuging for 5 min at 12,000 r.p.m. (in an Eppendorf centrifuge) the aqueous phase was transferred to a 1.5ml Eppendorf tube and re–extracted with an equal volume of phenol/chloroform/iso–amyl alcohol (25:24:1) for 5 min, inverting the sample by hand. After centrifugation, the aqueous phase was again transferred to a fresh Eppendorf tube to which was added 0.1 volume of 3M sodium acetate (pH 4.8) and an equal volume of iso–propanol. This was left at room temperature for 10 min to precipitate the nucleic acids and then centrifuged at 12,000 r.p.m. for 15 min. The supernatant was discarded and the pellet washed twice with 1 ml of 70% ethanol (aspirating between washes) and dried in a vacuum centrifuge for 5 min. The DNA pellet was resuspended in 50μl R40 and stored at –20°C.

3.5.4 Purification of DNA using CsCl gradients

The method was performed according to Maniatis *et al.* (1982). DNA was resuspended in 7ml TE buffer. CsCl (7.5g) was added to the DNA suspension and inverted repeatedly to dissolve the salt. Finally 0.5ml of a 10mg/ml solution of ethidium bromide was added. After thorough mixing, the sample was centrifuged to equilibrium at 40,000 r.p.m. for 40 hr. The DNA band was transferred to a 5 ml tube and extracted with water–saturated butanol until the pink colour (of the ethidium bromide) was no longer visible. The DNA was then diluted 50 fold with SDW, precipitated with ethanol and resuspended in 200μl R40.

3.5.5 Purification of DNA fragments using "Geneclean"

DNA fragments were isolated with a "Geneclean " DNA purification kit (Genclean II Bio 101 Product No. 3106, U.S.A.) following manufacturer's instructions. Briefly, the DNA was dissolved in 6M NaI. DNA was then bound to 5μl glass milk and the DNA–glass complexes were pelleted by centrifugation and washed with the solution (NEW WASH) provided. DNA was eluted in TE buffer at 50°C

3.5.6 Measurement of Purity of DNA Samples

The optical densities of 100 fold dilutions of DNA samples were determined using an LKB spectrophotometer. The purity of the DNA was assessed from the absorbance ratios A_{260}/A_{230nm} and A_{260}/A_{280nm} which were between 2.1 – 2.3 and 1.7 – 1.83 respectively, suggesting that they were essentially free of protein and carbohydrate. DNA concentrations were adjusted to give 0.15μg/μl TE. The DNA concentration was

calculated from the A_{260nm} using the information that a 1mg/ml solution of DNA in TE buffer has A_{260nm} = 20.

3.5.7 Restriction Endonuclease Digestion of DNA

DNA samples from parent isolates and single zoospore–derived progeny were digested with *Bam* HI, *Dra* I and *Hin*d III according to manufacturer's instructions. Restriction enzymes were obtained from Boehringer Mannheim and digests were performed in buffers supplied by the manufacturer. With the exception of genomic DNA, which was left to digest for 4 – 5 hr, all digests were performed for 1 –2 hours at 37°C The success of the restriction digestion was tested as described in Section 3.5.6.

3.5.8 Electrophoresis of DNA

Agarose gel electrophoresis was performed as described in Maniatis *et al.* (1982). Electrophoretic analyses of DNA were performed in 1.0% agarose gels using TAE buffer (0.04M Tris–acetate, 0.001M EDTA, pH 8.3) at a constant current of approximately 20 mA. One part of gel–loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 15% Ficoll {type 400}) was added to five parts of DNA sample prior to gel loading. After electrophoresis, gels were stained with ethidium bromide (1 μg/ml) and DNA bands were visualised over a UV transilluminator. Lambda fragments digested with *Hin*d III were used as size markers.

3.6 CONSTRUCTION OF DNA LIBRARY

The methods outlined here were modified from Maniatis *et al.* (1982) and Goodwin *et al.* (1989).

3.6.1 Preparation of *P. citrophthora* and Vector DNA

3μg of genomic DNA of *P. citrophthora* isolate C23 was prepared as described in Section 3.5.1 and digested with *Hin*d III and *Eco* RI. The digested fragments were then precipitated with sodium acetate and ethanol as described in Section 3.5.2 before being purified using Geneclean and resuspended in TE buffer. Similarly the plasmid, PTZ19R (supplied by Dr. P. Langridge) was digested with *Hin*d III and *Eco* RI and "gene–cleaned".

3.6.2 Ligation

Several different ratios of plasmid DNA to genomic DNA were tested to ascertain the optimal ratio for transformation frequency. A 1:4 ratio was prepared as follows: approx. 0.5μg of plasmid DNA was combined with 2μg of genomic DNA and ligated at room temperature for 2 – 3 hr using 1unit T4 DNA ligase in the recommended buffer, according to manufacturer's instructions. The ligation mix was then used to transform competent cells of *E. coli* (strain DH5α).

3.6.3 Preparation of Competent Cells

The bacterial starter culture (*Escherichia coli* strain DH5α) was grown overnight in 2ml Luria–Bertaini (LB) broth (For 1 litre: 10g Bacto tryptone; 5g yeast extract; 5g NaCl,

pH 7.5, autoclaved at 121°C for 15 min) and incubated at 37°C on an platform shaker at approx. 120 o.p.m. 500μl of this bacterial suspension was added to 50ml of SOB + Mg solution (For 1 litre: 20g Bacto tryptone; 5g yeast extract; 0.6g NaCl; 0.19g KCl; 10mM $MgSO_4.7H_2O$; 10mM $MgCI_2$) and placed on a 37°C shaker/incubator for approximately 2 hr until the OD_{600nm} was between 0.45 and 0.55. The cells were then transferred to 30ml glass corex tubes. From this step onwards, the cells were kept on ice. The cells were centrifuged at 3000 r.p.m. for 10 min at 4°C, the supernatant poured off and the pellet resuspended in 8.5ml cold TFB buffer (10mM MES, pH 6.3, 45mM $MnCl_2.4H_2O$; 100mM RbCI; 10mM $CaCl_2.2H_2O$; 3mM $HaCoCl_2$). After 10 – 15 min the pellet was resuspended and the centrifugation step was repeated but this time only 2ml TFB was added as well as 70μl DMSO (dimethyl sulfoxide). After 5 min, 157μl 1M DTT (dithiothritol) was added and after a further 10 min, 75μl DMSO was added to the tubes. The cells were ready for transformation 5 min later.

3.6.4 Transformation of *E.coli*

20μl of ligated DNA were precipitated with ethanol to remove any contaminants which might inhibit the transformation process then resuspended in 10μl TE. This was added to 420μl fresh competent cells. The cell/DNA mixture was left on ice for 30 min before being heat–shocked in a 42°C water bath for 2 min. To this, 600μl SOC solution (7μl sterile glucose solution {50% w/v} in 1ml SOB+Mg, see Section 3.6.3) was added and the mixture placed on a shaker at 37°C for 45 min. 250μl of this mixture was spread on LB agar + ampicillin (50μg/ml; Sigma) plates and incubated overnight at 37°C. Random colonies were selected and the plasmid DNA was extracted using the method described

in Section 3.6.6. The DNA was digested with *Pvu* II and subjected to electrophoresis on 1% agarose gels to check for the presence of inserts.

3.6.5 Storage of DNA Library

The bacteria were grown in 2ml LB + ampicillin (50μg/ml) overnight on a 37°C shaker/incubator. From this bacterial suspension, a 700μl aliquot was removed and mixed with 300μl sterile (100%) glycerol in 1.5ml Eppendorf tubes. These were snap–frozen for 20 sec in liquid nitrogen before being stored at – 80°C.

3.6.6 Isolation of DNA from Clones

Plasmid DNA was isolated by an alkaline mini–preparation procedure (Maniatis *et al.*, 1982). Bacteria were transferred to a sterile 1.5ml Eppendorf tube and centrifuged at 4000 – 5000 r.p.m. for approx. 3 min at room temperature. The supernatant was decanted and 100μl of Plasmid I Solution (50mM glucose, 25mM Tris–HCI, pH 8.0, 10mM EDTA) was added and the bacterial cells resuspended by vortexing. Cell walls were lysed by adding 10μl of fresh lysozyme solution (30mg/ml) and the bacterial suspension was incubated on ice. After 10 min, 200μl of Plasmid II Solution (0.2N NaOH, 1% SDS) was added and the solution incubated for a further 10 min on ice. Finally, 150μl of 3M sodium acetate (pH 4.8) was added. The solution was placed at –20°C for 10 min and then centrifuged at 12,000 r.p.m. for 15 min. The supernatant, containing the plasmid DNA, was poured into Eppendorf tubes to which an equal volume of phenol/chloroform/iso–amyl alcohol (25:24:1) solution was added and left for 5 min, inverting by hand to mix, before being centrifuged at 12,000 r.p.m. for 5 min. The supernatant was transferred to a clean tube and precipitated with 1ml ethanol (99%).

The supernatant then was snap–frozen for 20 sec in liquid nitrogen and centrifuged at 12,000 r.p.m. for 15 min. The supernatant was decanted and the DNA pellet washed twice with 1 ml of 70% ethanol, aspirating between washes. The pellet was dried in a vacuum centrifuge for 5 min before being resuspended in 20μl R40 and stored at –80°C until ready for use.

3.7 SOUTHERN HYBRIDIZATION PROCEDURES

3.7.1 Preparation of Membranes

This method was first developed by Southern (1975) and is designed to immobilise single stranded DNA fragments on a membrane following electrophoresis. The method reported here uses dextran sulphate in the hybridization solution, which increases the effective concentration of the DNA probe, according to Wahl *et al.* (1979).

Total DNA of *P. citrophthora* isolates was digested with either *Bam* HI, *Dra* I or *Hin*d III and electrophoretically separated on a 1% agarose TAE gel as described in Section 3.5.6. After staining with ethidium bromide the gel was soaked in 200ml denaturing solution (0.5M NaOH, 1.5M NaCl) and shaken on a rocking platform for 30 min. The denaturing solution was decanted and the gel shaken in 200ml neutralising solution (3M NaCl, 0.5M Tris–HCl, pH 7.2) for a further 30 min. On a plastic tray a sponge soaked in 20X SSC (3M NaCl, 0.3M Na citrate, pH 7.0) was placed. Four sheets of Whatman 3MM filter paper also soaked in 20X SSC were placed individually on top of the sponge and then covered with a frame cut to the size of the gel in Parafilm™ laboratory film. Making sure to eliminate any air bubbles trapped in the sponge/filter paper stack, the gel

was placed upside down on the stack within the Parafilm frame. A Hybond–N^+ membrane (Amersham) was carefully placed on the gel, again taking care to exclude air bubbles. Two layers of Whatman 3MM filter paper were soaked in 20X SSC and placed on the Hybond–N^+ membrane. A stack of paper tissues (10cm thick) was placed on top followed by a glass plate and a 300g weight. The gel was left to transfer for a minimum of 4 hr.

On dismantling the stack, the membrane was rinsed briefly with 5X SSC, blotted dry (with Whatman 3MM filter paper) and air–dried overnight at room temperature. The DNA was fixed to the membrane by soaking the membrane for 20 min in 0.4M NaOH. The membrane was placed in neutralising solution for a further 5 min before being rinsed in 5X SSC and blotted dry.

3.7.2 Dot Blots

To each 1.5 ml Eppendorf tube, approx. 1 μg of each DNA sample and 100μl of 0.4M NaOH were added and left for 30 minutes at room temperature. The DNA samples were dotted (using the 'dot blotting' technique of Kafatos *et al.* (1979) and a Schleicher and Schuell 'minifold' dot blot apparatus) onto a membrane (Hybond–N^+) whilst applying suction to the under–side of the membrane. The NaOH was neutralised by placing the membrane on a glass plate and gently flooding the membrane with neutralising solution. The membrane was air–dried then sealed in a plastic bag for the prehybridisation reactions.

3.7.3 Preparation of Labelled DNA Probes

Probes were prepared with an 'oligo priming' labelling kit (Rapid hybridization system – Multiprime RPN1517; Amersham). 12.5μl of the labelling mix was mixed with 3μl of alpha–^{32}P–dCTP (30 microcuries), 5μl boiled template DNA (approx.1 μg), 1μl Klenow enzyme (1 – 2 units) and 12.5μl of buffer and incubated in a 37°C water bath for 90 min. DNA used as probes, other than that prepared above, included as follows; maize ribosomal DNA pMR1 (9 kb *Eco* RI fragment in pBR328) from Dr. P. Langridge; mitochondrial DNA (isolated from *P. citrophthora*) from Dr H. Förster; and PCIT 4 and PCIT 15 genomic DNA (isolated from *P. citrophthora*) from Dr. P. Goodwin. Probes were digested with *Pvu* II, according to manufacturer's instructions. Note that PCIT 4 and 15 were digested with a combination of *Pst* I and *Hin*d III as they had been ligated into *Hin*d III and *Eco* Rl–digested Bluescript and this vector does not contain a sequence which can be cut with *Pvu* II.

To separate unincorporated radionucleotide from the labelled DNA the following method was employed: a mini Sephadex G100 column was packed into a pasteur pipette and equilibrated with TE buffer supplemented with 0.1% SDS and the multiprime reaction mix was passed through the column. 0.5ml fractions were collected and the first peaks of radioactivity were pooled. These were boiled (to denature the DNA) and added to the hybridization mix.

3.7.4 Hybridization Procedures

Membranes (15 x 17cm) were prehybridised in bottles for 4 hr at 65°C with 20ml solution containing 10ml NP (Nanopure) H_20, 6ml 5X HSB (3M NaCl, 0.1M Pipes, 0.02M Na_2EDTA, pH 6.8), 2ml 10x Denhardt's III (2% gelatin, 2ml Ficoll, 2% polyvinyl pyrollidone, 10% sodium dodecyl sulfate, 5% tetrasodium pyrophosphate) and 2ml of freshly boiled carrier DNA (10mg/ml, sonicated and denatured herring sperm DNA, Sigma). Hybridization was performed overnight at 65°C in a hybridization oven (Hybaid) with 10ml of a solution containing 2ml NP H_20, 3ml 5X HSB, 1 ml 10X Denhardt's III and 4ml of dextran sulfate (25% w/v) plus 200μl of freshly boiled carrier DNA and the ^{32}P labelled probe. At the end of the hybridization, four 20–min washes were carried out at 65°C as follows: 1) 2X SSC, 0.1% SDS; 2) 1X SSC, 0.1% SDS; 3) 0.5X SSC, 0.1% SDS and 4) 0.2X SSC, 0.1% SDS.

3.7.5 Autoradiography

The membranes were exposed to X–ray films (Fuji) at – 80°C using intensifying screens. The film was developed in a G 153 X–ray Developer.

3.8 ELECTROPHORETIC KARYOTYPING OF *P. CITROPHTHORA*

3.8.1 Preparation of Samples

Several methods were tested for preparing chromosomal DNA.

1. Spheroplast Preparation

Method 1 involved the preparation of spheroplasts as a preliminary step. *P. citrophthora* cultures were grown as described in Section 3.5.1 and the mycelia collected by filtration,

washed with buffer (1M mannitol, 7mM $MgSO_4$. 0.05M EDTA, pH 8.0; Lucas *et al.*, 1990) and suspended in the same buffer to which was added either Novozym 234 (Novo Laboratories), Zymolyase (Seikagaku Koguo Co. Ltd.), Cellulase (Sigma) or Macerozym (Yakult Co. Ltd.) at a final concentration of 5mg ml^{-1}. After shaking at 100 r.p.m. for 2 – 4 hr at 30°C, the mycelial debris was removed by filtration through glass wool and the spheroplasts were collected by centrifugation (3000g for 5 min) and washed several times with the above buffer. Spheroplasts were resuspended in 200 μl of the buffer mixed with an equal volume of 50°C low melting point agarose (Sea Kem Le) in ET buffer (1 mM Tris–HCl, pH 8.0, 50mM EDTA) the final agarose concentration being 0.5%. The molten mixture was poured into a mould and allowed to set at 4°C for 30 min. The agarose plugs were then removed and incubated in the lysis solution (10mM Tris–HCl, pH 8.0, 500mM EDTA, 1% sarkosyl and 1mg/ml proteinase K (Boehringher, Mannheim) at 53 – 55°C for 24 hr. Treatment with proteinase K and sarkosyl removed the protein and lipids associated with the chromosomal DNA. The integrity of the DNA molecules was maintained by the addition of a chelating agent (EDTA) which helps to protect the DNA from nucleases. Furthermore, the fungal material was embedded in agarose prior to digestion to avoid mechanical shearing during subsequent treatments. The plugs were then rinsed several times with 0.5M EDTA (pH 8.0) over several hours and either used immediately or stored in this buffer at 4°C.

2. Embedded Zoospore Preparation

Method 2 was based on McClusky *et al.* (1990). *P. citrophthora* cultures were grown as described in Section 3.5.1 and zoospores prepared as described in Section 3.1.5. The

zoospores were filtered through 20μm nylon cloth to remove any large mycelial fragments and sporangia. The zoospores were transferred to a 10ml Eppendorf tube and the zoospore concentration was estimated with a haemocytometer. The zoospores were rinsed several times with SDW before being spun down at 3000g for 10 min. The supernatant was poured off and the zoospores were resuspended in 1volume 25mM TRIS, pH 7.5, 1M sorbitol and 25mM EDTA before being placed at 37°C to equilibrate. The zoospore suspension was mixed with an equal volume of ET molten agarose and the method continued as described for Method 1. The final zoospore concentration in the agarose plugs was approx. 2 x 10^7 zoospores/ml.

3. Embedded Mycelium Preparation

Method 3 was based on Guidet and Langridge (1992). *P. citrophthora* cultures were harvested as described in Section 3.5.1. The mycelium was collected by filtration then ground in the presence of liquid nitrogen with a mortar and pestle. The ground mycelium was cast directly into agarose plugs and the method continued as described for Method 1. The grinding action results in damage to the mycelial wall and the membrane which is left intact is amenable to digestion by the combined action of sarkosyl and a proteolytic enzyme (proteinase K) *in situ*.

3.8.2 Digestion of Samples

The aliquots were digested with *Mlu* 1 and *Not* 1 (according to manufacturer's recommendations). The day before digestion, the plugs containing lysed mycelium were soaked in 10ml TE buffer in a Petri dish and stored overnight at 4°C. The next day the

plugs were transferred individually to Eppendorf tubes and incubated in two changes of 4.5ml SDW plus 500μl 10X TE buffer at room temperature on a rocking platform. They were then transferred to tubes containing 20μl of 10X restriction buffer, 5μl of a 10mg/ml acetylated nuclease-free bovine serum albumin (BSA), 50 units of restriction endonuclease and 120μl SDW. They were kept on ice for 30 min in order for the enzyme to penetrate the agarose and were then incubated overnight at 37°C. The reaction was stopped by the addition of 500μl of ET (1mM Tris-HCI pH 8.0, 50mM EDTA). The plugs were either stored at 4°C or used immediately.

3.8.3 Conditions for CHEF Electrophoresis

Chromosomal DNA was resolved using a contour-clamped homogenous electric field (CHEF DR II; Bio-Rad Laboratories, U.S.A.) using horizontal electrodes separated by 30 cm and a pulsewave 760 switcher (Bio-Rad, U.S.A.) which could be programmed to vary switching intervals over a given time period. Agarose gels were cast in 0.5X TBE (Tris-borate-EDTA, Maniatis *et al.*, 1982) on pyrex plates (12.5 x 14cm) and electrophoresed in 0.5X TBE which was cooled to 15°C. The agarose concentrations, field strengths, switching intervals and run times are described in the relevant section (Section 8). After electrophoresis, gels were stained in ethidium bromide (1μg/ml) for 45 min and destained in distilled water for 2 hr.

Yeast chromosomes (*Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* chromosomes, Bio-Rad U.S.A. 170.3605 and 170.3633 respectively) were used as size markers. The chromosomes ranged in size from 245 kb to.5.7 Mbp .

3.9 DNA AMPLIFICATION BY PCR

3.9.1 Primers

Random plant intron splice junction primers (developed for genome mapping in cereals) were used and included: R1 (5'-GTCCATTCAGTCGGTGCT-3', Weining and Langridge, 1991); R2 (5'-TCGCGCTGCCAGAACCCC-3', Langridge, unpublished); E4 (5'-ACGTCCACCTTAAGG-3', Weining and Langridge, 1991);C1 (5'-GACCGTCATTGCTCTCTT-3', Taylor, unpublished); and C2 (5'-GGCCCAATGTTCTTCTCT-3', Taylor, unpublished). These had been synthesised on an Applied Biosystems 381ADNA synthesiser and purified on an OPC cartridge supplied by the manufacturer.

3.9.2 PCR Reaction

The polymerase chain reactions were carried out in a 25μl volume containing 0.2 – 0.5μg/μl of genomic DNA template in TE buffer, 0.2μM of each primer (see 3.9.1), 200 μM each of dATP, dCTP, dGTP and dTTP, 50mM KCl, 10 mM TRIS HCl (pH 8.3), 1.5 mM MgCl2, 0.001% gelatin and 0.2 – 0.5 units of Taq polymerase (Promega). The reaction mixture was overlayed with 60 μl silicon oil to prevent evaporation. The PCR was performed in an Intelligent Heating Block (Hybaid). The first six cycles were programmed at 94°C for 1 min, 40°C for 2 min, and 72°C for 2 min. This was followed by a further 28 cycles of 94°C for 1 min, 58°C for 2 min and 72°C for 2 min. One-fifth of the PCR products were analysed on 3% agarose gels and visualised under U.V. light following ethidium bromide staining. Lambda DNA fragments digested with *Hin*d III were used as size markers.

3.10 STATISTICAL ANALYSIS

Genstat 5 was used for all analyses in this study (Payne, 1987).

"There is no such thing as typical or atypical but merely variability of living things"

(Leonian, 1934).

4. MORPHOLOGICAL VARIATION IN *P. CITROPHTHORA*

4.1 INTRODUCTION

Morphological characters are the main criteria used for classification of species within the genus *Phytophthora*. Waterhouse (1963) segregated the genus into six main groups that were later retained by Newhook *et al.* (1978) in the tabular key. *P. citrophthora* is classified in Group II and is identified by having: 1) thickening of the sporangium apex; 2) caducous sporangia and a short (<5μm) pedicel (although it is now accepted that there are also non-caducous isolates, Ferguson, 1976; Ho, 1981); and 3) amphigynous antheridia. For further classification into species, more characters are needed, as problems have arisen when one "type" isolate in a species has been used to represent the species as a whole.

Most taxonomic keys have included minimum, optimum and maximum temperatures at which growth occurs. In general, the optimum and upper temperature limits cited in the literature have agreed with those first reported by Tucker (1931). *P. citrophthora* is reported to have an optimum of 24 - 27°C, with no growth above 32°C (Ferguson, 1976; Waterhouse, 1963). The inability to grow at 35°C is the character which distinguishes *P. citrophthora* from other *Phytophthora* spp. such as *P. parasitica*. *P. citrophthora* will grow at temperatures below 5°C (Ribeiro, 1978).

The length to breadth ratio has often been used in describing the sporangia of *Phytophthora* species. Ferguson (1976) found that the average length-breadth ratios

for *P. citrophthora* isolates were slightly smaller than those listed in taxonomic keys (e.g. Stamps *et al.*, 1990). Wide variation between isolates of *P. citrophthora* in the shape of the sporangia was also observed. Other morphological characters that were examined included the presence of chlamydospores and hyphal swellings and these again were found to be highly variable between isolates. One of the criticisms of Ferguson's work is that the sporangia compared had been produced under different conditions for different isolates. It is well documented that the breadth of sporangia is affected by external conditions and substrate (Waterhouse *et al.*, 1983). In addition, the isolates were not taken exclusively from Citrus but also from other hosts, including *Theobroma cacao*; therefore, some of the isolates may have belonged to another species (Förster *et al.*, 1990a).

Colony morphology is determined by the growth and branching pattern of the hyphae, but little is known about how hyphal branching is regulated. It is thought that hyphal branching may depend to a certain extent on the culture medium used (Waterhouse *et al.*, 1983). Examples of media which give distinctive patterns at the periphery of a colony are corn meal agar, potato dextrose agar and carrot agar. The main types of colony morphology in *Phytophthora* include; 1) uniformly radiate hyphae; 2) chrysanthemum (narrow petaloid sectors); 3) rose or camellia (broad, round petaloid sectors); 4) stellate (intermediate between radiate and chrysanthemum); and 5) uniform (without any obvious pattern). *P. citrophthora* is characterised by having finely radiate growth on corn meal agar (Ribeiro, 1978).

As evident above, the culture medium affects many characters. Despite recommendations from early work (Rosenbaum, 1917), standardisation of media and growing conditions remains an unfulfilled objective. There are, of course, limits to achieving uniformity because different species within a genus may have different requirements.

There are no details in the literature about the capacity of single zoospore–derived progeny to vary in *P. citrophthora.* Such information is essential before an attempt can be made to evaluate the potential for variation in this fungus. Considerable variation has been found in single zoospore–derived progeny of other *Phytophthora* spp. in terms of colony morphology and growth rates (e.g. Caten and Jinks, 1968) as well as pathogenicity (e.g. Kennedy *et al.*, 1986).

In addition, knowledge of the number of nuclei per zoospore is essential for any study on the mechanisms of variation and in studies on inheritance (dealt with in Chapter 8) genetically pure strains and thus uninucleate single zoospores should be used. There have been some contradictory reports in the literature about the number of nuclei in zoospores of *Phytophthora* spp., and no work has been done on *P. citrophthora* specifically. For example, while some authors indicate that zoospores of *P. infestans* are predominantly uninucleate (e.g. Shepherd and Pratt, 1974) others note that there is a high frequency of multinucleate zoospores (e.g. Clayson, 1958). Again, different cultural conditions used to liberate the zoospores will produce

varying results. Furthermore, differences have been shown to exist between isolates (Caten and Jinks, 1968).

In this study, variation between both parent isolates and single zoospore–derived progeny of *P. citrophthora* was examined for a range of characters, including growth rate at 25°C, sporangium length and breadth, culture pattern on agar media and the number of nuclei per zoospore. Single zoospores were isolated from parent cultures that had a high probability of containing uninucleate zoospores. Growth rates were compared between parent isolates at only one temperature, 25±1°C. This temperature was chosen because it is used as the standard incubation temperature for all the experiments in this project and is within the reported optimum range. Therefore, any variation in growth rates at 25°C observed between isolates may be correlated with other parameters, such as pathogenicity. Parent and single zoospore–derived progeny were compared for both sporangium breadth and length and colony morphology.

4.2 EXPERIMENTAL PROCEDURES

4.2.1 Number of Nuclei in Zoospores

Comparison of the number of nuclei in zoospores from 18 different isolates was based on a randomised block design. Zoospore nuclei were stained using the fluorochrome Dapi as described in Section 3.1.8 and scored as either uninucleate, binucleate, or multinucleate. The experiment was repeated four times. Each time approximately 1000 zoospores were counted per isolate.

A binomial model was fitted to each of the uninucleate, binucleate and multinucleate pooled data sets. For each isolate, the difference between replicated experiments was also tested for significant variation in the proportion of uninucleate, binucleate and multinucleate cells.

4.2.2 Probability of Isolating a Uninucleate Single Zoospore

The aim was to find a effective and non-destructive technique which would identify single zoospores that were uninucleate. Unfortunately, while the Dapi stain was not found to adversely affect the viability of the zoospores, the UV fluorescence which is required to visualise the stain, irrespective of the exposure time, did affect germination (data not shown). Other vital stains were examined (methylene blue and neutral red), but were found to be less effective for staining nuclei and also reduced zoospore viability. Nomarksi optics were unsuitable because it was difficult to distinguish the nuclei due to the granular surface of the zoospores.

In view of the above, it was decided to use only those isolates that statistically were more likely to be uninucleate and to isolate individual zoospores without prestaining the cells. It was also decided to isolate only 3 uninucleate zoospores per parent culture as this would be a statistically valid number of replicates to use to gain some indication of variability within single spore-derived cultures. Therefore to calculate

the probability (p) of selecting a single uninucleate zoospore (r) from a number of zoospores (n) for three replicates, the following formula was used:

$$P(X=r) = \binom{n}{r} p^r (1-p)^{n-r}$$

$$= \frac{n!}{r!(n-r)!} p^r (1-p)^{n-r}$$

where $n! = n(n-1)(n-2)(n-3).....(n-a)...3, 2, 1$

Single zoospores were derived from those isolates which were found to have a high probability of being uninucleate. Single zoospores were isolated using the method described in Section 3.1.9, plated onto CMA and incubated at 25°C.

4.2.3 Assessment of Growth at 25°C

The growth of each isolate was determined using the following method: 18 parent isolates were grown on CMA (see Section 3.1.2) and when the mycelium colony covered approximately 75% of the diameter of the petri dish, 8mm diameter plugs were taken from the edge of the actively growing margin. Each disc was placed in the centre of a CMA plate and the plate sealed with parafilm. Each treatment was replicated three times and placed at 25°C in the dark. Measurements were made on 3, 5 and 7 days after inoculation. The mean colony diameter for each treatment was calculated.

This was a 2-way factorial experiment (growth x time) laid out in a completely randomised design. The data were analysed using analysis of variance on the log transformation of the growth values.

4.2.4 Assessment of Colony Morphology on CMA

For comparisons of colony morphology, 8mm discs were taken from an actively growing margin and placed singly in the centre of Petri dishes (90mm in diameter) containing 17ml of CMA. Three replicate plates per isolate were used and the plates incubated at 25°C in the dark. When the cultures were 14 days old, colony morphology was characterised according to Ribeiro (1978).

For single zoospore–derived cultures, the only exception to the above was that the mycelial disc was replaced with a single zoospore.

4.2.5 Measurement of Sporangial Breadth and Length

Measurements of length and breadth are of little diagnostic value unless standard conditions are used (Ribeiro, 1978). *P. citrophthora* isolates, both parent and single zoospore–derived cultures, were grown on CMA at 25°C. Sporangia were prepared as described in Section 3.1.5. Sporangia were mounted on slides and stained with lacto–phenol cotton blue. Slides were examined at a magnification of 100x using a Leitz Orthoplan compound microscope.

Measurement of sporangia was based on a completely randomised design with 10 sporangia examined per isolate. For each isolate, sporangia from both parent and single zoospore–derived progeny were examined. Length and breadth measurements for each sporangium were taken and the length to breadth ratio calculated. The analysis of variance was used to compare variation between parent isolates and variation between parent and single zoospore–derived progeny.

Plate 4.1 Zoospores of *P. citrophthora* stained with the fluorochrome Dapi.

Zoospores were obtained using the agar disc method (Section 3.1.5). Zoospores were stained with Dapi at 0.1 μg/ml in TE buffer and nuclei examined under a fluorescence microscope epi–illuminated with wavelength 360 nm. The micrographs on the left show the nuclei in bright field and on the right in UV fluorescence. Note the large nucleus and peripheral DNA within the mitochondria **(A)**. Note the large size of the multinucleate zoospore (arrowed) in comparison with the uninucleate zoospores **(B)**. Scale bar represents 5 μm.

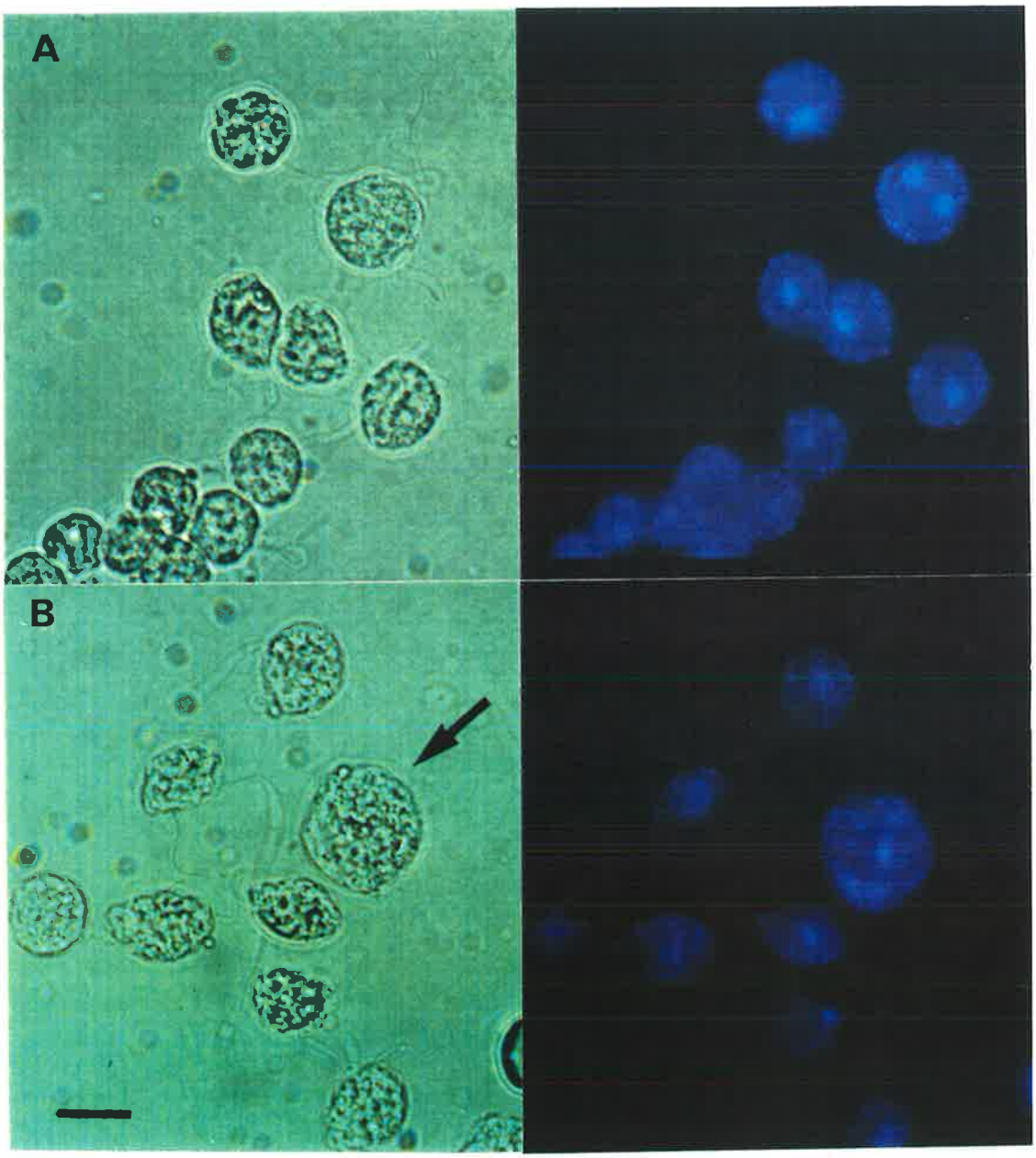

A
B

Table 4.1 Percentage of uninucleate, binucleate, and multinucleate zoospores for *P. citrophthora* isolates.

Zoospores from 18 isolates were examined, as described in Section 4.2.1. The number of uninucleate (1), binucleate (2), and multinucleate (>2) zoospores was expressed as a percentage of the total number of zoospores for that isolate. The data are based on the mean values for a total of four experiments. The overall significant difference between isolates was P<<0.001(not indicated).

	Percentage of Nuclei per Zoospore		
Isolate	**1**	**2**	**>2**
C23	99.76	0.24	0.0
C6	99.81	0.19	0.0
D64660	99.76	0.24	0.0
P3418	99.66	0.34	0.0
P3582	99.91	0.09	0.0
P6826	99.77	0.2	0.03
P3693	99.89	0.11	0.0
P6698	99.81	0.2	0.0
P3382	99.88	0.12	0.0
P3435	98.96	0.85	0.19
P3434	98.98	0.89	0.12
P0318	99.75	0.25	0.0
P6806	99.85	0.15	0.0
P1152	99.95	0.05	0.0
P1163	99.94	0.06	0.0
P3367	99.79	0.21	0.0
P6708	99.86	0.14	0.0
P3078	93.63	3.82	2.5

4.3 RESULTS

4.3.1 Number of Nuclei in Zoospores

Results are presented in Table 4.1 and the mean number of zoospores with one, two or more nuclei expressed as a percentage. In all isolates over 90% of zoospores were uninucleate. Overall there were highly significant differences between the 18 parent isolates examined in the number of nuclei per zoospore ($P<<0.001$). No statistical analysis was made for individual isolates as this may lead to false significant values, however, it is clear that P3078 is the more variable isolate. The stained nuclei within zoospores are shown in Plate 4.1. In this study it was found that larger zoospores tended to be multinucleate.

There was no significant difference between the replicates within isolates, irrespective of treatment or time, with the exception of isolate P3434 where there was a significant difference between the four replicates in the numbers of uninucleate and binucleate cells ($P<0.05$ in both cases). This, however, may be explained by technical problems with the microscope when one of the replicates was examined; if this variant is excluded there is no significant difference between the replicates.

4.3.2 Probability of Isolating a Uninucleate Zoospore

The probability of choosing a uninucleate zoospore was very high particularly when the number of replicates was increased from one to three (i.e. 9.9850×10^{-1}, see Table 4.2). Probabilities were not calculated for those isolates which were found to contain multinucleate zoospores and which also had higher binucleate:uninucleate

Table 4.2. The probability of choosing a uninucleate single zoospore.

The probability of selecting a single uninucleate zoospore from a number of zoospores, for 0 to 3 replicates (X=0, X=1, X=2, X=3), is presented in Section 4.2.2. These probabilities were not calculated for isolates P6826, P3435, P3434, P3078 because they were found to contain multinucleate zoospores and had higher binucleate:uninucleate ratios compared to the other isolates.

Isolate	Probability			
	P(X = 0)	P(X = 1)	P(X = 2)	P(X = 3)
C23	1.3824×10^{-8}	1.7239×10^{-5}	7.1655×10^{-3}	9.9282×10^{-1}
C6	6.8590×10^{-9}	1.0809×10^{-5}	5.6784×10^{-3}	9.9431×10^{-1}
D64660	1.3824×10^{-8}	1.7239×10^{-5}	7.1655×10^{-3}	9.9282×10^{-1}
P3418	3.9304×10^{-8}	3.4562×10^{-5}	1.0131×10^{-2}	9.8983×10^{-1}
P3582	7.29×10^{-10}	2.4278×10^{-6}	2.6951×10^{-3}	9.9730×10^{-1}
P3693	1.3310×10^{-9}	3.6260×10^{-6}	3.2927×10^{-3}	9.9670×10^{-1}
P6698	6.859×10^{-3}	1.0809×10^{-5}	5.6784×10^{-3}	9.9431×10^{-1}
P3382	1.7280×10^{-9}	4.3148×10^{-6}	3.5914×10^{-3}	9.9640×10^{-1}
P318	1.5625×10^{-8}	1.8703×10^{-5}	7.4625×10^{-3}	9.9252×10^{-1}
P6806	3.375×10^{-9}	6.7399×10^{-6}	4.4865×10^{-3}	9.9551×10^{-1}
P1152	1.25×10^{-10}	7.4963×10^{-7}	1.4985×10^{-3}	9.9850×10^{-1}
P1163	2.16×10^{-10}	1.0794×10^{-6}	1.7978×10^{-3}	9.9820×10^{-1}
P3367	9.261×10^{-9}	1.3202×10^{-5}	6.2736×10^{-3}	9.9371×10^{-1}
P6708	2.744×10^{-9}	5.8718×10^{-6}	4.1882×10^{-3}	9.9581×10^{-1}

Table 4.3 Comparison of parent with single zoospore-derived cultures of *P. citrophthora*.

Both parent isolates and single zoospore-derived progeny were grown on CMA at 25°C in the dark. There were three replicate plates per culture and the plates compared when the cultures were approximately 14 days old. Note that the term 'sectorial' refers to a non-uniform pattern of growth.

Isolate	% Zoospores Forming Colonies	Similar To Parent	Comments
C6	64	Yes	
C23	41	Yes	
D64660	69	No	very fine sectorial growth
P3582	65	No	sectorial though not as fine as D64660
P3693	42	No	variation within this isolate ie.some like the parent, others more fluffy and petaloid
P6698	80	Yes	
P3382	58	Yes	similar but more petaloid
P1163	55	No	cobweb-like aerial mycelium very fine sectorial growth
P1152	85	No	cobweb-like aerial mycelium fine sectorial growth
P6708	60	Yes	very similar to parent but with larger "petals"

Table 4.4. Average linear growth (mm) of *P. citrophthora* isolates at different time intervals.

For each isolate an 8mm diameter disc was taken from the edge of the actively growing margin and placed in the centre of a CMA plate. The fungus was grown at 25°C in the dark. Measurements were made on Days 3, 5 and 7. The diameters of the colonies of each of the three replicates were averaged and means are presented. a and b indicate where there are significant differences between isolates (within columns) at the $LSD_{0.05}$ level.

	Growth (mm)		
Isolate	**Day 3**	**Day 5**	**Day 7**
C6	30.0 [a]	56.7[a]	80.0
C23	38.2 [b]	70.5[b]	80.0
D64660	28.2 [a]	55.5[a]	80.0
P0318	40.3 [b]	71.2[b]	80.0
P3367	33.5	60.5[a]	80.0
P3435	34.2	64.0[a]	80.0
P1152	35.9	64.8[ab]	80.0
P1163	37.3	69.0[b]	80.0
P3078	29.0 [a]	55.3[a]	75.3
P3382	39.8 [b]	71.8[b]	80.0
P6698	33.7	57.3[a]	80.0
P6708	38.5 [b]	70.3[b]	80.0
P6806	37.7 [b]	69.8[b]	80.0
P6826	34.2	61.3[a]	80.0
P3693	36.2	69.3[b]	80.0
P3582	39.5 [b]	73.8[b]	80.0
P3434	31.0 [a]	60.0[a]	80.0
P3418	39.0 [b]	74.5[b]	80.0

ratios (P6826, P3435, P3434, P3078) as single zoospore cultures were not derived from these isolates. In this study the proportion of single zoospores that developed into colonies varied from 41 to 85% depending upon the particular parent isolate from which they were derived (Table 4.3).

4.3.3 Growth at 25°C

Mean growth for all isolates is presented in Table 4.4. Significant differences were observed in growth between isolates at a particular time point (LSD = 9.21). The experiment was repeated several times with consistent results. However, only one of these experiments was analysed, as a comparison between experiments was not recommended (L. Giles, pers comm.) due to possible confounding effects (e.g. differences in time, environmental conditions).

4.3.4 Colony Morphology on CMA

The overall appearance of the fungal colony on cornmeal agar for all parent isolates was finely radiate. However, there was variation within the character of "finely radiate" observed between isolates which distinguished them into four groups (see Table 4.5): Group 1 had a petaloid pattern of growth; Group 2 was more radiate; Group 3 showed characteristically finely radiate growth; and Group 4 showed a distinctive fluffy mycelium, quite atypical of *P. citrophthora*.

Table 4.5 Grouping of *P. citrophthora* isolates according to colony morphology.

For each isolate, 8 mm discs were taken from an actively growing margin and placed singly in the centre of a CMA plate. There were three replicate plates per isolate. Plates were incubated at 25°C in the dark and assessed after 14 days. The 18 isolates fell into 4 groups according to colony morphology : Group 1 = petaloid growth; Group 2 = radiate, slightly petaloid growth; Group 3 = finely radiate growth; Group 4 = radiate with fluffy aerial mycelium. Descriptions based on Ribeiro (1978).

GROUP			
1	**2**	**3**	**4**
P6708	P1152	C6	C23
P6806	P6698	P3435	P3582
P6826	P3434	P3693	
P3382	D64660		
P1163	P0318		
	P3367		
	P3418		
	P3078		

The single zoospore–derived progeny differed from their parents mainly in colony morphology (Table 4.3) as illustrated in Plate 4.2. All colonies from single zoospores were similar to each other irrespective of the parents. The variation observed between parent isolates and single zoospore–derived progeny could be related to differences in the morphology of the hyphae. The hyphae of single zoospore–derived progeny were much branched and had a knobby appearance. This contrasted with the smooth, seldom branched, thin (5 – 7 μm) hyphae of the parent isolates (Plate 4.3). When the single zoospore–derived progeny were further subcultured, this pattern of hyphal growth disappeared and the colony morphology reverted back to that of the parents.

4.3.5 Measurement of Sporangial Breadth and Length

Significant differences between the parent isolates for both breadth and length were found ($P<0.001$ in both cases, Table 4.6). For example, isolate C23 had the greatest breadth and length compared to the other isolates and, although isolate P1152 had the smallest breadth it had a large mean length measurement. The overall s.e.d. values for parent isolates were 1.32 and 2.90 for breadth and length respectively (LSD = 2.65 and 5.83 respectively).

In general, there were no significant differences for sporangial breadth and length measurements between parent isolates and single zoospore–derived progeny, with the exception of two isolates; there were significant differences within P1152 for both sporangial breadth and length ($P<0.05$) and within P1163 for sporangial length. The length to breadth ratios were determined for all the above isolates and results (Table 4.7) showed similar trends in variation, including significant differences within P1152 and P1163 ($P<0.05$). The overall length to breadth ratio was 1.5:1 (41.52 x 28.94). Plate 4.4 illustrates the range of sizes and shapes of sporangia found in both parent isolates and single zoospore–derived progeny. The principal shapes were rounded, ovoid to obpyriform and occasionally dumbbell shapes. The terminology for describing sporangium shape is taken from Blackwell (1949).

Plate 4.2 Differences in cultural characteristics between parent isolate (A) and single zoospore–derived progeny (B) of *P. citrophthora* isolate D64660.

Single zoospore–derived progeny were prepared according to the method outlined in 4.2.2. All cultures were grown on CMA incubated at 25°C in the dark for 14 days. Note the difference between the radial and uniform growth of the mycelium in the parent isolate **(A)** compared to the non–uniform sectoring observed in the single zoospore–derived progeny **(B)**.

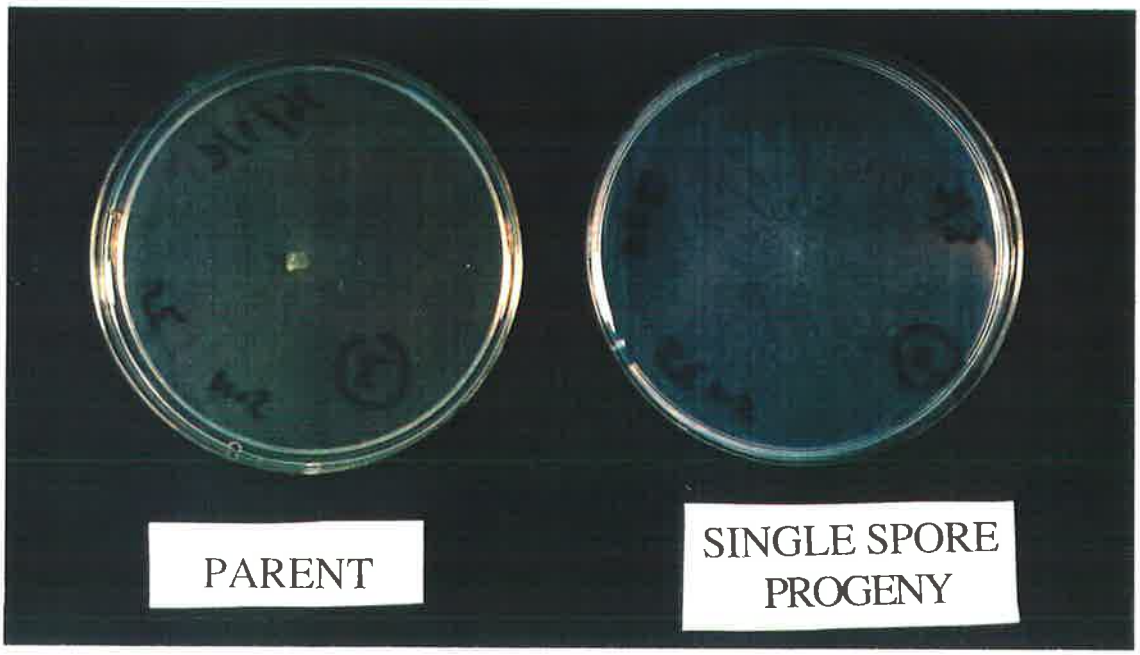
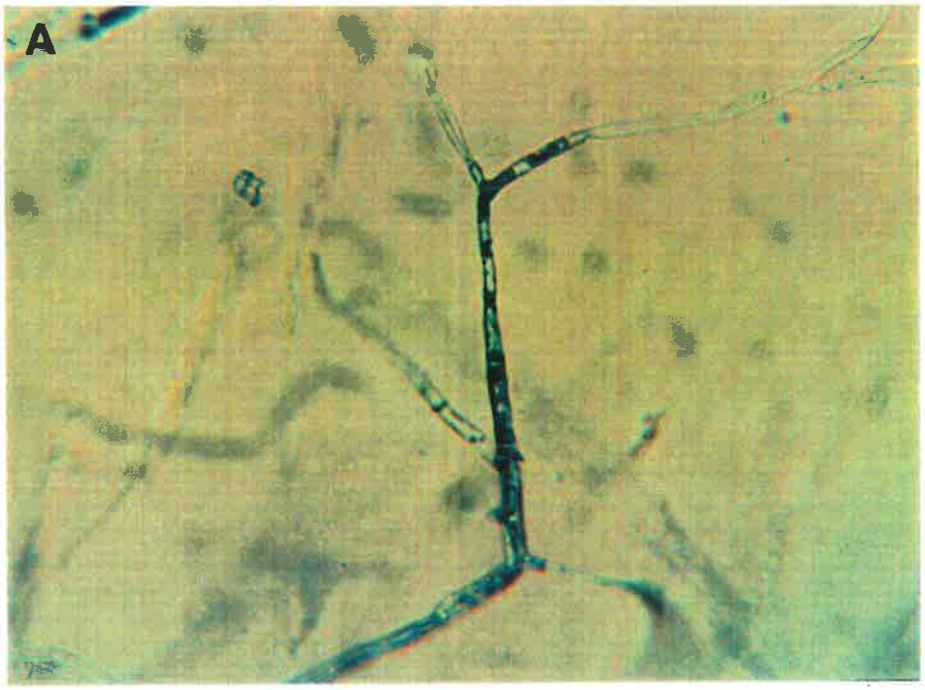

PARENT
SINGLE SPORE
PROGENY

Plate 4.3 Differences in hyphal morphology between parent isolate (A) and single zoospore–derived progeny (B) of *P. citrophthora* isolate D64660.

Single zoospore–derived isolates were prepared according to the method outlined in Section 3.1.9. All isolates were grown on CMA incubated at 25°C in the dark for 14 days. Note the smooth, seldom branched hyphae of the parent isolate **(A)** in contrast to the much branched and knobbly appearance of the hyphae in the single zoospore–derived progeny **(B)**. Scale bar represents 20 μm.


A


B

Table 4.6 Sporangial breadth and length measurements of both parent isolates and single zoospore-derived progeny.

Sporangia were prepared by first growing the fungus on CMA for 5 – 7 days. Discs 8 mm in diameter were removed from the margin of an actively growing colony, placed in a petri dish and flooded with sterile distilled water. The flooded discs were placed under white fluorescent lights for 48 hr, after which time sporangia had formed. A minimum of 10 sporangia per isolate were measured. Sporangial breadth (A) and length (B) were measured under 250x magnification using a Leitz Orthoplan compound microscope. The means are presented. – indicates missing values. SS1, SS2 and SS3 refer to the three single zoospore-derived progeny taken for that particular parent isolate. The s.e.d. values between parent isolates and single zoospore-derived progeny are presented and * indicates where these were significant at the $LSD_{0.05}$ level.

A.	Sporangia Breadth (μm)				
Isolate	**P**	**SS1**	**SS2**	**SS3**	**av. s.e.d.**
C23	31.12^{b}	31.27	30.51	30.09	2.02
C6	26.30^{ab}	24.93	25.56	24.83	2.58
D64660	26.62^{ab}	27.25	26.09	27.46	3.54
P3382	29.35^{ab}	30.21	–	29.63	1.54
P1152	22.72^{a}	23.14	25.06	24.41	1.42*
P1163	29.84^{ab}	29.46	29.46	29.04	1.13
P6708	25.98^{ab}	25.85	27.14	27.14	1.46
P3582	28.75^{ab}	–	–	29.89	1.50
P3693	27.14^{ab}	26.93	26.93	26.30	1.43
P6698	28.19^{ab}	29.25	29.04	27.98	1.60

B.	Sporangia Length (μm)				
Isolate	**P**	**SS1**	**SS2**	**SS3**	**av. s.e.d.**
C23	54.35^{b}	51.74	54.39	52.71	4.63
C6	35.87^{a}	37.03	37.66	37.03	5.27
D64660	34.72^{a}	35.98	36.50	37.66	3.77
P3382	40.61^{ab}	41.03	–	41.38	1.5
P1152	45.55^{ab}	45.24	41.70	39.87	2.36*
P1163	47.15^{ab}	39.87	41.89	47.45	3.12*
P6708	36.82^{a}	35.62	36.61	35.66	1.84
P3582	41.20^{a}	–	–	41.12	3.97
P3693	38.92^{a}	37.87	37.87	37.45	3.0
P6698	37.87^{a}	36.93	37.14	36.93	1.87

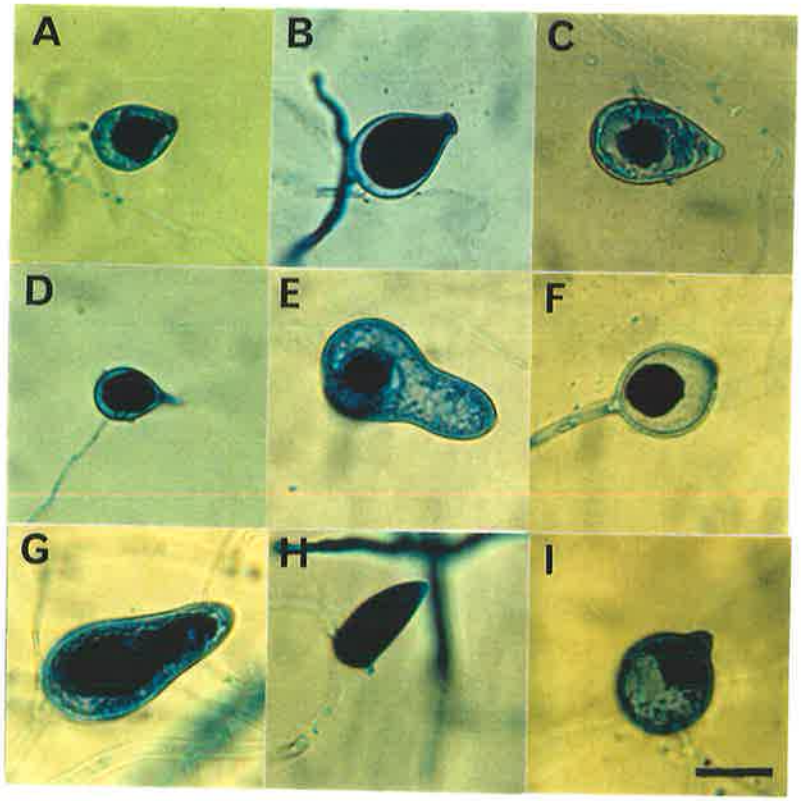
Table 4.7 Sporangial length to breadth ratios for both parent and single zoospore-derived progeny of *P. citrophthora* isolates.

Sporangia were prepared by first growing the fungus on CMA for 5 – 7 days. Discs 8mm in diameter were removed from the margin of an actively growing colony, placed in a petri dish and flooded with sterile distilled water. The flooded discs were placed under white fluorescent lights for 48 hr, after which time sporangia had formed. The sporangial length and breadth measurements were used to calculate the length to breadth ratio. – indicates missing values. SS1, SS2 and SS3 refer to the three single zoospore-derived progeny taken for that particular parent isolate. Differences between parent isolates and single zoospore-derived progeny are presented and * indicates significant differences at the $LSD_{0.05}$ level.

	Length to Breadth Ratios				
Isolate	**P**	**SS1**	**SS2**	**SS3**	**av s.e.d.**
C23	1.76[ab]	1.67	1.78	1.76	0.13
C6	1.37[a]	1.50	1.48	1.49	0.096
D64660	1.33[a]	1.34	1.42	1.39	0.106
P3382	1.41[a]	1.36	–	1.41	0.122
P1152	2.03[b]	2.06	1.68	1.69	0.156*
P1163	1.58[b]	1.36	1.43	1.65	0.115*
P6708	1.42[a]	1.39	1.36	1.33	0.077
P3582	1.43[a]	–	–	1.38	0.108
P3693	1.45[a]	1.41	1.54	1.42	0.126
P6698	1.35[a]	1.29	1.29	1.34	0.098

Plate 4.4 Variation in sporangial size and shape in isolates of *P. citrophthora*.

Sporangia were prepared by first growing the fungus on CMA for 5 – 7 days. Discs 8 mm in diameter were removed from the margin of an actively growing colony and placed in a petri dish flooded with sterile distilled water. The flooded discs were placed under white fluorescent lights for 48 hr, after which time sporangia had formed. The sporangia were stained with lacto–phenol cotton blue and examined under 250x magnification using a Leitz Orthoplan compound microscope. Sporangia shown were taken from the following isolates : **(A)** D64660 p ; **(B)** P3693 p; **(C)** P3382 p; **(D)** D64660 p; **(E)** P1163 p; **(F)** P1163 ss; **(G)** C23 p; **(H)** P1152 p: **(I)** P6698 p. Note that p = parent isolates and ss = single zoospore–derived progeny. Scale bar represents 20 μm.


A
B
C
D
E
F
G
H
I

4.4 DISCUSSION

The development of useful and meaningful taxonomic criteria is dependent on the identification of the morphological features of the fungus which are the most stable, and on a greater knowledge of the variation which exists in these features. Many variants from the original type species described were found among the *P. citrophthora* isolates from Citrus hosts from diverse geographic locations examined in this study. Differences were found in a range of characters including growth rates at 25°C, colony morphology, sporangium length and breadth and the number of nuclei per zoospore.

The wide variation in sporangium size observed in this study suggests that this is not a useful taxonomic criterion. Variation in size and shape of sporangia within other *Phytophthora* spp. has frequently been noted (see Erwin *et al.*, 1963). The length to breadth ratio was also found to be variable between isolates. The length to breadth ratio from this and other work (Ferguson 1976; Ribeiro, 1978) indicates that *P. citrophthora* has a small length to breadth ratio i.e. less than 1.6:1 and not, as originally suggested by Waterhouse (1963), a ratio of 1.6:1. This is in contrast to *Phytophthora* species with a distinctively large length to breadth ratio (greater than 1.6:1), such as *P. hibernalis* (Waterhouse *et al.*, 1983).

Growth is considered to be a good character to distinguish *Phytophthora* species (Waterhouse *et al.*, 1983). In this study, significant variation between isolates in growth was found, but since growth was only examined at one temperature, 25°C, some variation may (at least in part) be a reflection of differing optimal requirements by different isolates. Differences in temperature requirements for optimal growth

between isolates have been accounted for in terms of regional differences. For example, research in both the northern and the southern hemispheres indicates considerable variation in response to temperature in *P. cinnamomi* isolates (Shepherd and Pratt, 1974; Zentmyer *et al.*, 1976). However, results here indicate that the growth differences observed were independent of the geographic origin of the isolates. For example, isolates C6 and C23 were collected only a few kilometres from one another and had significantly different growth rates. This suggests that micro-environmental influences within a specific area could be important in the adaptation of *P. citrophthora* isolates and therefore not only temperature but other factors (e.g. type of medium, humidity, pH) may also affect the growth response. This may be of relevance to pathogenicity studies and should be considered further. A positive correlation was observed between fungal growth and pathogenicity (see Chapter 6).

Colony morphology also varied between isolates but in spite of this variation, the radiate pattern was still apparent for all parent isolates examined, suggesting this may be a more stable character to use for taxonomic purposes than, for example, sporangial size. However, other authors note that because variation is commonly observed among isolates of the same species, colony morphology should not be relied upon exclusively for species determination (Kellam and Zentmyer, 1986a).

In this study, differences in sporangium length and breadth of uninucleate single zoospore-derived progeny were statistically significant for only two of the isolates ($P<0.05$). The main differences observed were in colony morphology of the single

zoospore–derived progeny compared to that of the parents. Caten and Jinks (1968) found that single zoospore–derived colonies of *P. cinnamomi* displayed extensive variation in colony morphology. In contrast, Ann and Ko (1990) found that zoospore progeny of isolates taken from *P. parasitica* were very uniform and similar to their respective parents. In this study, some of the variation in morphology observed could be related to the differences in colony morphology of the hyphae. The hyphae of many of these variants were extensively branched and had a gnarled, knobbly appearance; this observation has been previously reported (Caten and Jinks, 1968).

Most reports indicate that zoospores of various *Phytophthora* spp. are predominantly uninucleate with only a few binucleate (e.g. Shepherd and Pratt, 1974), but others note a multinucleate condition (e.g. Gallegly and Eichenmuller, 1959). The results here suggest that the zoospores of *P. citrophthora* are predominantly uninucleate. The results also support the suggestion that multinucleate zoospores are larger than the uninucleate ones (Shepherd and Pratt, 1974).

Often single zoospores have been isolated assuming that they were all uninucleate. The results in this study indicate that some isolates of *P. citrophthora* may contain a higher percentage of binucleate and/or multinucleate zoospores and thus the probability of randomly selecting a uninucleate zoospore decreases significantly. The single zoospore–derived progeny in this study were only isolated from parents that had a high probability of being uninucleate. In studies examining fungal variation, care should be taken to screen isolates for the number of nuclei per zoospore; this was undertaken by Caten and Jinks (1968). Caten and Jinks could therefore exclude

heterokaryosis as a mechanism accounting for the variation between single spore–derived progeny observed in their study, because the zoospores of the isolate examined were uninucleate. This is of particular relevance to the work reported in Chapter 8, which examines genetic variation using RFLPs, whereby the use of progeny derived from a uninucleate zoospore eliminates any confounding effects due to factors other than inheritance.

It is clear that there are many examples of variation in morphology observed among single zoospore derivatives of parent cultures of different *Phytophthora* spp. but the question remains as to the mechanisms responsible for generating this variation. This study was limited to three single zoospore–derived progeny taken from each of 10 different parent isolates. Other studies have examined hundreds of single zoospore–derived progeny usually taken from only one or two parent isolates (e.g. Caten and Jinks, 1968). In these studies, the variation observed in growth rates and colony morphology, was over successive generations and it was suggested that some underlying genetic control mechanism, either cytoplasmic or nuclear (Caten and Jinks, 1968), was responsible. In this study, however, because the cultures reverted back to the parent state after subculturing, the phenotypic changes observed in colony morphology of single zoospore–derived progeny were probably brought about by the influence of environmental factors.

The following Chapters examine rapid methods for screening Citrus hosts for disease resistance to *P. citrophthora*. In these screening experiments isolates were further examined for variation in pathogenicity.

5. EVALUATION OF RESISTANCE OF CITRUS SEEDLINGS TO *P. CITROPHTHORA*

5.1 INTRODUCTION

Evaluations of Citrus cultivars for resistance to infection by *Phytophthora* were first published in 1923 by Fawcett, in conjunction with the results of his experiments establishing the parasitic nature of *Phytophthora* spp. on Citrus. As mentioned previously in the literature review, there are several *Phytophthora* spp. which attack Citrus but the two most important are *P. citrophthora* and *P. parasitica*.

A number of inoculation methods which enable the evaluation of resistance of Citrus rootstocks to these species of *Phytophthora* have been reported. These include: (a) transfer of seedlings to incubation beds infested with pathogenic isolates of *Phytophthora* with periodic waterlogging (Carpenter and Furr, 1962; Grimm and Hutchison, 1973; Tsao and Garber, 1960); (b) growth of plants in aerated solutions with the addition of *Phytophthora* on pieces of lucerne stems (Broadbent, 1971; Klotz *et al.*, 1958); (c) planting in infested field sites (Klotz *et al.*, 1965; 1967); (d) inoculation of intact root systems of seedlings growing in silica sand with a zoospore suspension (Cameron *et al.*, 1972; Klotz and DeWolfe, 1965) (e) inoculation of wounded stems by using mycelial agar discs (Broadbent, 1971; Klotz *et al.*, 1958; Uppal and Kamat, 1936); or (f) by applying a zoospore suspension into a watertight collar around the stem (Whiteside, 1971; 1974).

Citrus cultivars were assessed for root rot infection either by expressing rotted roots as a percentage of total root length (Blaker and MacDonald, 1986); staining the roots with the vital stain, 2,3,5–triphenyltetrazolium chloride (TTC) (Cameron *et al.*, 1972; Klotz and Wolfe, 1965) or alternatively giving a disease rating of 1 to 5 based on damage to roots (Broadbent, 1971; Grimm and Hutchison, 1973; Sawant *et al.*, 1984). In collar rot experiments, in contrast to root inoculation experiments, lesion length was used as the principal indicator of resistance or susceptibility (e.g. Broadbent, 1971).

The range of reactions of Citrus rootstocks to *Phytophthora* infection varied from highly resistant in Trifoliate Orange, through moderately resistant in Citranges, to susceptible reactions in Rangpur Lime and Sweet Orange rootstocks. However, it has been noted by several authors that there is often a poor correlation between resistance to root rot and collar rot (Broadbent, pers. comm ; Timmer and Menge, 1988). Different selections of a particular rootstock cultivar also varied. It has been found, for example, that cultivars of Rough Lemon differed in their susceptibility to root rot and collar rot (Broadbent, 1971; Hough, in press; Whiteside, 1971).

Environmental factors were also found to influence the course of the disease in the host, particularly where resistance is not complete, as was found with the Citranges (Carpenter and Furr, 1962). Because *P. citrophthora* is a root pathogen, the soil environment is very important and characteristics such as soil temperature, soil oxygen, soil fertility and soil moisture content have all been shown to affect root and collar rot infection either

by affecting the growth of the pathogen or by affecting seedling vigour and root development, as discussed previously (See Chapter 2).

A major problem with many of the screening experiments outlined above is that the seedling material used was between 6 months and 3 years old and so it was a matter of years before some assessments could be completed. This has stemmed partly from the notion that juvenility following seed propagation can modify important characteristics in Citrus, including disease resistance (Prasad and Raghavendra Rao, 1985). However, Broadbent (1969), in her studies with 10cm Citrus (approx 2 – 3 months old) seedlings, has shown that the resistance response is evident quite early.

Therefore, the principal aims of this study were to develop simple and fast *in vivo* screening techniques which would allow the examination of responses of Citrus cultivars to both collar and root rot. For the development of an economically efficient screening technique this would mean that plants should be assessed at the earliest possible opportunity both in terms of the age of the plant and time of harvest at which the difference between resistant and susceptible plants can be detected. In addition, the inoculum level would need to distinguish clearly between resistant and susceptible cultivars and give both predictable and repeatable results. Pilot experiments were conducted to optimise experimental conditions, in particular to identify sources of variation related to the vigour of the host or to soil conditions. Results obtained from growth cabinet experiments were used as a basis for developing tissue culture screening techniques (see Chapter 6).

5.2 EXPERIMENTAL PROCEDURES

Two sets of comparisons, between a resistant and a susceptible cultivar, were made in the following experiments: 1) Carrizo Citrange and McKillops Rough Lemon; and 2) Carrizo Citrange and Symons Sweet Orange, respectively. Citrus seedlings of approximately 10 cm height were used in all experiments. The plants were 2 – 3 months old, depending on growth conditions and the time of year. All experiments were performed in a growth cabinet under the conditions described in Section 3.2.4. The *P. citrophthora* isolate used for all inoculation experiments was C23.

In all experiments where there was a significant interaction, the LSD was calculated at the 0.05 level in addition to analysis of variance (Snedecor and Cochran, 1967). At harvests, randomly selected pieces of either infected stem or root tissue were plated onto selective medium as described in 3.1.2 or examined microscopically to detect the presence of *P. citrophthora* (Plate 5.1B).

For assessment of root rot, a rating system based on Grimm and Hutchison (1973) was used to distinguish symptoms on tap roots from feeder roots, as follows:

Rating	Feeder roots	Tap roots
1	No visible symptoms	No visible symptoms
2	A few roots with symptoms	Symptoms only at tip
3	Majority of root system with symptoms	Diseased from tip to 1/4 length of root
4	All roots infected, cortex sloughed	Diseased more than 1/4 length of root from major roots
5	Roots dead	Tap root dead

The scores for tap roots and feeder roots for harvests 1 and 2 were summed to give a total out of the maximum number obtainable (where 10 plants were measured this number was 50). A binomial model was then fitted to each of the data sets. The data are presented in terms of the mean proportion of assessments and then averaged over the number of replicates.

5.2.1 Experiment 1 : Comparison of Two Root Inoculation Methods

The inoculation methods compared in this experiment, root–dipping and root–drenching, are described in detail in Section 3.4.1. Several inoculum levels including a control were used (0, 10^2 and 10^4 zoospores per ml). A moderately resistant cultivar (Carrizo Citrange) and a highly susceptible cultivar (McKillops Rough Lemon) were compared. The experiment was a completely randomised design and the treatments were laid out as a three–way factorial: 2 root inoculation methods x 3 inoculum levels x 2 Citrus cultivars. There were four replicates per treatment. Plants were harvested on Days 7

and 21. Non-flooded controls were also included for the root-dipping method to check that symptoms observed were due to infection and not to waterlogging. The roots were assessed for infection as described in Section 5.2. Results were analysed as described using analysis of variance with the modification that log transformation was applied to the data from the second harvest.

5.2.2 Experiment 2: Comparison of Different Inoculum Levels Using the Root-Dipping Method

This experiment was an extension of that described for Experiment 1 (Section 5.2.1). Using only the root-dipping method, the aim was to optimise conditions for infection by adding peat to the recycled soil (1:1 w/w) firstly to decrease waterlogging and hence its confounding effect on the controls and secondly to test whether a higher inoculum level (10^5, rather than 10^4 zoospores per ml), would better distinguish resistant from susceptible Citrus cultivars. The experiment was designed as a 2-way factorial which included a control and two inoculum levels (0, 10^4 and 10^5 zoospores per ml) x two Citrus cultivars (Carrizo Citrange and McKillops Rough Lemon). In addition, the number of replicates was increased from four to ten because of the high variability in results found in Experiment 5.2.1. Root assessment was as described in 5.2. Log transformation was applied to the data for plant growth for both harvests and an analysis of variance was then used to test for interaction between cultivar and inoculum.

5.2.3 Experiment 3: Comparison of Carrizo Citrange and Symons Sweet Orange using the Root–Dipping Method

The aim of this experiment was to compare the responses of Carrizo Citrange and Symons Sweet Orange to inoculation using the root dipping technique. The experimental design was randomly arranged as a 2–way factorial, but only included a control plus one inoculum level (0 and 10^4 zoospores per ml) x 2 cultivars. The experiment was analysed as described in Section 5.2 2.

5.2.4 Collar Rot Experiments (Experiments 4 and 5)

The principal aim of these two experiments was to test whether a collar inoculation method (described in Section 3.4.2) could be used on young seedlings to distinguish between resistant and susceptible Citrus cultivars. The only difference between them was that the susceptible cultivars which were compared with the resistant Carrizo Citrange were different in the two experiments (McKillops Rough Lemon in Experiment 4 and Symons Sweet Orange in Experiment 5). The experimental design was completely randomised with the inoculated and control treatments and the two cultivars arranged as a 2–way factorial. The control treatment consisted of agar minus fungus. It was included to assess the effect of wounding on the host plant. For both collar rot experiments there were 20 replicates per treatment.

Lesion length was used as the principal indicator of resistance or susceptibility. Plants were examined for lesion development on Days 14 and 28. On Day 28 the plants were harvested, washed free of soil and separated into roots and shoots. Plant growth

was measured by recording the shoot and root fresh weights and shoot and root dry weights (g). Dry weights were determined after plant material had been oven–dried at 80°C for 24 h . were excluded from the lesion length analysis because they showed no effect of wounding.

5.3 RESULTS

The results of each experiment have been divided into various sections detailing the effects of infection on the different plant growth parameters as well as assessing the extent of root or collar rot. A summary of results has also been included at the end of each section.

5.3.1 Experiment 1: Comparison of Two Root Inoculation Methods

The procedure for this experiment was outlined in Section 5.2.1.

A. Shoot Fresh Weight

At the first harvest (7 days) there were no significant differences in shoot fresh weights for Carrizo Citrange treatments but for McKillops Rough Lemon there was a significant decrease in shoot fresh weight for both inoculum levels (10^2 and 10^4 zoospores per ml) compared with the control (Figure 5.1A LSD = 1.04). There was no significant difference between the two inoculum levels themselves. The interaction between cultivar and inoculum was significant ($P<0.001$). There was no significant difference between root–dipped and root–drenched treatments. In contrast, at Harvest 2 (21 days), for both cultivars, the root–dipped plants had significantly lower shoot fresh weights than those of the root–drenched plants ($P<0.01$) but there was no interaction between cultivar and

inoculum level as seen in Harvest 1. There were large standard errors associated with the shoot fresh weights using the root–drenching method compared with the lower standard errors using the root–dipping method. The controls showed reduced shoot growth at Harvest 2 compared to Harvest 1 due to waterlogging.

B. Root Fresh Weight

Figure 5.1B shows that for both methods at Harvest 1 there was a significant decrease in root fresh weights of the susceptible cultivar, McKillops Rough Lemon, compared with the control, at 10^4 zoospores per ml (LSD = 0.539). This was not observed in Carrizo Citrange. The interaction between cultivar and inoculum was significant ($P<0.05$). There was no significant difference in root fresh weight between 0 and 10^2 or 10^2 and 10^4 zoospores per ml. At Harvest 2, there was no significant interaction but a separate effect of root treatment ($P<0.01$) and inoculum ($P<0.05$); the root–dipping treatment resulted in significantly lower root fresh weights for both cultivars than the root–drenching method and this effect was significantly greater at higher inoculum levels (10^2 versus 10^4 zoospores per ml). The controls showed reduced root growth at Harvest 2 compared to Harvest 1 due to waterlogging.

Figure 5.1 Plant growth measurements comparing two root inoculation methods.

Data taken from Experiment 1. Carrizo Citrange and McKillops Rough Lemon were grown under growth cabinet conditions described in Section 3.2.4. Roots were inoculated with *P. citrophthora* isolate C23 as described in Section 3.4.1. There were two inoculum levels (10^2 and 10^4 zoospores per ml) plus a control (0) and two root inoculation methods (M1 = root–dipping and M2 = root drenching.). Plants were harvested at Day 7 (Harvest 1) and Day 21 (Harvest 2). Values are the means of four replicates. Standard errors of the means are indicated by bars : **(A)** = shoot fresh weight ; and **(B)** = root fresh weight.

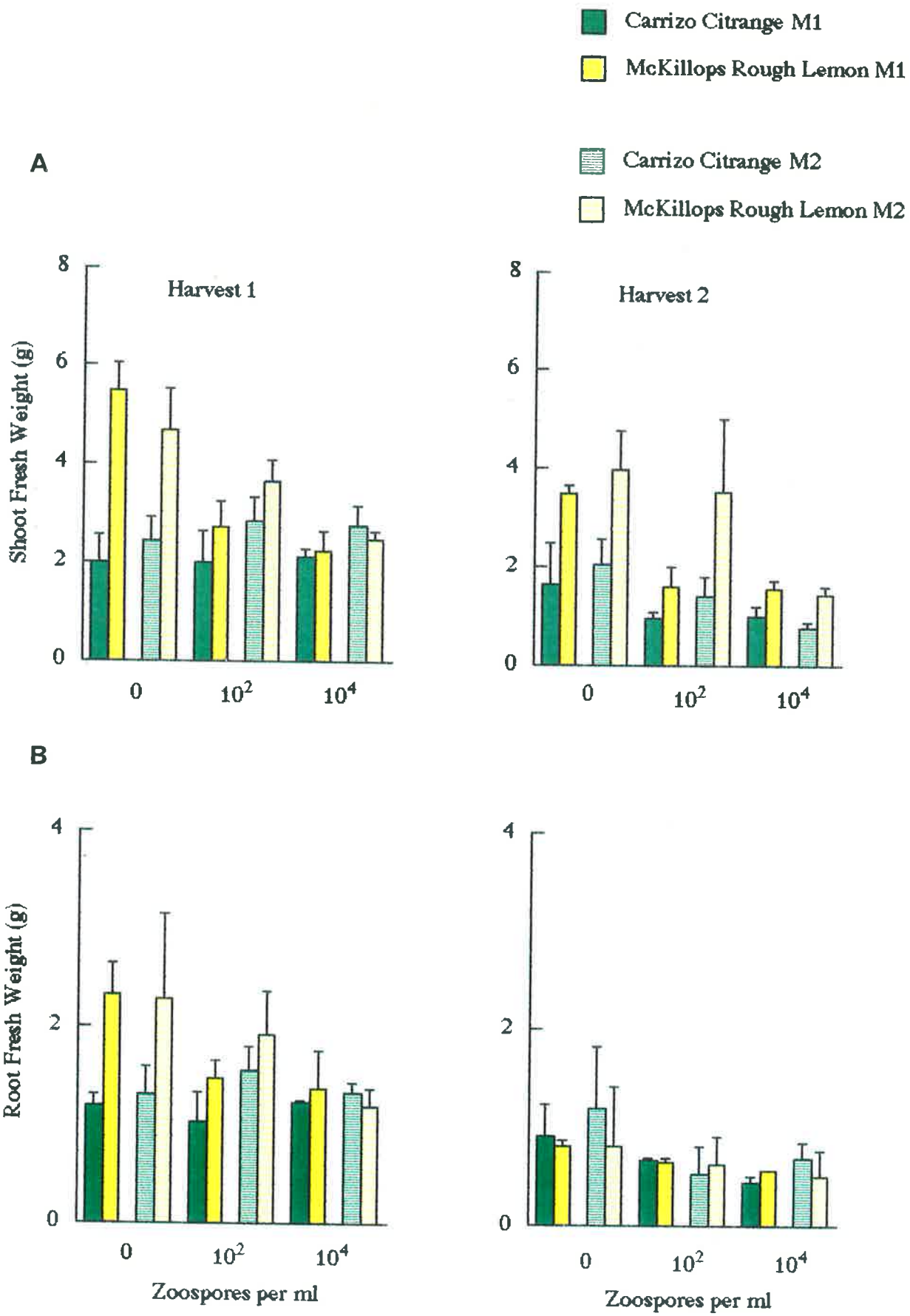

Carrizo Citrange M1
McKillops Rough Lemon M1
Carrizo Citrange M2
McKillops Rough Lemon M2
A
Harvest 1
Harvest 2
Shoot Fresh Weight (g)
8
6
4
2
0
0
10²
10⁴
B
Root Fresh Weight (g)
4
2
0
Zoospores per ml

Table 5.1 Root Rot Assessment: Comparison of two root inoculation methods.

Data for tap and feeder roots for Experiment 1. Two methods of inoculating roots, root-dipping and root-drenching (described in 3.4.1) were used. Plants were inoculated with *P. citrophthora* isolate C23. There were two different inoculum levels (10^2 and 10^4) plus a control (0). There were two harvests, at Day 7 and Day 21. Tap (T) and feeder (F) roots were assessed separately using the rating system described in 5.2. The data are presented in terms of mean proportion of assessments and then averaged over the number of replicates. Mean values for this experiment were taken from four replicates. ± indicates the standard errors. *Significant main effect of root treatment P<0.05 **Significant main effects for inoculum level and root treatment P<0.001.

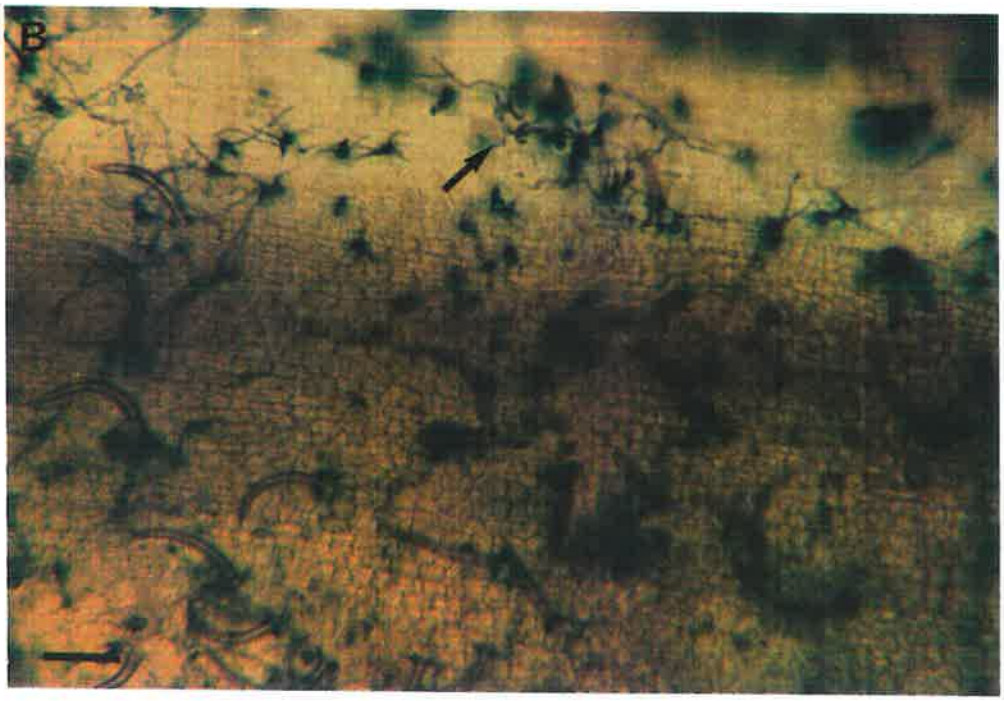
	0**		10^2**		10^4**	
	T	F	T	F	T	F
HARVEST 1						
Root-Drenching*						
Carrizo Citrange	0.33 ±0.07	0.33 ±0.07	0.33 ±0.13	0.40 ±0.12	0.47 ±0.07	0.73 ±0.07
McKillops Rough Lemon	0.27 ±0.07	0.27 ±0.07	0.20 ±0.00	0.30 ±0.10	0.53 ±0.07	0.67 ±0.07
Root-Dipping*						
Carrizo Citrange	0.33 ±0.13	0.40 ±0.12	0.47 ±0.07	0.60 ±0.00	0.60 ±0.12	0.73 ±0.07
McKillops Rough Lemon	0.27 ±0.07	0.33 ±0.07	0.60 ±0.00	0.60 ±0.00	0.80 ±0.00	0.80 ±0.00
HARVEST 2						
Root-Drenching**						
Carrizo Citrange	0.30 ±0.10	0.40 ±0.20	0.53 ±0.07	0.60 ±0.00	0.73 ±0.07	0.80 ±0.00
McKillops Rough Lemon	0.20 ±0.00	0.30 ±0.10	0.40 ±0.12	0.47 ±0.13	0.70 ±0.10	0.90 ±0.10
Root-Dipping**						
Carrizo Citrange	0.30 ±0.30	0.40 ±0.20	0.80 ±0.00	1.00 ±0.00	0.80 ±0.00	0.90 ±0.10
McKillops Rough Lemon	0.30 ±0.10	0.20 ±0.00	0.80 ±0.00	0.93 ±0.07	0.80 ±0.00	1.00 ±0.00

Plate 5.1 Infection of Citrus by *Phytophthora citrophthora*.

(A) Severe infection on tap roots and feeder roots 21 days after dipping the roots of a susceptible Citrus cultivar (McKillops Rough Lemon) in a zoospore suspension (10^4 zoospores per ml) of *P. citrophthora*.

(B) Colonization of stem tissue of a susceptible Citrus cultivar, Symons Sweet Orange, after collar inoculation, revealing the presence of the *P. citrophthora* (arrowed). Scale bar represents 50 μm.


A


B

C. Root Assessment Scores

The results for both tap roots and feeder roots are summarized in Table 5.1: At Harvest 1 there was a difference between the two root inoculation methods in terms of their effects on disease severity; the higher the inoculum level the greater disease severity on the roots ($P<0.01$ and $P<0.001$ for tap roots and feeder roots respectively). There was a significant difference ($P<0.05$) between the root–dipping and the root–drenching methods. Clearly the root–dipping method gave more uniform infection of plant roots.

By Harvest 2 the difference between the two root inoculation methods had increased ($P<0.001$). As in Harvest 1, there was an inoculum effect for both tap roots and feeder roots ($P<0.05$ and $P<0.01$ respectively). The controls in both harvests showed some symptoms of rot on the roots which were presumably attributable to the effects of waterlogging and which may have masked any significant interaction between inoculum level and cultivar. Plate 5.1A illustrates severe rotting symptoms on both tap roots and feeder roots.

D. Summary of Results

The root–drenching method was not as effective as the root–dipping method for causing infection of the cultivars. Statistically, either of the root inoculation methods could distinguish between resistant and susceptible cultivars. However, the root–dipping method was more effective at lower inoculum levels (10^2 zoospores per ml) than the root–drenching method. In addition, the root–drenching method gave more variable

results than the root–dipping method as indicated by the large standard errors associated with both the shoot and fresh weights in Harvest 2 (see Figure 5.1).

Results in this study were confounded by the effects of waterlogging on control plants which reduced shoot FW (Harvest 2 compared to Harvest 1) and caused symptoms of root rot. Therefore, in order to minimize waterlogging, a mixture of recycled soil:peat in a 1:1 ratio (v/v) was used in subsequent experiments. Although the water holding capacity of this recycled soil plus peat is greater, it also enables water to drain more efficiently and therefore reduces the incidence of root rot due to waterlogging due to increased air-filled pore space.

5.3.2 Experiment 2: Comparison of Different Inoculum Levels Using the Root–Dipping Method

The procedure for this experiment was outlined in Section 5.2.2.

A. Shoot Fresh Weight

Figure 5.2A shows that for the cultivar, Carrizo Citrange, there was no significant difference between control (0) and inoculum treatments (10^4 or 10^5 zoospores per ml) on shoot fresh weight at Harvest 1 (Day 7). In contrast McKillops Rough Lemon showed a significant effect of the fungus in reducing shoot FW but there was no significant difference between 10^4 and 10^5 inoculum levels (LSD = 0.15). This interaction between cultivar and inoculum was highly significant ($P<0.001$).

At Harvest 2 (Day 21), the interaction between cultivar and inoculum was again highly significant ($P<0.001$). The same trends were observed as in Harvest 1. However, this

time the fungus did reduce shoot fresh weights in Carrizo Citrange compared to the control, although this difference (LSD = 0.139) was not as great as that observed in McKillops Rough Lemon.

B. Shoot Dry Weight

Data for Harvest 1 (Figure 5.2B) showed that *P. citrophthora* infection at either inoculum level (10^4 or 10^5) reduced shoot dry weights in McKillops Rough Lemon compared to the uninoculated control treatment; this difference was significant (LSD = 0.075). The fungus did not affect the shoot growth in Carrizo Citrange at Harvest 1. The interaction between cultivar and inoculum was significant ($P<0.05$). By the time of the second harvest, this interaction was no longer significant and both cultivars showed reduced shoot dry weights as a result of infection.

C. Root Fresh Weight

The data obtained at Harvest 1 (Figure 5.2C) show that both Carrizo Citrange and McKillops Rough Lemon controls yielded similar root fresh weights. However, there was a significant decrease in root fresh weight for inoculated treatments, although for Carrizo Citrange this effect was not as great as that observed with McKillops Rough Lemon ($P<0.01$ compared to $P<0.001$). This interaction between cultivar and inoculum was significant ($P<0.05$). The LSD value (0.078) also confirmed that the differences between control and inoculum treatments were marginally significant for Carrizo Citrange in contrast to the highly significant difference observed for McKillops Rough Lemon. By the second harvest, the interaction between cultivar and inoculum was no

Figure 5.2 Plant growth measurements of *P. citrophthora*–inoculated and uninoculated Citrus cultivars: Carrizo Citrange and McKillops Rough Lemon.

Data from Experiment 2. Carrizo Citrange and McKillops Rough Lemon were grown under growth cabinet conditions described in 3.2.4. Roots were inoculated with *P. citrophthora* isolate C23 as described in Section 3.4.1. There were two inoculum levels (10^4 and 10^5 zoospores per ml) plus a control (0). Plants were harvested at Day 7 (Harvest 1) and Day 21 (Harvest 2). Values are the means of 10 replicates. Standard errors of the means are indicated by bars : **(A)** = shoot fresh weight ; **(B)**= shoot dry weight; and **(C)** = root fresh weight.

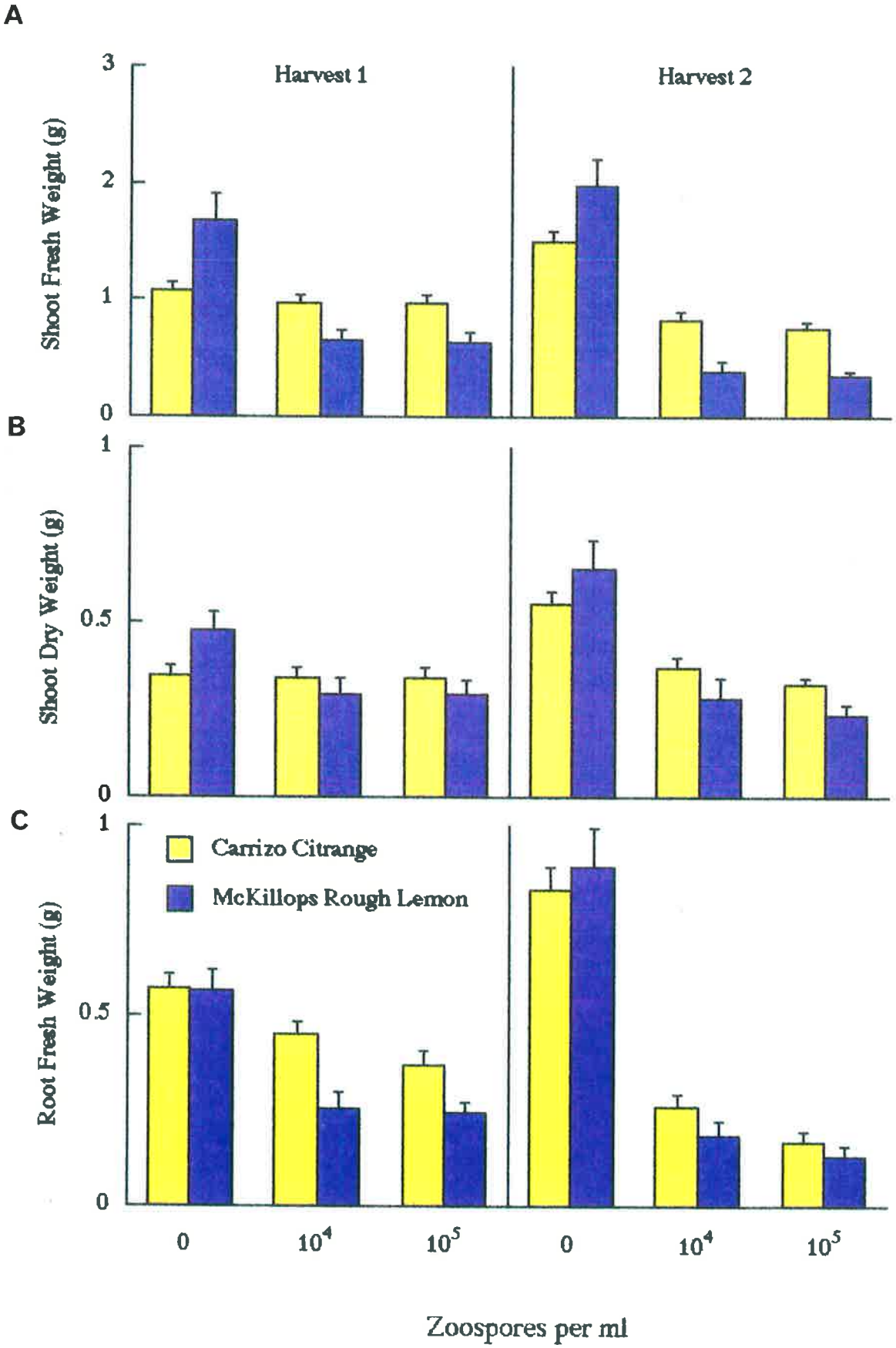

A
B
C
Harvest 1
Harvest 2
Shoot Fresh Weight (g)
Shoot Dry Weight (g)
Root Fresh Weight (g)
Carrizo Citrange
McKillops Rough Lemon
0
0.5
1
2
3
0
10^4
10^5
Zoospores per ml

Table 5.2. Root Rot Assessment: Comparison of *P. citrophthora*-inoculated and uninoculated *Citrus* cultivars, Carrizo Citrange and Rough Lemon (McKillops), using the root-dipping method.

Tap and feeder root data from Experiment 2. There were two harvests, at Day 7 and Day 21. Plants were inoculated with *P. citrophthora* isolate C23. There were two different inoculum levels (10^4 and 10^5) plus a control (0). Tap (T) and feeder (F) roots were assessed separately using the rating system described in 5.2. The data are presented in terms of mean proportions of assessments and then averaged over the number of replicates. Mean values for this experiment were taken from 10 replicates. ± indicates the standard errors. a and b indicate where there are significant differences in disease severity on tap roots between the different cultivars at the $LSD_{0.05}$ level.

	0		10^4		10^5	
	T	F	T	F	T	F
HARVEST 1						
Carrizo Citrange	0.20 ±0.00	0.28 ±0.03	0.69[a] ±0.04	0.73 ±0.05	0.78[a] ±0.02	0.76 ±0.04
McKillops Rough Lemon	0.22 ±0.02	0.30 ±0.03	0.92[b] ±0.03	0.82 ±0.02	0.93[b] ±0.03	0.84 ±0.03
HARVEST 2						
Carrizo Citrange	0.20 ±0.00	0.26 ±0.03	0.90 ±0.03	0.88 ±0.03	0.88 ±0.03	0.98 ±0.02
McKillops Rough Lemon	0.20 ±0.00	0.26 ±0.03	0.98 ±0.02	0.92 ±0.03	0.96 ±0.03	1.00 ±0.00

longer significant; *P. citrophthora* infection significantly reduced root fresh weight in both cultivars (P<<0.001).

D. Root Rot Assessment Scores

Table 5.2 shows that for Harvest 1, root rot severity was significantly greater in the tap roots of susceptible cultivars compared with those of the resistant cultivar (P<0.01). There was no difference in root rot severity on feeder roots between McKillops Rough Lemon and Carrizo Citrange (P<0.01). The data from the second harvest show that there were no longer significant differences between cultivars. Differences between uninoculated and inoculated roots were highly significant for both harvests (P<0.001). There was no difference between inoculum levels (10^4 or 10^5 zoospores per ml).

E. Summary of Results

It can be concluded from the results of this experiment (including both the growth parameters measured and the root assessment) that, using the root–dipping method, 2 to 3 month old seedlings of resistant and susceptible Citrus cultivars could be clearly distinguished as early as 7 days after inoculation with *P. citrophthora* isolate C23. By the time of the second harvest (21 days) this was no longer the case because the fungus had overgrown the small seedlings and overcome resistance. There was no significant difference in host response using different inoculum levels (10^4 or 10^5 zoospores per ml). Symptoms of waterlogging on control roots were greatly reduced by the use of the recycled soil/peat mixture as indicated by the growth of control plants compared to Experiment 1.

5.3.3 Experiment 3: Comparison of Carrizo Citrange and Symons Sweet Orange using the Root–Dipping Method.

The procedure for this experiment was outlined in Section 5.2.3.

A. Shoot Fresh Weight

At Harvest 1 (14 days, Figure 5.3) there was no significant difference in the effect of *P. citrophthora* inoculation on shoot fresh weight in Carrizo Citrange compared to controls in contrast to Symons Sweet Orange where inoculated plants (10^4 zoospores per ml) had significantly lower shoot fresh weights compared to the uninoculated controls (LSD = 0.4034). This interaction between cultivar and inoculum was highly significant ($P<0.001$). At Harvest 2 (28 days) the interaction was again highly significant ($P<0.001$). The same shape of response was observed as in Harvest 1, except that the difference between Symons Sweet Orange control and inoculated treatments were larger. There was also a significant difference between Carrizo Citrange control and inoculated treatments (LSD = 0.308).

B. Shoot Dry Weight

At Harvest 1, the shoot dry weight of Symons Sweet Orange was significantly reduced compared to the uninoculated control (LSD = 0.149, see Figure 5.3B).but there was no such effect observed for Carrizo Citrange. The interaction between cultivar and inoculum was significant ($P<0.01$). By Harvest 2, the interaction was no longer significant and the shoot dry weights of both inoculated cultivars were significantly reduced compared to the uninoculated controls.

Figure 5.3 **Plant growth measurements of *P. citrophthora*–inoculated and uninoculated Citrus cultivars: Carrizo Citrange and Symons Sweet Orange.**

Data from Experiment 5.3.3. Carrizo Citrange and Symons Sweet Orange were grown under growth cabinet conditions described in Section 3.2.4. Roots were inoculated with *P. citrophthora* isolate C23 as described in Section 3.4.1. There was one inoculum level (10^4 zoospores per ml) plus a control (0). Plants were harvested at Day 7 and Day 21. Values are the means of 10 replicates. Standard errors of the means are indicated by bars.: **(A)** = shoot fresh weight ; **(B)** = shoot dry weight ; and **(C)** = root fresh weight.

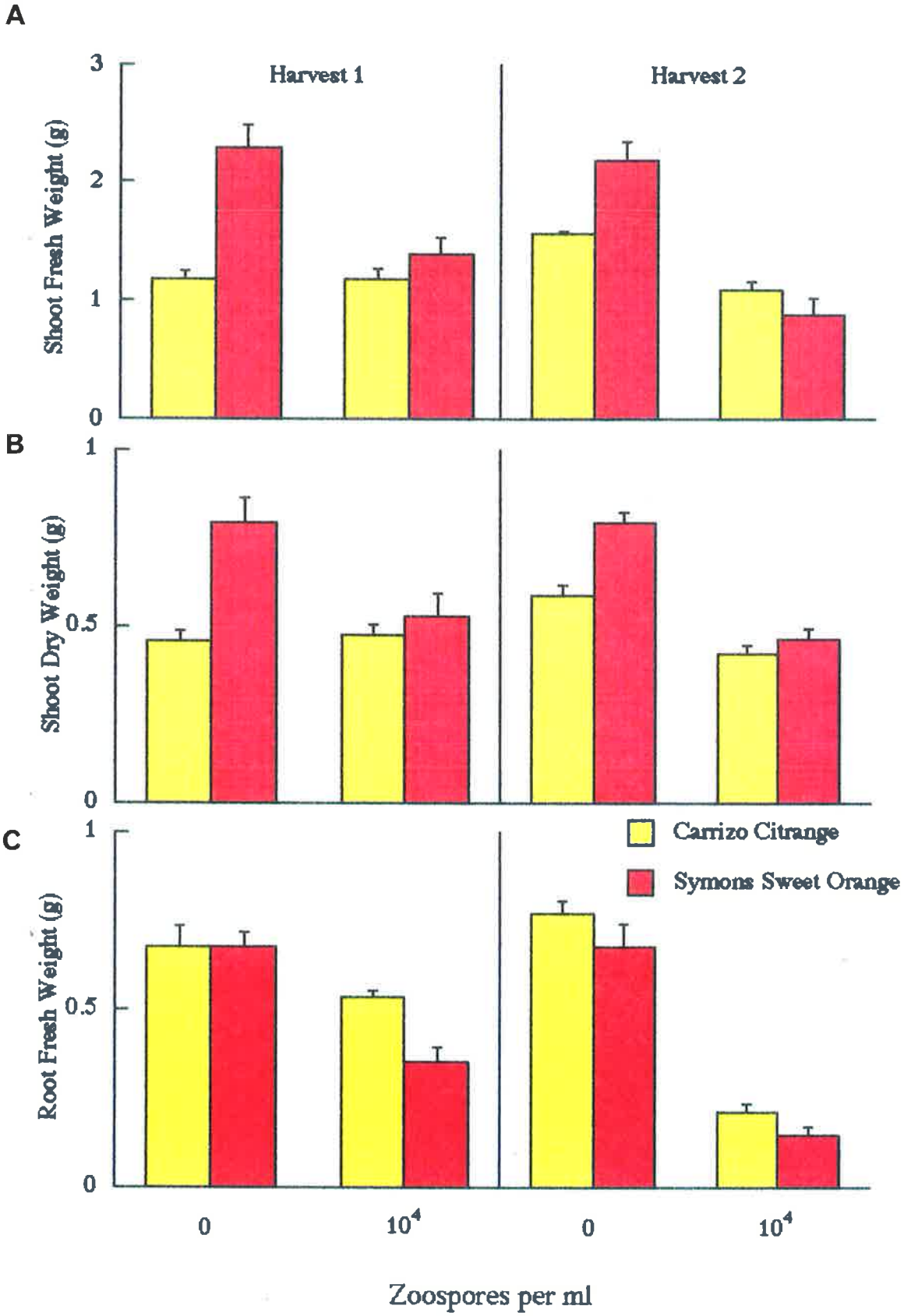

A
Harvest 1
Harvest 2
Shoot Fresh Weight (g)
3
2
1
0
B
Shoot Dry Weight (g)
1
0.5
0
C
Root Fresh Weight (g)
1
0.5
0
Carrizo Citrange
Symons Sweet Orange
0
10^4
0
10^4
Zoospores per ml

Table 5.3 Root Rot Assessment: Comparison of *P. citrophthora*-Inoculated and Uninoculated *Citrus* Cultivars: Carrizo Citrange and Symons Sweet Orange, Using the Root-Dipping Method of Inoculation.

Data from Experiment 3. There were two harvests at Day 7 and Day 21. Plants were inoculated with *P. citrophthora* isolate C23. Tap roots and feeder roots of Carrizo Citrange and Symons Sweet Orange were assessed separately using the rating system described in 5.2 . The data are presented in terms of mean proportions of assessments and then averaged over the number of replicates. Mean values for this experiment were taken from 10 replicates. ± indicates the standard errors. a and b indicate where there are significant differences in disease severity on tap roots between the different cultivars at the $LSD_{0.05}$ level.

	0		10^4	
	T	F	T	F
HARVEST 1				
Carrizo Citrange	0.20 ±0.00	0.28 ±0.03	0.60[a] ±0.06	0.72 ±0.04
Symons Sweet Orange	0.22 ±0.02	0.28 ±0.03	0.84[b] ±0.03	0.84 ±0.03
HARVEST 2				
Carrizo Citrange	0.20 ±0.00	0.24 ±0.00	0.91 ±0.04	0.91 ±0.03
Symons Sweet Orange	0.20 ±0.03	0.24 ±0.04	0.96 ±0.04	0.96 ±0.03

C. **Root Fresh Weight**

At Harvest 1 the inoculated treatments significantly reduced root fresh weight compared to the uninoculated control and this difference was more marked for Symons Sweet Orange than for Carrizo Citrange (LSD = 0.139, see Figure 5.3C), resulting in a significant interaction between cultivar and inoculum ($P<0.05$). By the second harvest, the interaction between cultivar and inoculum was no longer significant although the difference between the control and inoculated treatments was still highly significant ($P<0.001$).

D. **Root Rot Assessment Scores**

At Harvest 1 (Table 5.3), root rot severity was significantly greater in the tap roots of the susceptible cultivar compared with those of the resistant cultivar ($P<0.01$). The data from the second harvest indicated that there were no longer significant differences between cultivars but differences between inoculum levels were still highly significant ($P<<0.001$). There was no significant difference found in disease severity between cultivars for feeder roots at either harvest.

Plate 5.2 Effect of *P. citrophthora* inoculation on root-dipped seedlings of Carrizo Citrange (resistant) and Symons Sweet Orange (susceptible) cultivars.

Roots were inoculated with *P. citrophthora*, isolate C23, by the root-dipping method described in Section 3.4.1. There were two inoculum levels: 0 (control) and 10^4 zoospores per ml. **(A)** Shows the effect of infection on shoot growth 7 days after inoculation (the plants here are 98 days old) and **(B)** 21 days after inoculation (112 days old). Susceptible and resistant plants could be clearly distinguished.

A


CARRIZO CITRANGE
CONTROL
98 DAYS
CARRIZO CITRANGE
10^4
98 DAYS
SYMONS SWEET ORANGE
CONTROL
98 DAYS
SYMONS SWEET ORANGE
10^4
98 DAYS

B

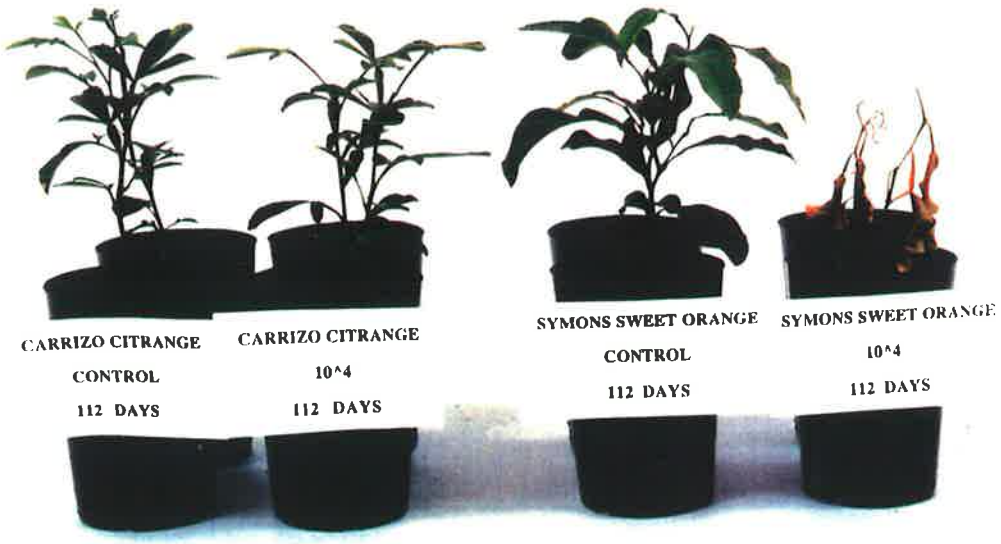

CARRIZO CITRANGE
CONTROL
112 DAYS
CARRIZO CITRANGE
10^4
112 DAYS
SYMONS SWEET ORANGE
CONTROL
112 DAYS
SYMONS SWEET ORANGE
10^4
112 DAYS

Plate 5.3 Seedlings of Carrizo Citrange and Symons Sweet Orange cultivars, 21 days after root inoculation with zoospores of *P. citrophthora* isolate C23.

The root dipping method for inoculating plant roots is given in Section 3.4.1. **(A)** Carrizo Citrange control and inoculated plants. **(B)** Symons Sweet Orange control and inoculated plants. Please note that feeder roots did not show-up clearly on Symons Sweet Orange

A

CARRIZO CITRANGE
CONTROL
112 DAYS

CARRIZO CITRANGE
10^4
112 DAYS

B

SYMONS SWEET ORANGE
CONTROL
112 DAYS

SYMONS SWEET ORANGE
10^4
112 DAYS

E. Summary of Results

As found in Experiment 5.2, both resistant and susceptible responses could be distinguished by the first harvest using the root–dipping technique. Although these differences were no longer significant by the second harvest (see figure 5.3) Carrizo Citrange appeared more "healthy" and was less water–stressed compared with susceptible cultivars. (see Plate 5.2 and 5.3) The results from the tap roots and feeder roots supported those of Experiment 2 (compare Table 5.2 and Table 5.3) and suggest some type of correlation between reduced infection on tap roots and positive plant growth in resistant cultivars.

5.3.4 Collar Rot Experiments

The procedure for these experiments were outlined in Section 5.2.4.

Experiment 4: Comparison of Carrizo Citrange and McKillops Rough Lemon

The size of the lesions which developed was significantly greater on McKillops Rough Lemon than on Carrizo Citrange compared to the respective controls ($P < 0.001$; Table 5.4). The controls showed no discolouration around the original wound. There was no significant difference in lesion lengths observed on Day 14 compared with those observed on Day 28.

McKillops Rough Lemon inoculated (+P) plants had significantly lower shoot and root fresh weights (LSD = 0.369 and 0.216 respectively, Table 5.5 A) and lower shoot and root dry weights than uninoculated (–P) plants (LSD= 0.161 and 0.065 respectively, see Table 5.5 B). No significant difference was observed between Carrizo control and

inoculated treatments. There was no significant interaction observed between cultivar and inoculum except for shoot dry weight ($P<0.05$).

Experiment 5: Comparison of Carrizo Citrange and Symons Sweet Orange

Mean lesion length was significantly greater in Symons Sweet Orange than Carrizo Citrange compared to the controls (see Plate 5.4). As shown in Table 5.6 results did not significantly vary on Days 14 and 28.

For the susceptible cultivar Symons Sweet Orange, total plant growth was reduced (Table 5.7). Symons Sweet Orange control plants had significantly higher means than Symons inoculated plants for both shoot and root fresh weight data (LSD = 1.54 and 0.378 respectively) and shoot and root dry weight data (LSD =0.36 and 0.07 respectively). There was no difference between Carrizo control and inoculated treatments for any of the growth parameters examined. The interaction was significant between cultivar and inoculum for both the shoot and root fresh weight data ($P<0.05$ and $P<0.001$, respectively). The dry weight data was more variable compared to the fresh weight data.

C. Conclusions

There was a significant difference in lesion length between resistant and susceptible cultivars in both experiments and this is illustrated in Plate 5.4. However, there was still wide variability in lesion length within cultivars for both experiments (as indicated by the standard errors) in spite of the large number of replicates (20).

Table 5.4 Mean length of necrosis on collar region 28 days after inoculation: Carrizo Citrange and McKillops Rough Lemon.

Data taken from Experiment 4. The collar region of the different Citrus cultivars was inoculated with a mycelial agar disc of *P. citrophthora* isolate C23 as described in Section 3.4.2. Agar discs without the fungus were used in the controls but results are omitted because no necrosis was observed. Lesion length was used as the principal indicator of resistance or susceptibility. Assessment was made on Days 14 and 28. Values are the means of 20 replicates. ± indicates the standard errors. a and b indicate where there are significant differences in lesion length between the different cultivars at the $LSD_{0.05}$ level. Please note that details of plant preparation are given in Section 3.2.2.

	Lesion Length (mm): Mean±SE	
	Carrizo Citrange	**McKillops Rough Lemon**
Day 14	9.45[a] ±0.80	17.73[b] ±1.61
Day 28	10.50[a] ±0.98	18.89[b] ±2.05

Table 5.5 Growth data from the collar rot inoculation experiment comparing Carrizo Citrange and McKillops Rough Lemon : Root and shoot fresh weight and dry weight.

Data taken from Experiment 4. Plants were grown under growth cabinet conditions as described in Section 3.2.4. Collar inoculation with *P. citrophthora* isolate C23 was described in Section 3.4.2. On Day 28, the plants were measured for shoot and root fresh **(A)** and dry **(B)** weights. Root and shoot material was separated and fresh and dry weights measured. Values are the means of 20 replicates. −P = uninoculated plants (control) +P = inoculated plants. ± indicates the standard errors. a and b indicate significant differences between uninoculated and inoculated treatments at the $LSD_{0.05}$ level.

A.	Fresh Weight (g)			
	Shoots		Roots	
	−P	+P	−P	+P
Carrizo Citrange	2.12 ±0.09	2.27 ±0.095	1.31 ±0.07	1.19 ±0.09
McKillops Rough Lemon*	3.67^a ±0.15	3.33^b ±0.18	1.59^a ±0.05	1.31^b ±0.09

B.	Dry Weight (g)			
	Shoots		Roots	
	−P	+P	−P	+P
Carrizo Citrange	0.80 ±0.04	0.86 ±0.03	0.36 ±0.02	0.31 ±0.025
McKillops Rough Lemon	1.46^a ±0.09	1.28^b ±0.06	0.42^a ±0.018	0.33^b ±0.03

Plate 5.4 Lesions in 98 day-old stems of two Citrus cultivars: Carrizo Citrange (resistant) and Symons Sweet Orange (susceptible) 28 days after collar inoculation with *P. citrophthora* isolate C23.

(A) Shows the effect of collar rot inoculation on plant growth. Plant growth in Symons Sweet Orange inoculated with *P. citrophthora* was severely affected compared with Carrizo Citrange.

(B) Shoot were excised from the above plants to show the difference in lesion length between Carrizo Citrange and Symons Sweet Orange. Please note that the lesion girdled the stem of Symons Sweet Orange

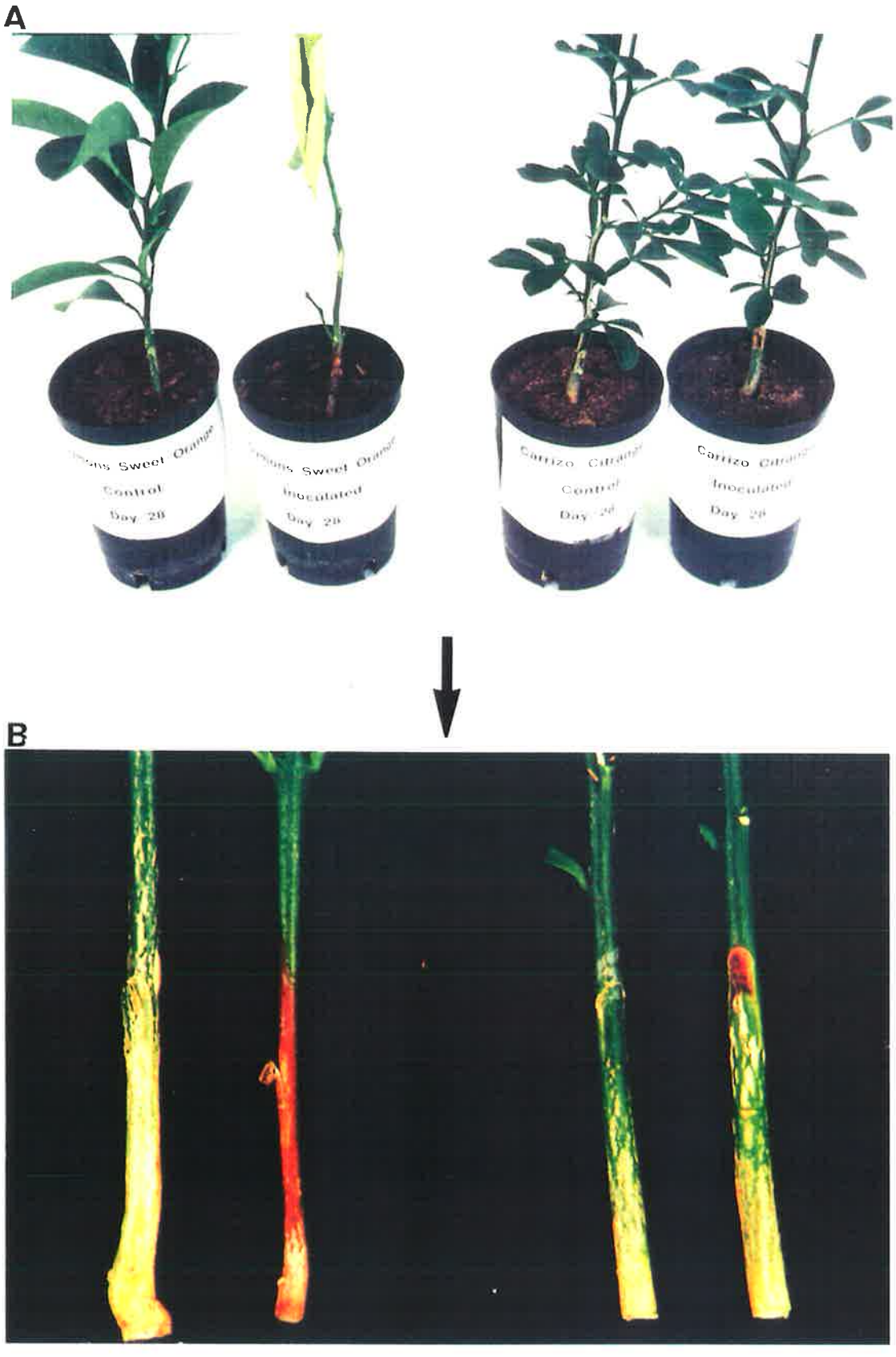

A
Sweet Orange
Control
Day 28
Sweet Orange
Inoculated
Day 28
Carrizo Citrange
Control
Day 28
Carrizo Citrange
Inoculated
Day 28
B

Table 5.6 Mean length of necrosis on collar region 28 days after inoculation: Carrizo Citrange and Symons Sweet Orange.

Data taken from Experiment 5. The collar region of the different Citrus cultivars was inoculated with a mycelial agar disc of *P. citrophthora*, isolate C23, as described in Section 3.4.2. Agar discs without the fungus were used in the controls but results are omitted because no necrosis was observed. Lesion length was used as the principal indicator of resistance or susceptibility. Assessment was made on Days 14 and 28. Values are the means of 20 replicates. ± indicates the standard errors. a and b indicate where there are significant differences in lesion length between the different cultivars at the $LSD_{0.05}$ level.

	Lesion Length (mm): Mean±SE	
	Carrizo Citrange	**Symons Sweet Orange**
Day 14	7.90 ±0.58	22.10 ±1.81
Day 28	8.65 ±0.61	23.45 ±1.99

Table 5.7 Growth data from the collar rot inoculation experiment comparing Carrizo Citrange and Symons Sweet Orange : Shoot and root fresh weight and dry weight.

Data taken from Experiment 5. Plants were grown under growth cabinet conditions as described in Section 3.2.4. Collar inoculation with *P. citrophthora* isolate C23 was described in Section 3.4.2. On Day 28, the plants were measured for shoot and root fresh (A) and dry (B) weights. Root and shoot material was separated and fresh and dry weights measured. Values are the means of 20 replicates. –P = uninoculated plants (control) +P = inoculated plants. ± indicates the standard errors. a and b indicate significant differences between uninoculated and inoculated treatments at the $LSD_{0.05}$ level.

A.	Fresh Weight (g)			
	Shoots		Roots	
	–P	+P	–P	+P
Carrizo Citrange	4.80 ±0.12	4.63 ±0.16	3.21 ±0.12	3.24 ±0.106
Symons Sweet Orange	10.68[a] ±0.29	7.66[b] ±1.06	4.02[a] ±0.14	3.27[b] ±0.17

B.	Dry Weight (g)			
	Shoots		Roots	
	–P	+P	–P	+P
Carrizo Citrange	1.40 ±0.04	1.40 ±0.03	0.56 ±0.02	0.55 ±0.02
Symons Sweet Orange	2.72[a] ±0.08	2.24[b] ±0.24	0.64[a] ±0.03	0.53[b] ±0.03

Generally, differences in plant growth between control and inoculated treatments were not significant for Carrizo Citrange but were significant for the susceptible cultivars and this was more marked in Symons Sweet Orange than in McKillops Rough Lemon.

5.4 DISCUSSION

This study successfully developed a rapid and reproducible method for screening Citrus rootstocks for resistance to *Phytophthora* root and collar rot. Young seedlings, 2 to 3 months old, were inoculated either by dipping roots into a zoospore suspension (10^4 zoospores per ml) or by inserting mycelial agar discs into a wound in the collar region of the stem, and evaluated 7 or 14 days later. The results were similar to those obtained in tests conducted by others over a wide range of inoculum densities with plants 6 months and older over experimental periods ranging from 6 weeks to several months (see Section 5.1). Recently Graham (1990), using chlamydospore–infested soil to infect 3 month–old *Citrus* rootstocks, also found that the ratings of rootstock resistance were the same as when older seedlings were used.

The root–dipping method was chosen to assess root rot. The root–drenching method was considered less suitable because it was not as sensitive as the root–dipping method for distinguishing differences between resistant and susceptible cultivars at lower inoculum levels. This is more of a problem for isolates that do not produce high zoospore yields. In any case the results obtained using the root–drenching method, were highly variable.

Although both the root–dipping and the collar inoculation methods were equally successful, the root–dipping method would be less likely to discern subtle differences between susceptible or resistant cultivars than the collar inoculation technique. This is because the method of assessing disease severity using root inoculation is highly subject to factors not associated with the fungus, such as environmental influences. For this reason authors have stressed the importance of preceding screening with pilot experiments in order to identify sources of variation while testing the experimental methods (Phelps *et al.*, 1991). Such 'pilot' experiments were shown to be valuable where, for example, in Experiment 1, soil conditions were predisposed to waterlogging and caused symptoms on the host similar to those produced by the fungus. Once this confounding effect was eliminated by the addition of peat to the potting mix, the tap root data revealed a significant difference in root rot symptoms between resistant (Carrizo Citrange) and susceptible cultivars (McKillops Rough Lemon and Symons Sweet Orange).

Although not compared directly in a single experiment, results from the collar rot experiments, suggested that Symons Sweet Orange was more susceptible than Rough Lemon. A higher degree of susceptibility to *Phytophthora* spp. has been revealed in most Sweet Oranges, in contrast to the Rough Lemons where the range of responses can extend from moderately resistant to highly susceptible (Broadbent, 1977).

Although collar inoculation gives rise to greater variability in disease development (Smith *et al.*, 1987; Whiteside, 1971), in this study the collar inoculation technique was not subjective and thus allowed less margin for error than the 1 - 5 rating scale used with the root-dipping method.

The type of inoculum used in collar inoculation may influence disease severity. Mycelial agar discs produced significantly greater lesions than did zoospores and chlamydospore-infested soil (Smith *et al.*, 1987). It was suggested that mycelial agar discs may have a higher inoculum potential than zoospores or chlamydospores. In addition, rootstock vigour, rapid tissue regeneration and seed source have been suggested as other sources of variability in response to collar inoculation. Smith *et al.* (1987) reduced this variability by adjusting lesion length to remove the effects of differential growth rates and vigour between rootstocks. Their work indicated that the same results were obtained whether this measurement or the standard manner of measuring lesion length was used. In this study, differences related to the host, were minimised by using plants of the same physiological age grown under conditions which data from preliminary experiments indicated would provide the same relative growth rates for both susceptible and resistant cultivars. In this study, the most variable factor was the difference in response of individual plants within a cultivar. This type of variation reflects the field situation.

In this study, the susceptible and resistant ratings of commercial rootstocks using collar and root inoculation techniques were comparable. Generally, however, in the literature there is not necessarily a correlation between resistance to root rot and collar rot. For example, field tolerance is higher for collar rot than root rot with Carrizo Citrange (Afek *et al.*, 1990; Grimm and Hutchison, 1977; Hutchison, 1985 (Graham, 1990)). Some of this variation may be accounted for by the method of root assessment. In contrast to the feeder roots, the tap root data, which were assessed separately for both experiments, showed a positive correlation between plant growth and infection at the first harvest but by the second

harvest this correlation was negative. It should be noted that because the seedlings used in this study were very young, it is likely that any resistance would be overcome more quickly than if using older seedling material. Therefore, for young plant material earlier and more frequent harvests would overcome this disadvantage.

In contrast to the tap roots, the feeder roots exhibited a high degree of susceptibility to infection. This may account for some of the controversy as to whether the Citranges are resistant or susceptible to *Phytophthora*. If feeder root rot is used as an indicator then the Citranges have been found to be very susceptible. For example, Klotz *et al.* (1958) found the Citranges to be quite susceptible in percentage of fibrous roots showing decay caused by *P. citrophthora* and *P. parasitica*. Carpenter and Furr (1962) attributed the variability in their results to differences between old and young root systems. Hough (in press) noted that 8 month–old Carrizo and Troyer Citranges lost more than 50% of their feeder roots. In contrast, other studies, where the tap root data have been included with the feeder root data, indicated that the Citranges are moderately resistant to infection by *Phytophthora* spp. (Grimm and Hutchison, 1973).

It is not clear why the tap roots are more resistant than the feeder roots. The tap roots may be more resistant because they produce less root exudates which may result in a differential chemotropic response by the fungus or because of their regenerative ability (Menge, unpublished data) or because they may physically be more difficult to penetrate due to suberisation of hypodermal cells (Walker *et al.*, 1984).

In addition, it was noted in this study, particularly in the root inoculation trials, that Carrizo Citrange was less prone to water–stress than susceptible cultivars which have larger and broader leaves and may be less efficient in terms of transpiration rates. Such physiological factors may influence the degree of susceptibility. This applies particularly to the root inoculation experiments where Carrizo grew well in spite of a badly infected feeder root system. The relatively healthy tap roots of these plants were unlikely to be sufficient to maintain such growth. Recently Menge (unpublished data) found that the Citranges produced adventitious roots in response to infection but on closer examination these "apparently healthy roots" were found to be infected, although they showed no symptoms at the time of sampling. The effects of root or collar inoculation on plant growth and physiology would be an area worth further investigation.

The success of screening experiments depends on maintaining conditions favourable for disease development. It has been suggested, therefore, that screening experiments for disease resistance, should take place under growth cabinet conditions similar to conditions optimal for infection in the field (J. Menge, pers. comm.). In this study, the experiments took place at 25°C which is considered the optimal temperature for growth of *P. citrophthora* (Ribeiro, 1978). Normally, in South Australia, it is the winter months with lower temperatures (around 15°C) and higher rainfall which are conducive for growth and sporulation of *P. citrophthora*. During the summer months, when conditions are hotter and drier, the population declines. Therefore, it may be interesting to repeat the above experiments at a lower temperature, because it is also well–documented that

increases or decreases in temperature may alter plant resistance to fungal pathogens (Bell, 1981).

Both the collar and root inoculation techniques used in this study can be applied to screening Citrus for disease resistance to *P. citrophthora* in tissue culture. Inoculation techniques relying on zoospore production have proved in the past to be both labour–intensive and difficult to standardise. However, the technique developed for *P. citrophthora* in Section 3.1.5 produces both large quantities of zoospores and uniform yields between isolates. Techniques using zoospores rather than mycelium are considered advantageous because they are most likely to imitate infection in the field and the inoculum density is not as high. However, this also requires an additional step in tissue culture, that being the production of roots (see Section 6.2.1). Probably the simplest approach would be initially to use a modified collar rot evaluation of shoots in culture. As evident from these studies this approach has additional advantages; first, in discerning variability within cultivars; and second, in producing less ambiguous results for moderately resistant cultivars such as Carrizo Citrange which can be difficult to assess using root inoculation techniques. Details of the tissue culture screening work are presented in the next chapter.

6. THE APPLICATION OF TISSUE CULTURE TECHNIQUES TO SCREEN CITRUS ROOTSTOCKS FOR RESISTANCE TO *P. CITROPHTHORA* ISOLATES.

6.1 INTRODUCTION

Plants micropropagated by tissue culture techniques are currently being incorporated into conventional breeding programmes to both complement and shorten screening for many traits, including disease resistance. Sharma and Skidmore (1988) reported that partial resistance to *Phytophthora palmivora* could be identified in shoot cultures of papaya (*Carica papaya* L.) following inoculation of shoots excised from tissue culture with sporangial suspensions of the fungus. Scott *et al.* (1992) screened micropropagated shoots of almond rootstocks for resistance to *P. cambivora*, *in vitro*. Differences in aggressiveness were observed between *P. cambivora* isolates which agreed with results obtained from field experiments. The advantages and disadvantages of this approach for use in screening for disease resistance have been discussed in Section 2.6.3; the principal point of concern is that such assessment of micropropagated material should be confirmed *in vivo*.

Tissue culture techniques can also be used to increase our understanding of the host/pathogen interaction. For example, it has been suggested (Menge, unpublished data) that the Citranges may be more resistant to *Phytophthora* infection because they produce less root exudates than do susceptible Citrus hosts and this may result in a differential chemotactic response by the fungus (see Section 5.4). Chemotaxis, both negative and positive, is undoubtedly an

important factor in *Phytophthora* infection (Carlile, 1983). Several authors have examined the response of zoospores to resistant and susceptible roots of hosts (including Citrus), by immersing root systems (wounded and non-wounded) in zoospore suspensions and observing events microscopically (Zentmyer, 1961; Broadbent, 1969). However, such studies were limited because of confounding factors, for example, the nutritional status of the plant (Carlile, 1983). Tissue culture techniques, on the other hand, offer advantages in that environmental and nutritional factors can be strictly controlled (Section 2.6.3).

Therefore, there were several aims in this study. The principal aim was to establish whether a modified excised twig assay technique (Scott *et al.*, 1992) could be used to distinguish Citrus cultivars known to be resistant or susceptible to *P. citrophthora*. The responses to infection observed in tissue culture experiments were compared with those observed in whole plants *in vivo* (Chapter 5). Once repeatable tissue culture screening techniques had been established, *P. citrophthora* isolates, including single zoospore-derived progeny, were examined for variation in pathogenicity on resistant and susceptible Citrus hosts. Tissue culture techniques were also used to examine the question of whether there is a differential chemotropic response by *P. citrophthora* towards different Citrus cultivars. However, certain criteria should be established for Citrus cultivars before any experiments can be conducted; these include 1) that shoots can be multiplied within a short time period 2) that shoots can form roots and 3) that shoots are no less than 1.5cm long before they are inoculated (E. Scott, pers. comm.). Therefore a further aim was to optimise conditions for multiplying and rooting resistant

and susceptible Citrus cultivars in culture. Although Citrus has been micropropagated principally by means of shoot multiplication, different cultivars have been shown to vary in their requirements (Barlass and Skene, 1986).

6.2. EXPERIMENTAL PROCEDURES

6.2.1 Experiment 1: Determination of Optimal Procedures for Tissue Culture of Citrus

Several resistant and susceptible cultivars were examined in terms of their response to different hormone concentrations in multiplication media, their rooting capacity and their potential to elongate in culture. Details of tissue culture media and procedure are given in Section 3.3.

A. Multiplication of Citrus Cultivars

Forty shoots of each of the following cultivars 1/31, 28/25, Carrizo Citrange, Symons Sweet Orange and McKillops Rough Lemon, were individually transferred to small tubs (30ml Polycarbonate Specimen Container, Disposable Products) containing MS medium (10ml per tub) supplemented with either 0.2 or 2mgl^{-1} BA. After 42 days, the number of new shoots per explant was recorded. These shoots were then either placed on basal medium for 14 days prior to use in experiments or placed directly on rooting medium (see below).

In addition, other media supplements were tested including: casein hydrolysate (0.1%); malt extract ($0.5gl^{-1}$); IAA (0.1, 0.5 and $1mgl^{-1}$); zeatin riboside (1 and $10mgl^{-1}$); and MS medium supplemented with $0.4gl^{-1}$ NH_4NO_3 (Lee, 1986)

B. Procedure for Inducing Rooting of Citrus Cultivars

Forty–eight Symons Sweet Orange and Carrizo Citrange shoots of between 1.5 and 2cm long were individually transferred to small tubs containing MS medium (10ml per tub) plus $5mgl^{-1}$ NAA. Shoots were kept on this medium for 28 days. They were then transferred to basal medium to permit elongation of the roots and later scored for the percentage of rooted shoots. Rooted shoots were then used in chemotropic experiments (see Section 6.2.5)

6.2.2 Experiment 2: Development of Tissue Culture Techniques for Screening for Resistance *In Vitro*.

For all tissue culture screening experiments (2A, 2B, 3, and 4) healthy Citrus shoots of between 1.5 – 2 cm in length were excised from cultures on multiplication medium and transferred to basal medium where they were grown for 10 days to dilute out any residual BA from the shoots. Large leaves were carefully excised taking care not to damage the stem. Four partially defoliated shoots were placed upright around the margin of a large tub (250ml Polycarbonate Specimen Container, Disposable Products) containing 50ml CMA colonised with *P. citrophthora* isolates and incubated at 25°C in the dark (see Plate 6.2A). An uninoculated CMA control was included in all experiments.

Experiments 2A and 2B were devised to examine whether a modified twig assay (Scott *et al.*, 1992) could be used to distinguish susceptible (Symons Sweet Orange) from resistant (Carrizo Citrange) Citrus cultivars. For both experiments, the experimental design was completely randomised with treatments arranged as a 2–way factorial; *P. citrophthora* isolate C23, (plus the uninoculated control) x two cultivars (Carrizo Citrange and Symons Sweet Orange). However, there were some differences between the two experiments. In Experiment 2A there were three replicate tubs and in Experiment 2B there were five replicate tubs per treatment. In addition, the development of shoot necrosis (measured in terms of lesion length) was assessed daily for 5 days in Experiment 2A and for 6 days in Experiment 2B. This latter difference was due to the fact that in the second experiment shoots were placed in the tubs when the fungus was 10mm from the side of the tub whereas in the first experiment the fungus had already reached the side. Although this did not affect the results, it did take a day longer for infection to become established.

The possible effects of BA on the host/pathogen interaction were investigated by comparing shoots treated above with shoots transferred directly from multiplication medium to CMA colonised by the fungus.

Shoots were examined using a Wild stereo–zoom dissecting microscope to confirm visual assessments of infection and in some cases tissue segments were plated onto CMA to confirm the presence of the fungus. Analysis of variance was used to determine the

significance of differences in lesion length between the different cultivars. Data from each day were analysed separately. LSD was calculated at the 0.05 level.

6.2.3 Experiment 3: Comparison of Aggressiveness of Several *P. citrophthora* Isolates on Resistant and Susceptible Citrus Cultivars

The aim of this experiment was to compare several isolates of *P. citrophthora* for variation in aggressiveness on different Citrus hosts. This experiment was a 2–way factorial design with four isolates (plus the uninoculated control) x two cultivars, randomly arranged as described in Section 6.2.2. There were three replicate tubs per treatment. The isolates examined were: C23, C6*, P3693* and P1152. These isolates were chosen because they varied either in terms of mating type, (A1 or A2*, see Table 7.1), morphological characters (in particular C23 and P1152, see Table 4.6) or growth rate (see Table 4.4). The two cultivars were Carrizo Citrange and Symons Sweet Orange. Shoot necrosis was assessed daily for 6 days. Analysis of variance was used to examine differences in aggressiveness between these four *P. citrophthora* isolates on both Carrizo Citrange and Symons Sweet Orange at different times.

6.2.4 Experiment 4: Comparison of Parent and Single Zoospore–Derived Progeny for Differences in Aggressiveness on Resistant and Susceptible Citrus Cultivars.

In Experiment 4, a comparison was made between isolate P1152 and its single zoospore–derived progeny. Isolate P1152 was chosen because it was found to be morphologically highly variable compared to other isolates (see Chapter 4). The

experiment was a completely randomised but unbalanced design with treatments arranged as a 2–way factorial; four isolates (P1152 parent and three single spore–derived progeny and a control) x five different Citrus cultivars. The five cultivars included Carrizo Citrange, two additional resistant cultivars, 28/25 and1/31, and the susceptible cultivars, Symons Sweet Orange and McKillops Rough Lemon. There were three replicate tubs per treatment. There was insufficient material of 28/25 and 1/31 therefore a comparison between parent and single spore–derived progeny was made on only three of the cultivars, Carrizo Citrange, Symons Sweet Orange and McKillops Rough Lemon. Instead priority was given to examining the responses of all five Citrus cultivars to the parent isolate in order to see if other resistant and susceptible cultivars could be distinguished using this screening technique.

6.2.5 Experiment 5: Chemotropism of hyphae of *P. citrophthora* to different Citrus hosts in Tissue Culture

Ten shoots or rooted shoots of each cultivar (Carrizo Citrange and Symons Sweet Orange) were individually placed in separate 250ml tubs, containing 50ml MS basal medium, approximately 1.5 cm from one side. The medium was inoculated with a mycelial disc of isolate C23 placed 2 cm from the opposite side. The arrangement is shown in Plate 6.4. Growth of *P. citrophthora* isolate C23 was measured at four different orientations (clockwise) to the host which were either 1) adjacent to the shoot, 2) to the left of the shoot , 3) opposite to the shoot or 4) to the right of the shoot. The control was isolate C23 growing in the absence of a host shoot. Fungal growth was slow on MS medium, therefore, results were recorded after 7 and 10

days. Analysis of variance was used to analyse the effect of cultivar on the growth of the fungus. The experiment was conducted using both shoots (5A) and rooted shoots (5B).

6.3. RESULTS

6.3.1. Experiment 1: Determination of Optimal Procedures for Tissue Culture of Citrus

A: Multiplication of Citrus Cultivars

As evident from Table 6.1, the number of shoots produced per explant varied between cultivars ($P<0.01$) and was dependent on the concentration of BA in the medium. For example, shoot multiplication of Carrizo Citrange was significantly greater at $2.0mgl^{-1}$ than at $0.2mgl^{-1}$ in contrast to McKillops Rough Lemon where the reverse was true (LSD = 0.66 and 0.55 respectively). Although, in general, there was high shoot multiplication at $2mgl^{-1}$ BA, the shoots were sometimes stunted and distorted. These were unsuitable for screening experiments where shoots need to be tall. Thus, it was decided to use BA at $0.2mgl^{-1}$, which gave equally high multiplication of shoots but did not result in stunting of the plants (see Plate 6.1). Other treatments did not increase either growth or shoot multiplication. A difference was observed between resistant and susceptible cultivars; the latter group had lower shoot multiplication.

Plate 6.1 shows the different stages of culturing Citrus. Shoots were subcultured at 6 week intervals onto fresh MS medium containing BA or onto basal medium 10 days prior to experiments to dilute any residual hormone.

Table 6.1 Comparison of the number of shoots produced by explants of different Citrus cultivars.

Data from Experiment 1. Shoot explants from five different Citrus cultivars (28/25, 1/31, Carrizo Citrange, McKillops Rough Lemon and Symons Sweet Orange) were placed onto MS medium containing one of two concentrations of BA; 0.2 or 2 mgl−1. There were ten tubs containing four shoots per treatment. After 6 weeks, the number of new shoots per explant was recorded. Mean and standard errors of means (±) are presented. a and b indicate significant differences between BA concentrations (rows) at the $LSD_{0.05}$ level.

Cultivar	Mean No. of New Shoots per Explant	
	BA (mgl^{-1})	
	2.0	0.2
1/31	2.7 ±0.88	2.4 ±0.76
28/25	1.65 ±0.57	2.16 ±0.83
Carrizo Citrange	2.89^a ±0.78	2.00^b ±0.73
McKillops Rough Lemon	1.32^a ±0.60	1.86^b ±0.56
Symons Sweet Orange	2.15 ±1.03	2.2 ±0.70

Plate 6.1 Micropropagation of Citrus.

Some of the different stages of culturing Citrus. Media and conditions are described in Section 3.3.

(A) Proliferation of shoots on multiplication medium.

(B) Shoot arising from a nodal explant.

(C) Flowering in culture.


A


B


C

B: Rooting of Citrus Cultivars

Rooting responses varied with resistant cultivars tending to root more readily than susceptible cultivars. The number of Carrizo shoots which rooted was 88% compared to 58% in Symons Sweet Orange. Both frequencies were adequate for experimental purposes.

Shoots left for long periods on the rooting medium developed callus and were therefore unsuitable for use in screening experiments. This problem was eliminated by reducing the period on MS medium supplemented with NAA ($5mgl^{-1}$) from 28 to 14 days.

6.3.2 Experiment 2: Tissue Culture Techniques for Screening for Resistance to *P. citrophthora in Vitro*

The procedure for Experiments 2A and 2B is described in Section 6.2.2.

Experiment 2A

Necrosis developed rapidly in excised shoots of Carrizo Citrange and Symons Sweet Orange inoculated with *P. citrophthora* and disease assessment was completed within 5 days. However, because no measurable effect was observed in the first 24 – 48 hr, results from the last 3 days only were analysed. As evident in Table 6.2, lesion lengths on Symons Sweet Orange were significantly greater than on Carrizo Citrange (LSD values for Day 3 = 4.48, Day 4 = 6.7; and Day 5 = 1.06). A significant interaction was found for both cultivar and fungus for each day ($P<0.05$, $P<0.05$ and $P<0.001$ respectively).

Table 6.2 Length of lesions in micropropagated shoots of Carrizo Citrange and Symons Sweet Orange inoculated with *P. citrophthora* (isolate C23).

Data from Experiment 2A. Citrus shoots of 1.5 – 2 cm in length were placed upright in either 250 ml tubs containing 50ml CMA colonised with *P. citrophthora* isolate C23, or uninoculated CMA (control). The tubs were placed at 25°C, in the dark. There were three replicate tubs, each with four shoots, for each treatment. Shoots grown on basal medium (–BA) for 10 days were also compared with those that had been transferred directly from medium containing the cytokinin BA (+BA). Shoots were assessed daily for necrosis. The mean length of lesions ± standard errors of the means. a and b indicate significant differences between cultivars (rows) for particular days at the $LSD_{0.05}$ level. Note that with the +BA treatment there is no significant difference between cultivars after Day 3.

		Mean Lesion Length (mm)			
		Carrizo Citrange		Symons Sweet Orange	
		– BA	+BA	–BA	+BA
Day	3	0.50^a ±0.34	8.0^a ±0.82	5.42^b ±1.20	15.0^b ±0.00
	4	3.67^a ±1.11	12.25 ±1.60	10.4^b ±1.31	15.0 ±0.00
	5	7.25^a ±1.63	15.0 ±0.00	15.0^b ±0.00	15.0 ±0.00

Necrosis was significantly greater in shoots that had been taken directly from multiplication medium (with BA) than in shoots which had been cultured on basal medium (without BA) for 10 days prior to inoculation trials. The influence of residual BA on lesion length was significant ($P<0.001$).

The response of the two host cultivars to infection also varied. Necrosis was more evident at the tip for Symons Sweet Orange, whereas for Carrizo Citrange, the lesion extended from the base upwards (see Plate 6.2). The absence of the fungus from the shoot tips of Carrizo Citrange was verified by plating out segments of inoculated and uninoculated shoots onto CMA plates which were incubated at 25°C in the dark for 7 days, followed by microscopic assessment of the shoots for the presence of the fungus. Similar patterns of host response were observed in repeated trials (Experiment 2B, Experiment 3 and Experiment 4).

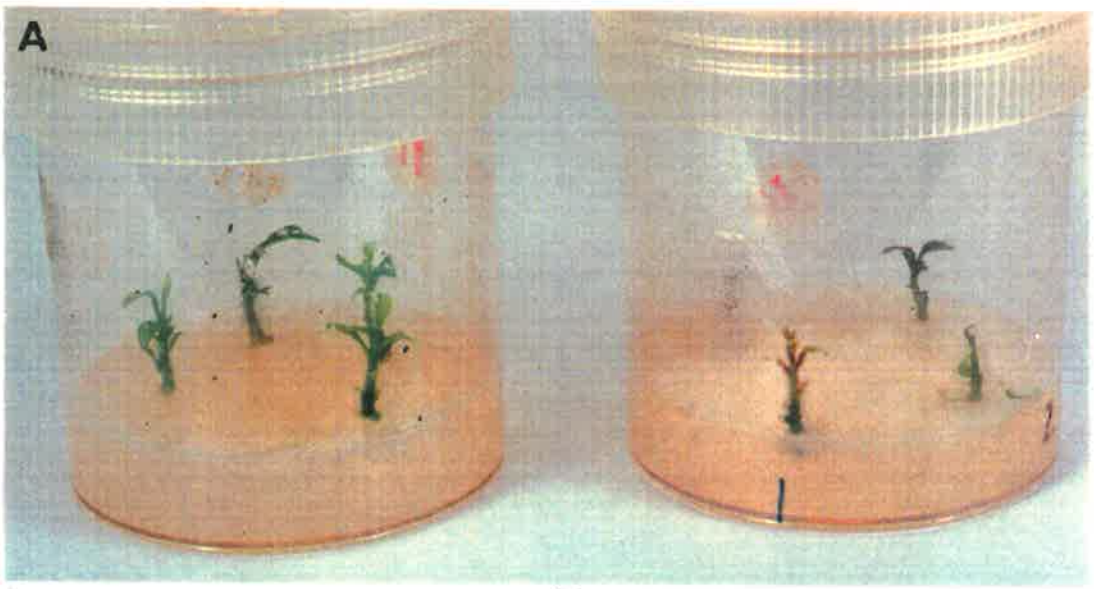
Experiment 2B

The results (Table 6.3) supported those obtained from Experiment 2A. No measurable difference in lesion length was observed in the first 24 – 48 hr. The last four days were analysed separately and significant interactions for fungus and cultivar were found for Day 3 ($P<0.05$), Day 4 ($P<0.001$), Day 5 ($P<0.001$) and Day 6 ($P<0.001$). LSD revealed significant differences between the two cultivars on all four days (Day 3 = 0.786, Day 4 = 1.51, Day 5 = 2.52, Day 6 = 1.31)

Plate 6.2 Pathogenicity of *P. citrophthora* isolate C23 to Carrizo Citrange (resistant) and Symons Sweet Orange (susceptible) Citrus cultivars.

(A) The tissue culture screening system. Four Citrus shoots of 1.5 – 2 cm in length were placed upright in 250 ml tubs containing 50ml of CMA colonised with *P. citrophthora* isolate C23 and placed at 25°C, in the dark.

(B) Shoots from the above tubs after 5 days, showing differences in necrosis between Carrizo Citrange and Symons Sweet Orange. Note that necrosis was more evident at the tip for Symons Sweet Orange, whereas for Carrizo Citrange, the lesion extended from the base upwards. C = uninoculated CMA (control) and I = inoculated CMA.


A


B
C
Carrizo
I
Citrange
C
Symons
I
Sweet Orange

Table 6.3 Length of lesions in micropropagated shoots of Carrizo Citrange and Symons Sweet Orange inoculated with *P. citrophthora* (isolate C23).

Data from Experiment 2B. Citrus shoots of 1.5 – 2 cm in length were placed upright in either 250 ml tubs containing 50ml CMA colonised with *P. citrophthora* isolate C23, or uninoculated CMA (control). The tubs were placed at 25°C, in the dark. There were five replicate tubs containing four excised shoots per treatment. Shoots were assessed daily for necrosis. The mean length of lesions ± standard errors of the means. a and b indicate significant differences between cultivars (rows) for particular days at the $LSD_{0.05}$ level.

		Mean Lesion Length (mm)	
		Carrizo Citrange	Symons Sweet Orange
Day	3	0.00^a ±0.00	1.15^b ±0.34
	4	0.55^a ±0.31	6.75^b ±0.57
	5	3.75^a ±0.77	10.10^b ±0.80
	6	8.70^a ±1.0	15.00^b ±0.00

6.3.3 Experiment 3: Comparison of Aggressiveness of *P. citrophthora* Isolates on Resistant and Susceptible Citrus Cultivars

The procedure for this experiment was described in 6.2.3. The experiment was assessed over 6 days but only the length of shoot necrosis from 4 days after inoculation is presented (Figure 6.1). Isolate C6 caused significantly less necrosis on Symons Sweet Orange than did the three other isolates tested for all three days, and on Carrizo Citrange this difference was observed only on Days 5 and 6 (LSD Day 4 = 0.7735, LSD Day 5 = 1.42, LSD Day 6 = 1.94, see Plate 6.3). The interaction between fungus and cultivar was significant ($P<0.01$, $P<0.05$ and $P<0.001$ respectively).

This difference in aggressiveness between the isolates could not be correlated with either mating type, RFLP banding patterns (Chapter 8) or differences in morphological characters (e.g. sporangia, Chapter 4). However, C6 grew significantly more slowly on CMA at 25°C than did the other three isolates, which also did not differ significantly from one another in growth rates (Chapter 4).

6.3.4 Experiment 4: Comparison of Parent and Single Zoospore–Derived Progeny for Differences in Aggressiveness on Several Resistant and Susceptible Citrus Cultivars

The procedure for Experiment 4 was described in Section 6.2.4. Results for Days 3, 4 and 5 were analysed separately. There were no significant differences in lesion length between the parent isolate P1152 and its single zoospore–derived progeny (Figure 6.2). However, significant differences in lesion length between the cultivars in their response

Figure 6.1 Comparison of lesion length between isolates of *P. citrophthora* on Carrizo Citrange and Symons Sweet Orange.

Data from Experiment 3. Four *P. citrophthora* isolates were examined, C23, C6, P3693 and P1152 on Carrizo Citrange (resistant) and Symons Sweet Orange (susceptible) Citrus cultivars. There were three replicate tubs containing four excised shoots per treatment. Shoots were assessed daily for necrosis. There were three replicate tubs each containing four excised shoots. The means are presented for Days 4, 5 and 6. Standard errors of the means are indicated by bars.

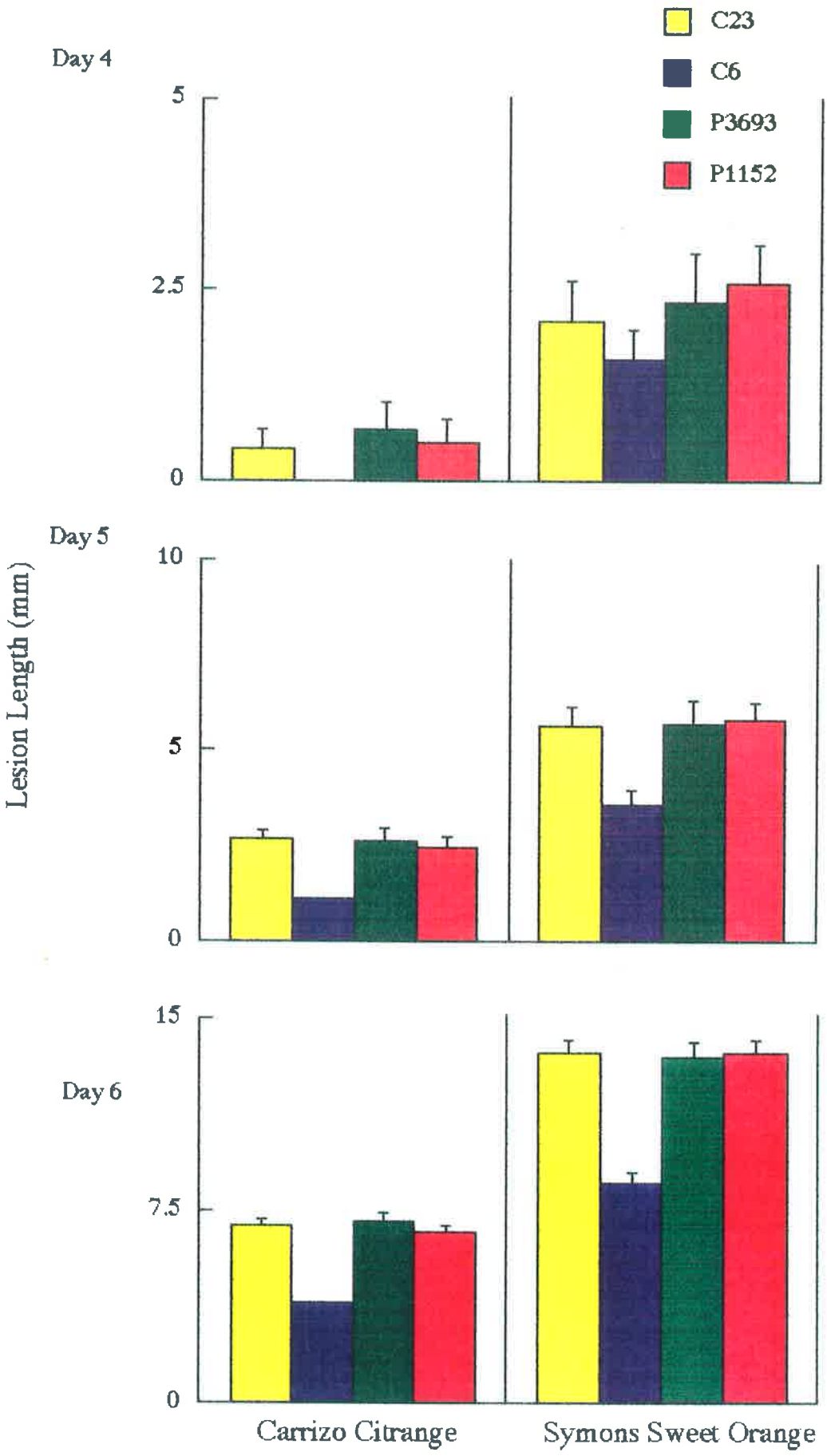

C23
C6
P3693
P1152
Day 4
Day 5
Day 6
Lesion Length (mm)
5
2.5
0
10
5
0
15
7.5
0
Carrizo Citrange
Symons Sweet Orange

Plate 6.3 Length of lesions in micropropagated shoots of Citrus cultivars, comparing differences in aggressiveness between isolates.

The tissue culture screening method has been previously described (Section 3.3). Isolate C6 can be seen to be less aggressive compared with isolate C23, on both Carrizo Citrange **(A)** and Symons Sweet Orange **(B)**.

A

Control C6 C23

Carrizo Citrange

B

Control C6 C23

Symons Sweet Orange

to the parent isolate and single spore–derived progeny were observed (LSD: Day 3 = 0.78; Day 4 = 1.7; Day 5 = 2.7; Table 6.4). The interaction between cultivars and isolate were significant on all three days ($P<0.01$, $P<0.001$, $P<0.001$ respectively).

As in previous experiments, there was a significant difference in lesion length between Carrizo Citrange and Symons Sweet Orange on all three days and in addition two groups could be distinguished; Group 1 comprised resistant plants, i.e. 28/25 1/31 and Carrizo Citrange and Group 2 comprised susceptible plants, i.e. Symons Sweet Orange and McKillops Rough Lemon. The significant difference in lesion length between Carrizo Citrange and McKillops Rough Lemon was only observed from Day 4. On all three days there were significant differences in lesion length between 1/31 and 28/25 compared with Symons Sweet Orange and McKillops Rough Lemon. There were no significant differences in lesion length between isolates within groups although it was evident that 28/25 was more resistant than Carrizo Citrange and Symons Sweet Orange was more susceptible than McKillops Rough Lemon.

6.3.5 Experiment 5: Chemotropism of hyphae of *P. citrophthora* to different Citrus hosts in Tissue Culture

The procedure for Experiment 5A and 5B was described in Section 6.2.5.

Experiment 5A: Shoots

Figure 6.3A shows that there was a significant difference observed between the control and the two cultivars for both Days 7 and 10 (LSD =1.37 and 0.99 respectively) which

Figure 6.2 Comparison of lesion length between parent and single zoospore–derived progeny on several Citrus cultivars.

Data from Experiment 4. Parent (P) and single zoospore–derived progeny (SS1, SS2 and SS3) taken from *P. citrophthora* isolate P1152 were examined to assess differences in pathogenicity on several different Citrus cultivars. The three cultivars included Carrizo Citrange (Carrizo), Symons Sweet Orange (Symons) and McKillops Rough Lemon (McKillops). The results for Days 4, 5, and 6 are presented. There were three replicate tubs each containing four excised shoots. Standard errors of the means are indicated by bars.

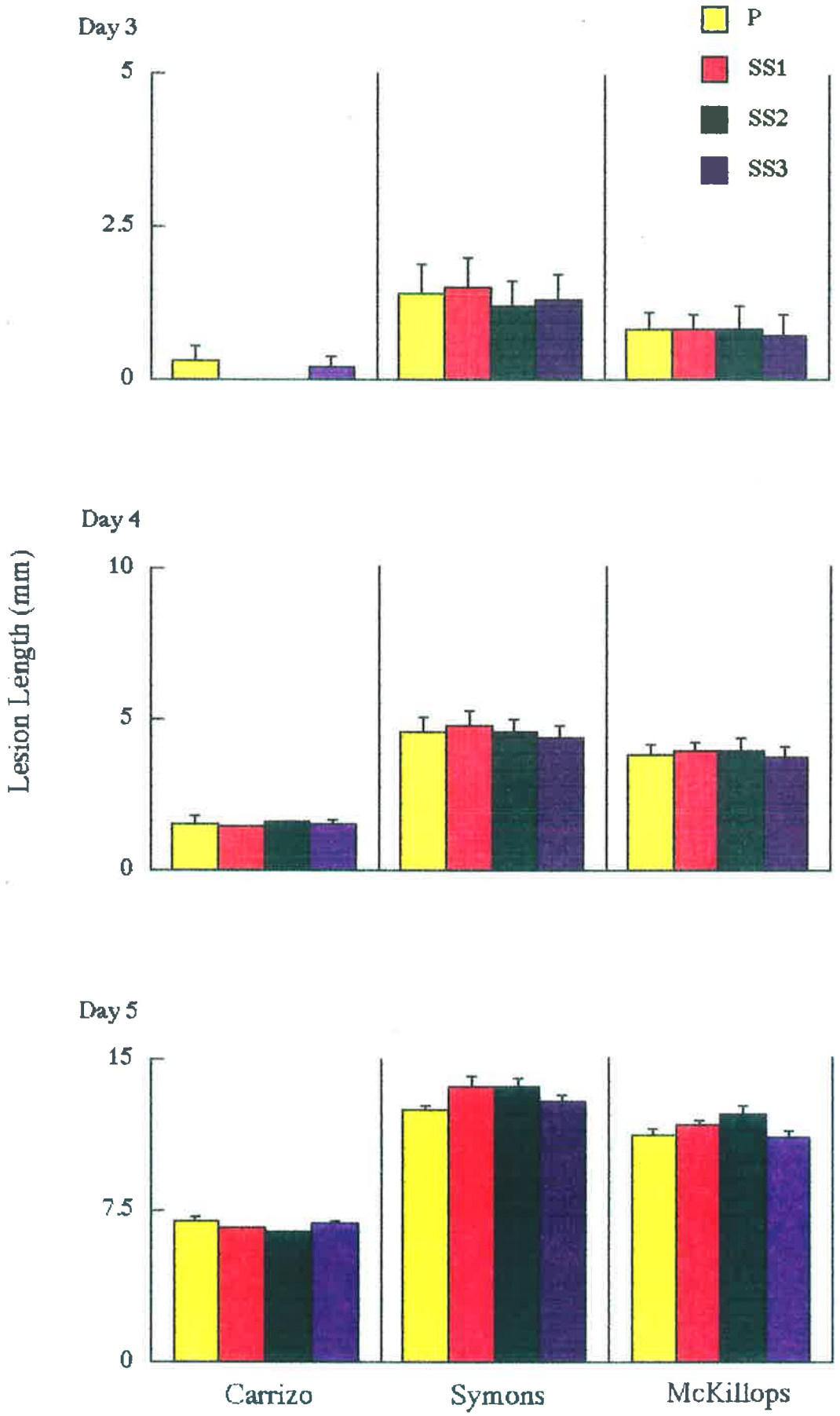

Day 3
P
SS1
SS2
SS3
5
2.5
0
Day 4
Lesion Length (mm)
10
5
0
Day 5
15
7.5
0
Carrizo
Symons
McKillops

Table 6.4: Comparison of lesion length in micropropagated shoots of several Citrus cultivars.

Shoot explants from five different cultivars (Symons Sweet Orange {Symons} McKillops Rough Lemon {McKillops} Carrizo Citrange {Carrizo}, 1/31 and 28/25) were compared for their response to infection by *P. citrophthora* (isolate P1152). There were three replicate tubs per treatment each containing four shoots. The mean and standard errors of the means are presented. a and b indicate significant differences in lesion length between cultivars (rows) at the $LSD_{0.05}$ level. Note that on Day 3 there was no significant difference in lesion length between Carrizo Citrange and McKillops Rough Lemon.

	Lesion Length (mm): Mean±SE				
Time	**Symons**	**McKillops**	**Carrizo**	**1/31**	**28/25**
Day 3	1.4^{a} ±0.48	0.8^{a} ±0.28	0.3^{ab}* ±0.25	0.0^{b} ±0.0	0.0^{b} ±0.0
Day 4	4.5^{a} ±1.08	3.8^{a} ±0.49	1.5^{b} ±0.42	1.0^{b} ±0.30	0.9^{b} ±0.42
Day 5	12.6^{a} ±0.92	11.2^{a} ±1.13	6.9^{b} ±0.69	5.7^{b} ±1.05	5.0^{b} ±0.90

indicated a significant directional growth response of the fungus towards the shoot. This interaction between the direction of fungal growth and cultivar was significant ($P<0.001$). No significant difference was observed between the two cultivars on Day 7 but on Day 10 a marginally significant difference was observed which indicated a slightly stronger growth response towards Carrizo Citrange. In addition, there was a significant interaction between time and fungal growth on Days 7 and 10.

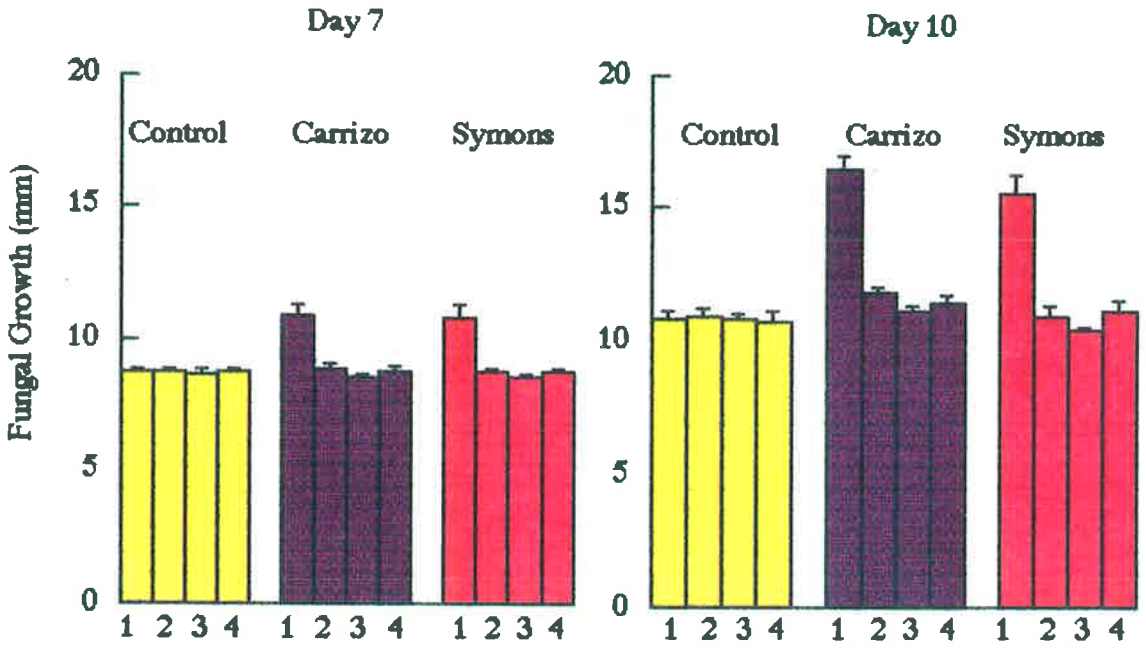
Experiment 5B: Rooted Shoots

When the experiment was conducted using rooted shoots, the results again showed a significant chemotropic effect on both Days 7 and 10 compared to the control (LSD = 0.58 and 0.90, respectively, Figure 6.3B). No significant difference was observed between resistant and susceptible cultivars on Day 7 but on Day 10 there was a marginally significant difference as observed in.5 A.

Figure 6.3 The chemotropic response of *P. citrophthora* (isolate C23) to Carrizo Citrange (resistant) and Symons Sweet Orange (susceptible) Citrus shoots.

Growth of *P. citrophthora* isolate C23 on MS basal medium was measured at four different orientations in relation to the shoots **(A)** and rooted shoots **(B)** of both Carrizo Citrange (Carrizo) and Symons Sweet Orange (Symons) Citrus cultivars: 1 = adjacent to the host; 2 = to the left of the host; 3 = opposite to the host; and 4 = to the right of the host. Control was the fungus growing in the absence of a host. Results were recorded on Days 7 and 10. The mean growth rates at each orientation are presented. Standard errors of the means are indicated by bars..

A


Day 7
Day 10
Control
Carrizo
Symons
Fungal Growth (mm)
0
5
10
15
20
1 2 3 4

B

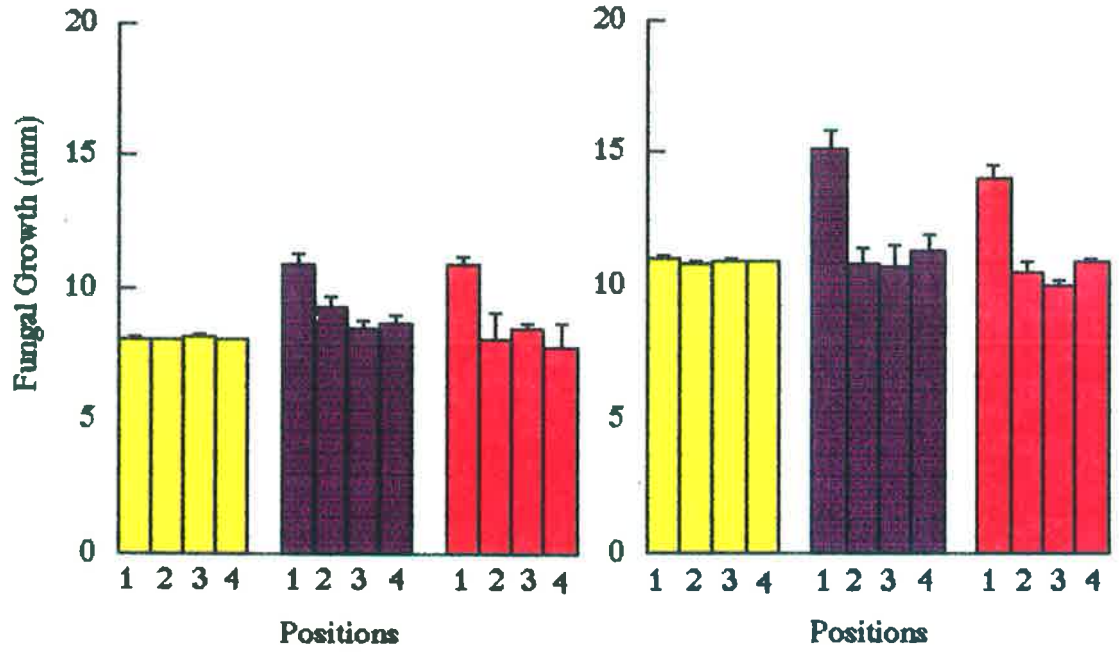

Fungal Growth (mm)
0
5
10
15
20
1 2 3 4
Positions

Plate 6.4 Chemotropic response in the presence of a Citrus host.

Rooted *Citrus* shoots were individually placed in tubs containing MS basal medium approximately 1.5 cm from one side and inoculated with a disc of agar colonised by mycelium of isolate C23 placed 2 cm from the opposite side. Control was the fungus minus the plant. Growth of the fungus was measured at 4 different orientations (clockwise) to the host plant; 1 = adjacent to the shoot, 2 = to the left of the shoot ; 3 = opposite to the plant; 4 = to the right of the shoot. The growth of the fungus is clearly greater in position 1 compared to the control where growth is uniform for all positions.


3
2
4
1

6.4 DISCUSSION

Several Citrus cultivars (Carrizo Citrange, 28/25, 1/31, Symons Sweet Orange and McKillops Rough Lemon), two of which (28/25 and 1/31) had not been previously cultured, were reliably multiplied from nodes excised from shoots. Results obtained using the modified shoot assay for screening for resistance to *Phytophthora* with micropropagated material of Carrizo Citrange, Symons Sweet Orange and McKillops Rough Lemon were in general agreement with those obtained in collar inoculation experiments in the growth cabinet (Chapter 4). In addition, the results obtained *in vitro* showed a clear distinction between rootstocks known to be susceptible (Symons Sweet Orange and McKillops Rough Lemon) and resistant (Carrizo Citrange, 28/25 and 1/31) to *P. citrophthora* infection in field and glasshouse trials (e.g. Broadbent and Gollnow, 1992).

The results in this study confirmed in part the findings of Broadbent's (1969) *in vivo* work which showed that chemotropism was evident towards both susceptible and resistant cultivars. This response was not greater towards the susceptible cultivar but the results using both rooted and non-rooted shoots suggested that by Day 10 there was a slight bias in fungal growth towards the resistant host. Therefore, it would seem unlikely that chemotropism plays a role in the susceptibility or resistance of Citrus to *Phytophthora* root or collar infection.

The modified excised shoot assay used for disease screening, was also successfully applied to examine pathogenicity of *P. citrophthora* isolates. This is the first detailed

study of this kind on the pathogenicity of *P. citrophthora* isolates. The four isolates of *P. citrophthora* tested were pathogenic on both Carrizo Citrange and Symons Sweet Orange but C6 was less aggressive than the other three isolates. This isolate had a lower growth rate on CMA at 25°C than the other three isolates (see Chapter 4). This correlation between growth rate and aggressiveness has previously been noted for isolates of *P. cinnamomi* from different host species (Robin, 1991) and for *P. cambivora* from almond (E. Scott, pers. comm.). Isolates of *P. cinnamomi* were also shown to be genetically distinct using RFLP analysis (e.g. Robin, 1991). The difference in aggressiveness between isolates was not related to differences in RFLP banding patterns, mating type, or morphological characters, observed in this study. As mentioned in Chapter 5, it may be important to examine further the effect of temperature on both pathogenicity and host resistance. In addition, any results from these tissue culture experiments should be confirmed *in vivo*.

There were no differences in pathogenicity between single spore–derived progeny compared to the parent isolate. However, further work should involve the use of a large number of single spore–derived cultures in order to confirm this finding.

Clearly there are advantages in using a tissue culture system in screening. Cultures can be maintained on defined media without the presence of contaminating organisms and under conditions which will give predictable and rapid responses. The use of mycelial inoculum in tubs, permitting four or more shoots per tub to be tested, makes this particular method efficient in terms of labour and resources. However, some problems

were encountered, particularly regarding the variability of results and the difficulty in assessing pathogenicity in tissue culture. These are discussed below.

Some of the variability in results observed can be attributed to the particular assay used for screening for resistance (Scott *et al.*, 1992). Alternative methods may be used such as applying a disc of mycelium to a wound made on the stem of an excised shoot, but this is difficult and time-consuming and may give more variable results than the modified excised shoot assay (E. Scott, pers. comm.). Alternatively zoospore inoculum may be used. The inoculation of individual shoots with zoospore suspensions has been criticised as being labour intensive and difficult to standardise (Scott *et al.*, 1992). However the technique developed in Section 3.1.5 produces good and uniform zoospore yields for isolates of *P. citrophthora*. Zoospores can be counted with a haemocytometer, washed with buffers or water, and then applied in appropriate concentrations to liquid MS medium containing rooted Citrus shoots. Recently, soybean cultivars were successfully evaluated for resistance to *Phytophthora* root rot caused by *P. megasperma* f. sp. *glycinea* based on monitoring lesion expansion on taproots inoculated with zoospores *in vitro* (Smith *et al.*,1991b). Information from this type of assay could be compared with *in vivo* root inoculation studies similar to those discussed in Chapter 5.

Variability in results may also occur because of slight changes in cultural conditions either during the culturing of the fungus or during the dual-culture phase (McComb *et al.*, 1987; Sharma and Skidmore, 1992). In the present study, results of experiments carried out at different times were not significantly different although the environment

may have altered slightly each time. Only the rate of infection was affected (slower or faster), not the relationships between the host and pathogen.

The main problem in using this type of tissue culture assay related to the difficulty in assessing pathogenicity. Development of necrosis in micropropagated shoots was more rapid in tissue culture due to the nature of the material (excised shoots) which, presumably, lacked the structural and biochemical barriers to infection operating in woody material. Tissues showed macroscopic evidence of infection only 3 days after inoculation. In susceptible cultivars there was a complete covering of the tissue pieces with mycelium within 5 or 6 days. It is important, therefore, that development of necrosis be monitored closely as shoots could rapidly be overgrown by fungal hyphae masking resistance and thereby making assessment difficult. This disadvantage has also been pointed out by Scott *et al.* (1992).

In addition, the infected host may not always produce obvious symptoms. In tissue culture this problem is accentuated because only a part of the plant is being evaluated. For example, some shoots were initially scored as 'uninfected' because they showed no necrotic symptoms but upon closer examination the pith tissue had degenerated to a watery pulp. Microscopic examination also showed evidence of infection.

An interesting observation in this study was the different ways in which the resistant and susceptible cultivars responded in culture. In the susceptible cultivars, the symptoms were evident early on at the stem tip with necrosis and browning of the leaves. The

resistant hosts rarely showed symptoms at the tip, but infection was observed at the base of the stem and developed upwards. Microscopic examination and plating segments of stem tissue onto selective medium revealed that infection in susceptible cultivars progressed much more quickly than in resistant cultivars. Further microscopic evaluation of the infection process, comparing resistant and susceptible plants, may help to clarify the mechanisms of resistance responsible.

The conditions for detecting expression of resistance in cultured tissue may vary with the host and the pathogen. The performance of the host is dependent upon various factors including genotype, the time of year the explant was collected and culture conditions. Studies with *P. parasitica* var. *nicotianae* grown on tobacco callus culture support this suggestion (Helgeson *et al.*, 1972). They revealed that 20°C gave the biggest difference between resistant and susceptible hosts to infection by *P. parasitica* var. *nicotianae*. The results from Chapter 5, however, suggest that pathogenicity testing should be done under conditions conducive to infection in the field in order to achieve a more realistic appraisal.

Growth conditions for the pathogen are also critically important. The pathogen must be maintained free of other microorganisms and on a medium which gives reasonable yields of mycelium and infective units (e.g. zoospores). Aggressiveness may vary if phytohormones are supplied in the medium. This was indicated by the results in Experiment 2A. Scott *et al.* (1992) found no such effect when assessing almond rootstocks for resistance to *P. cambivora*.

In general, this study indicates that tissue culture techniques can be used to shorten traditional procedures of screening for disease resistance. Material showing resistance or tolerance can be selected more quickly *in vitro* and material shown to be susceptible can be discarded. In this way, only putative resistant material would need to be further evaluated under glasshouse or orchard conditions. Tissue culture techniques were also successfully used to examine host–pathogen relationships. The further extension of tissue culture techniques to induce the sexual stage of *P. citrophthora* is discussed in the following chapter.

7. THE SEXUAL STAGE OF *P. CITROPHTHORA*

7.1 INTRODUCTION

In recent years great interest has been shown in elucidating the mechanisms by which variation occurs within a pathogen population, including somatic and sexual recombination (see Section 2.4). The major problem in studying mechanisms of variation in *P. citrophthora* has been generating the sexual stage. There have been only a few reports of the production of *P. citrophthora* oospores in culture. However, the majority of these described matings were either between isolates from hosts other than Citrus, such as Cocoa, and therefore may have involved other *Phytophthora* spp (Ferguson, 1976; Kellam and Zentmyer, 1986) or between interspecific crosses between *P. citrophthora* and other *Phytophthora* spp. (Ferguson, 1976; Savage *et al.*, 1968). There has been only one report which described oospores produced specifically in Citrus isolates of *P. citrophthora* (Telhada, 1990).

The characters of the sexual stage are of taxonomic importance in the classification of *Phytophthora* species. These include: 1) whether the isolate produces oospores in single (homothallic) or mixed (heterothallic) cultures; 2) the type of antheridium (whether paragynous or amphigynous); 3) the abundance of oogonia on defined media; 4) the size of oogonia and 5) the thickness of the oogonium wall. The culture medium has been shown to influence all of these characters (see Section 2.4.3). In most of the taxonomic keys there is no description of sex organs for *P. citrophthora* (Stamps *et al.*, 1990; Waterhouse, 1963) with the exception of Ribeiro (1978) in which *P. citrophthora* is

characterised as heterothallic, having amphygynous antheridia ranging in size from 14 – 14.5μm and oogonial diameter ranging from 26 – 36μm. However, these characters have been largely based on oospores derived from interspecific crosses. No detailed taxonomic study has been made of oospores produced from intraspecific matings of *P. citrophthora* because of the problem of inducing the sexual stage in this species. Thus the use of morphological criteria to classify *P. citrophthora* has been limited to characters of the asexual stage.

In general, it is accepted that for conditions to be conducive for the production of oospores, matings should take place in the dark at temperatures below those optimum for growth (Elliot, 1983). Investigations involving *P. citrophthora* have used complex media such as lima bean (Savage *et al.*, 1968), carrot (Kellam and Zentmyer, 1986ab) or hemp seed agar (Ferguson, 1976), although Telhada (1990) used a synthetic medium for the formation of oospores. Hemp seed agar was used because this natural medium contains large amounts of sterols. It has been suggested that both the nitrogen source and an exogenous source of sterols (of the 3–beta–hydroxy type) are important for the sexual stage to occur (Elliott, 1983). There has been some interest in using naturally infected host material to induce the formation of oospores in heterothallic species; Kellam and Zentmyer (1986a) inoculated Cocoa pods to produce oospores of *P. citrophthora* , *P. palmivora* and *P. capsici.* For some fungal species, tissue culture techniques have been used with varying success to induce the sexual stage; for example, callus culture was used to induce the different stages of the life cycle in *P. brassicae* (Tommerup and Ingram, 1971). The advantage in this technique is that spores could be obtained free

from contaminating organisms, with the plant providing a natural source of nutritional requirements.

Low and inconsistent frequency of germination of oospores is a further obstacle in genetic studies of *Phytophthora* (see Section 2). In general, oospores of self–inducing (homothallic) species are easier to germinate than those of cross–inducing (heterothallic) species (Zentmeyer and Erwin, 1970). Ann and Ko (1988) developed a technique for the heterothallic species, *P. parasitic*a, in which more than 90% of oospores germinated. Treatment with $KMnO_4$, exposure to light during maturation and germination and the use of a defined medium appeared to be the most important factors.

The main aim of this study was to establish mating types for specific *P. citrophthora* isolates from Citrus using previously tested techniques as well as evaluating the potential of using tissue culture techniques to produce oospores. An additional aim was to compare the morphological characteristics of the sexual stage in order to examine the extent of variation in size and abundance of oogonia and size of oospores and antheridia and the usefulness of these characters as taxonomic indicators for the species. A final aim was to induce germination of oospores of *P. citrophthora* which has not previously been attempted.

7.2 EXPERIMENTAL PROCEDURES

7.2.1 Development of Techniques for Oospore Production

P. citrophthora isolates previously defined (see Table 3.1) as belonging to either A1 or A2 mating type were crossed with isolates for which the mating type was unknown. Two methods for producing oospores on plates were compared. In the first method, an uninoculated CA disc was "sandwiched" between two isolates growing on CA discs (see Section 3.1.6). There were four stacks of discs per cross. The experiment was repeated using a thicker stack of carrot agar discs (individual discs were 4 instead of 3mm) and the presence of oospores was assessed after 56 instead of 28 days. In the second method, isolates were grown together on hemp seed agar (HSA) plates and assessed after 28 and 56 days (see Section 3.1.6). There were four HSA plates per cross. Controls to test for homothallic oospore production were included for all isolates in both experiments.

A pilot study was also undertaken to test if oospores could be produced in tissue culture of Citrus. Carrot agar discs containing *P. citrophthora* of different mating types were used to inoculate a susceptible Citrus cultivar, Symons Sweet Orange (see Plate 7.1). The method is described in detail in Section 3.1.6. Only one mating combination was tested (C6 x P3382). These isolates were chosen because they had produced abundant oospores in culture using the carrot agar sandwich method and had been characterised in terms of RFLP markers. A susceptible Citrus cultivar was used because it was considered less likely to contain inhibiting metabolites that may be present in resistant cultivars. A two-way factorial experiment was performed where there were 3 mating

Plate 7.1 The production of oospores in tissue culture.

Two carrot agar discs each containing a different *P. citrophthora* isolate (or for controls, the same isolate) were placed at the base of a Symons Sweet Orange shoot (rooted) in 0.5% water agar (indicated by an arrow). Inoculated plant material was placed at 24°C for 24 hr in the dark and then incubated at 21°C in the dark for 56 days.

combinations (including 2 controls) x 2 treatments (rooted and unrooted shoots). There were 4 replicates per treatment. The presence of oospores was examined after 56 days. The number of oospores was estimated as follows: Agar or agar plus plantlet was transferred to a sterile petri dish and sterile coverslips were placed over the top. The number of oospores per mating combination was counted at 100x magnification using a Leitz Orthoplan compound microscope. The following rating scale was applied. No statistical analysis was performed on the data.

abundant	= >50 oospores per replicate
intermediate	= 10 – 50 oospores per replicate
scarce	= 1 – 10 oospores per replicate

7.2.2 Analysis of Oospore Morphology

Based on the results in 7.3.2, isolates were classified into 2 groups (A1 or A2 mating type). From successful matings between these two groups, the oospore, oogonial and antheridial breadth and length were measured. On average 10 replicates were examined.

Not every combination of A1 and A2 mating types was paired so when fitting the statistical model the unbalanced nature of the experimental design was taken into account. Thus, in each case the following models were fitted:

$$y_{ijk} = A_{1i} + A_{2j} + (A_1A_2)_{ij} + E_{ijk}$$

Where y_{ijk} = response variate
A_{1i} = A1 isolates (13)
A_{2j} = A2 isolates (9)
$(A_1A_2)_{ij}$ = interaction between A1 and A2 isolates
E_{ijk} = error term
k = no. of replicates

The reciprocal model was also fitted where the order of inclusion of A1 and A2 isolates was interchanged. The terms are defined as for the above:

$$y_{ijk} = A_{2i} + A_{1j} + (A_1A_2)_{ij} + E_{ijk}$$

The oospore, oogonial and antheridial breadth and length measurements were analysed using analysis of variance.

7.2.3 Germination of Oospores

The experimental design was completely randomised with six different mating combinations and three different light treatments arranged as a two–way factorial. The

mating pairs were chosen at random from the material available in the carrot agar sandwich experiment. The six different isolate combinations were as follows: PAL41 x P318, PMO52 x C6, PAL 7 x C6, P318 x P3367, PAL 41 x C6 and C6 x P3367. The oospores were isolated and plated onto S+L medium according to the method of Ann and Ko (1988, refer to Section 3.1.7). Oospores were then incubated at 20 –21°C under blue light (NEC FL15BL, 300 – 40nm), white light (NEC 15W) or in darkness. There was a maximum of four plates of each mating combination per treatment containing an average of 50 oospores per plate. The number of germinated and non–germinated oospores was counted at 7 and 42 day intervals. For the analysis, a binomial model was fitted to test for the effects of the different treatments.

A problem with isolating oospores produced in tissue culture of Citrus was that the concentration of oospores was very low and they were embedded in 10 ml of water agar. Therefore, Ann and Ko's (1988) technique for isolating oospores could not be used. Instead, the oospores were located microscopically under 100x magnification and, under aseptic conditions, a wire "spoon" was used to "scoop out" single oospores. One oospore was placed on a plate containing S+L medium (as described in Section 3.1.2) and incubated at 21°C under white light. In this way, the oospore was isolated from other fungal fragments or sporangia which could germinate and confound results. In addition, C6 and P3382 were also crossed using the CA sandwich technique as a positive control.

7.3 RESULTS

7.3.1 Evaluation of Methods for Oospore Production

It was found that only the CA sandwich method produced oospores. In general, depending on the mating combination, there was variation in the amount of oospores produced (see Table 7.1). No oospores were produced using the HSA method although many of the pairings showed interaction with the hyphae looping around one another.

Of the 117 pairings, 57 produced oospores. From the successful pairings, isolates could be assigned to A1 or A2 mating type. There were 14 A1 and 8 A2 classifications (Table 7.2). Isolates that could not be so characterised (P6806 and P6826) may have required more time, different mating combinations or different environmental conditions to produce oospores. For example, isolate P6698 produced oospores at 26°C (in one experiment the thermostat in the incubator failed and the temperature reached 26°C) but would not produce oospores at 21°C. However, this observation would need to be verified by further experimentation. In addition, one isolate, PAL 7, may have been incorrectly classified as an A2 mating type (refer to Table 3.1) because in this study, PAL 7 mated only with A2 isolates and therefore would appear to be an A1 mating type. All controls, testing for homothallic oospore formation, were negative, including for PAL 7. Therefore it is unlikely that the results were a consequence of this isolate being homothallic.

Table 7.1 Abundance of oospores in *P. citrophthora* matings

Agar discs from the CA sandwich technique described in Section 3.1.6 were transferred to a sterile petri dish and sterile coverslips were placed on top. The number of oospores per mating combination was counted at 100x magnification using a Leitz Orthoplan compound microscope using the following rating scale: A = abundant (>50 oospores per replicate); I = intermediate (10 – 50 oospores per replicate); S = scarce (1 – 10 oospores per replicate). – indicates missing values.

		A2 Mating Type							
		C6	D64660	P3078	P3693	P3434	PAL45	P0318	CBS289
A1 Mating Type	P3367	I	I	I	I	S	S	I	–
	C23	S	–	–	–	–	S	I	–
	P1152	I	–	–	–	–	S	I	–
	P1163	S	–	–	–	–	S	A	–
	P3382	A	–	–	–	–	I	I	–
	P6708	I	–	–	–	–	–	S	–
	P3418	S	–	–	–	–	–	S	–
	PAL7	A	A	I	A	A	I	A	–
	PAL41	A	I	S	S	I	I	S	–
	PMO52	S	I	S	I	S	S	S	–
	P3435	I	I	A	A	I	–	–	–
	PAL2	–	–	–	I	S	–	–	–
	P3582	S	–	–	–	–	–	–	–
	P6698	S	–	–	S	–	–	–	–

Table 7.2 Classification of *P. citrophthora* isolates derived from Citrus hosts into A1 and A2 mating types.

P. citrophthora isolates that had been characterised previously as belonging to either A1 or A2 mating types were crossed with isolates whose mating type was unknown. The carrot agar sandwich method was used as described in Section 3.1.6. Oospores formed only in matings where both compatibility types were present (A1 and A2). *PAL 7 was originally classified as an A2 mating type (refer to Table 3.1).

MATING TYPE	
A1	**A2**
P3367	P318
P3435	C6
PAL 41	PAL 45
PAL 2	P3693
PAL 7*	P3078
PMO 52	P3434
P3382	D64660
P3418	CBS 289
P1152	
P1163	
P6708	
C23	
P3582	
P6698	

Table 7.3 The production of oospores in tissue culture of Citrus.

The method and conditions for producing oospores in culture are given in Section 3.1.6. Only one mating combination (C6 x P3382) and controls (C6, P3382) were used to inoculate rooted and non-rooted Symons Sweet Orange shoots. There were four replicates per treatment. Each treatment was assessed individually for oospores using 100x magnification using a Leitz Orthoplan compound microscope and a rating system applied. +/− = the presence or absence of oospores; NR = non rooted and R = rooted shoots; Scarce = 1 – 10 oospores per replicate

Isolate	Replicate No.	Shoots NR	Shoots R	Oospore No.
C6	1	–	–	
	2	–	–	
	3	–	–	
	4	–	–	
P3382	1	–	–	
	2	–	–	
	3	–	–	
	4	–	–	
C6 x P3382	1	–	–	
	2	–	+	Scarce
	3	–	+	Scarce
	4	–	+	Scarce

Oospores were produced in tissue cultures of Citrus but only in the presence of rooted shoots (see Table 7.3). In addition, C6 and P3382, which produced abundant oospores using the CA sandwich technique, produced few in tissue culture (Table 7.1 compared with Table 7.3) suggesting that conditions need to be further improved.

7.3.2 Comparison of Oospore Characters between *P. citrophthora* Matings

Oospores formed in the region where the hyphae of the two isolates were in contact. Oospores were usually thick-walled, single in the oogonium and had amphigynous antheridia. There were significant differences between matings for all oospore characters examined. In general, oospores were highly variable in shape and size and while some had a golden brown pigment, others were non-pigmented (refer to Plate 7.2).

Oospore breadth and length varied between matings (see Table 7.4), although in some cases size may have been related to the maturity of the oospore. This variability in size was dependent on the specific combination of isolates used in matings and was termed "specific mating ability". For example, P6698 x C6 had the largest oospore mean breadth (27.5μm) and mean length (26.1μm) and PMO52 x P0318 had the smallest oospore mean breadth (16.07μm) and mean length (16.07μm) compared to other mating combinations. Differences in sizes between matings were also noted for the oogonial and antheridial breadth and length measurements (Table 7.5 and Table 7.6 respectively). Overall the results were highly significant ($P<0.001$) in all cases. However, no individual comparisons were made between matings as multiple t-tests used for this purpose may show false significant differences.

Plate 7.2 Variation in oospore morphology of *P. citrophthora*.

Oospores were produced as described in Section 3.1.6. Oospores were examined under 250x magnification using a Leitz Orthoplan compound microscope. **(A)** P3367 x P318; **(B)** PMO52 x P3434; **(C)** C6 x PAL 7 ; **(D)** PAL 41 x P3434. Note that **a** = the antheridium, **b** = the oogonium and **c** = the oospore. Bar represents 20 μm.

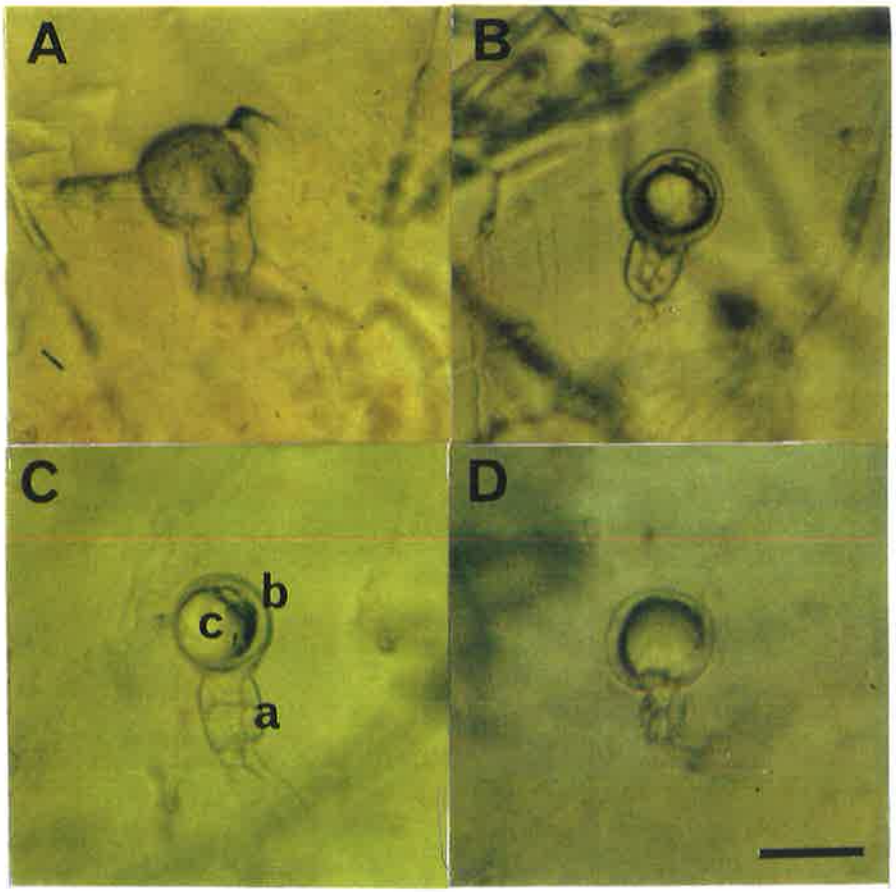

A
B
C
b
c
a
D

Table 7.4. Breadth and length of oospores of *P. citrophthora* produced in matings between A1 and A2 isolates

Oospores were produced as described in Section 3.1.6. Ten oospores were measured per cross. Oospore breadth (A) and length (B) measurements were made under 250x magnification using a Leitz Orthoplan compound microscope. The means and standard errors of the means (±) are presented. – indicates no oospores observed. Overall, the results were highly significant (P<0.001). No individual comparisons were made between matings.

A. Oospore Breadth (μm)

A1 Mating Type	A2 Mating Type: C6	D64660	P3078	P3693	P3434	PAL 45	P0318	CBS 289
P3367	17.83 ± 1.37	20.09 ± 0.72	23.27 ±0.95	20.30 ±1.08	20.30 ±1.08	19.03 ±1.77	23.14 ±1.17	–
C23	21.15 ±1.34	–	–	–	–	17.45 ±1.59	18.19 ±0.52	–
P1152	20.81 ±0.21	–	–	–	–	21.57 ±0.42	19.03 ±1.50	–
P1163	20.73 ±1.04	–	–	–	–	18.19 ±1.27	18.02 ±1.18	–
P3382	20.30 ±0.85	–	–	–	–	16.67 ±1.43	21.66 ±1.16	–
P6708	20.05 ±1.88	–	–	–	–	–	19.46 ±1.23	–
P3418	20.09 ±1.06	–	–	–	–	–	19.97 ±0.41	–
PAL 7	21.15 ±1.81	19.88 ±1.08	18.19 ±0.85	19.88 ±1.58	19.88 ±1.43	19.03 ±1.16	19.74 ±1.18	–
PAL 41	19.46 ±1.82	19.71 ±1.82	19.03 ±0.95	19.03 ±1.77	20.73 ±0.42	20.73 ±0.42	19.29 ±0.98	–
PMO 52	21.15 ±0.0	20.30 ±1.43	22.0 ±0.85	18.61 ±1.40	18.19 ±1.58	20.56 ±1.69	16.07 ±1.72	–
P3435	19.46 ±1.04	18.19 ±1.84	18.19 ±1.08	19.88 ±1.72	18.19 ±0.85	–	–	–
PAL 2	–	–	–	17.34 ±1.55	20.87 ±1.25	–	–	–
P3582	18.61 ±1.23	–	–	–	–	–	–	–
P6698	27.50 ±1.22	–	–	24.32 ±1.06	–	–	–	–

B. Oospore Length (μm)

A1 Mating Type	A2 Mating Type: C6	D64660	P3078	P3693	P3434	PAL 45	P0318	CBS 289
P3367	17.52 ±0.89	19.39 ±1.01	22.42 ±1.27	19.46 ±0.79	20.73 ±1.04	19.03 ±1.77	21.26 ±0.67	–
C23	18.19 ±2.28	–	–	–	–	17.45 ±0.53	18.61 ±1.04	–
P1152	21.57 ±0.79	–	–	–	–	22.0 ±0.52	19.03 ±1.34	–
P1163	18.61 ±0.79	–	–	–	–	19.88 ±0.52	17.60 ±1.50	–
P3382	20.0 ±0.52	–	–	–	–	17.34 ±1.04	21.40 ±1.43	–
P6708	19.80 ±0.77	–	–	–	–	–	19.03 ±1.16	–
P3418	20.09 ±1.06	–	–	–	–	–	20.81 ±0.21	–
PAL 7	22.21 ±1.62	19.46 ±0.79	18.61 ±1.23	19.03 ±0.67	20.30 ±1.43	20.30 ±0.52	19.25 ±1.30	–
PAL 41	18.19 ±1.58	19.88 ±1.27	20.30 ±1.08	21.15 ±1.16	22.84 ±1.04	20.13 ±0.82	18.61 ±1.23	–
PMO 52	20.73 ±0.42	19.88 ±1.43	20.30 ±0.85	17.77 ±1.27	17.51 ±1.23	20.13 ±0.82	16.07 ±1.72	–
P3435	19.46 ±1.23	18.19 ±1.43	16.50 ±0.42	19.46 ±1.94	19.03 ±0.95	–	–	–
PAL 2	–	–	–	16.50 ±1.82	21.85 ±0.71	–	–	–
P3582	19.46 ±1.23	–	–	–	–	–	–	–
P6698	26.09 ±1.87	–	–	23.26 ±0.0	–	–	–	–

Table 7.5 **Breadth and length of oogonia of *P. citrophthora* produced in matings between A1 and A2 isolates**
Oogonia were produced as described in Section 3.1.6. Ten oogonia were measured per cross. Oogonial breadth (A) and length (B) measurements were made under 250x magnification using a Leitz Orthoplan compound microscope. The means and standard errors of the means (±) are presented. – indicates no oogonia observed. Overall, the results were highly significant ($P<0.001$). No individual comparisons were made between matings.

A. Oogonial Breadth (μm)

A1 Mating Type	A2 Mating Type: C6	D64660	P3078	P3693	P3434	PAL45	P0318	CBS 289
P3367	24.47 ±1.21	27.0 ±0.78	30.46 ±0.85	25.38 ±0.67	24.53 ±0.85	25.38 ±0.67	28.93 ±0.87	–
C23	25.55 ±0.17	–	–	–	–	24.32 ±0.61	25.38 ±0.0	–
P1152	25.80 ±0.42	–	–	–	–	27.07 ±0.79	26.65 ±0.85	–
P1163	27.50 ±0.95	–	–	–	–	25.80 ±0.42	25.55 ±0.17	–
P3382	26.23 ±0.52	–	–	–	–	23.69 ±0.79	26.40 ±0.82	–
P6708	27.92 ±0.79	–	–	–	–	–	24.96 ±1.04	–
P3418	24.32 ±1.06	–	–	–	–	–	25.38 ±0.0	–
PAL 7	29.05 ±1.64	26.23 ±0.52	25.80 ±0.42	25.80 ±1.04	25.80 ±0.42	24.96 ±1.04	25.73 ±1.01	–
PAL 41	28.76 ±1.58	24.96 ±0.42	24.96 ±0.79	25.97 ±0.79	27.92 ±1.04	25.80 ±0.42	23.10 ±0.96	–
PMO 52	26.23 ±1.08	26.23 ±0.85	28.34 ±0.85	26.23 ±0.52	24.96 ±0.42	25.38 ±1.34	26.23 ±1.08	–
P3435	25.38 ±0.0	23.86 ±0.94	22.84 ±0.79	24.53 ±1.08	24.96 ±1.04	–	–	–
PAL 2	–	–	–	24.96 ±0.79	26.09 ±0.71	–	–	–
P3582	24.11 ±0.52	–	–	–	–	–	–	–
P6698	24.68 ±±0.71	–	–	20.09 ±3.17	–	–	–	–

B. Oogonial Length (μm)

A1 Mating Type	A2 Mating Type: C6	D64660	P3078	P3693	P3434	PAL45	P0318	CBS 289
P3367	23.87 ±1.10	24.68 ±0.71	29.61 ±1.16	24.53 ±0.52	26.23 ±1.08	25.28 ±0.67	26.23 ±0.77	–
C23	26.23 ±0.85	–	–	–	–	23.79 ±1.01	25.38 ±0.67	–
P1152	25.80 ±0.42	–	–	–	–	27.50 ±0.95	26.65 ±1.08	–
P1163	25.38 ±1.16	–	–	–	–	27.07 ±0.79	26.23 ±0.52	–
P3382	26.23 ±0.52	–	–	–	–	24.53 ±0.85	26.23 ±0.85	–
P6708	27.07 ±1.40	–	–	–	–	–	25.80 ±0.42	–
P3418	26.44 ±3.17	–	–	–	–	–	25.38 ±0.0	–
PAL 7	29.75 ±1.57	26.23 ±0.52	25.80 ±0.42	24.96 ±0.79	25.80 ±0.42	26.23 ±0.52	25.03 ±1.01	–
PAL 41	27.50 ±0.95	24.11 ±0.52	24.96 ±0.79	27.07 ±0.79	27.50 ±0.95	25.38 ±0.67	22.84 ±1.04	–
PMO 52	25.80 ±0.79	26.23 ±0.85	27.50 ±0.95	24.96 ±0.79	24.96 ±0.42	25.38 ±0.67	26.23 ±1.08	–
P3435	26.65 ±0.85	22.84 ±0.79	22.42 ±0.52	24.11 ±1.27	25.80 ±1.14	–	–	–
PAL 2	–	–	–	24.62 ±1.14	24.68 ±1.87	–	–	–
P3582	23.69 ±0.42	–	–	–	–	–	–	–
P6698	21.85 ±0.71	–	–	20.09 ±3.17	–	–	–	–

Table 7.6 **Breadth and length of antheridia of *P. citrophthora* produced in matings between A1 and A2 isolates**
Antheridia were produced as described Section 3.1.6. Ten antheridia were measured per cross. Antheridial breadth (A) and length (B) measurements were made under 250x magnification using a Leitz Orthoplan compound microscope. The means and standard errors of the means (±) are presented. – indicates no antheridia observed. Overall, the results were highly significant (P<0.001). No individual comparisons were made between matings.

A. Antheridial Breadth (μm)

A1 Mating Type	A2 Mating Type: C6	D64660	P3078	P3693	P3434	PAL45	P0318	CBS 289
P3367	15.71 ±0.63	13.40 ±0.45	13.54 ±1.08	13.96 ±0.89	14.38 ±1.04	14.81 ±1.64	17.89 ±0.43	–
C23	16.50 ±1.40	–	–	–	–	14.49 ±0.92	16.50 ±0.42	–
P1152	14.97 ±0.54	–	–	–	–	13.54 ±0.58	14.13 ±1.24	–
P1163	14.81 ±0.95	–	–	–	–	13.96 ±1.27	13.71 ±0.47	–
P3382	17.34 ±1.04	–	–	–	–	12.27 ±0.42	13.96 ±0.52	–
P6708	12.69 ±0.27	–	–	–	–	–	14.55 ±0.72	–
P3418	14.81 ±2.11	–	–	–	–	–	15.23 ±0.42	–
PAL 7	15.16 ±1.01	13.37 ±0.90	11.59 ±0.92	13.03 ±0.21	13.71 ±0.82	14.81 ±0.67	13.54 ±0.48	–
PAL 41	14.81 ±0.67	13.54 ±1.08	14.55 ±0.72	14.38 ±0.79	13.54 ±0.85	15.65 ±0.85	13.37 ±0.32	–
PMO 52	16.92 ±0.67	13.11 ±0.42	13.96 ±1.08	14.38 ±1.04	13.28 ±0.49	14.81 ±0.95	13.79 ±0.93	–
P3435	16.50 ±0.42	14.38 ±1.04	13.79 ±0.92	14.81 ±0.647	12.44 ±0.49	–	–	–
PAL 2	–	–	–	13.28 ±0.79	16.22 ±2.54	–	–	–
P3582	14.81 ±0.95	–	–	–	–	–	–	–
P6698	15.51 ±0.70	–	–	12.69 ±0.80	–	–	–	–

B. Antheridial Length (μm)

A1 Mating Type	A2 Mating Type: C6	D64660	P3078	P3693	P3434	PAL45	P0318	CBS 289
P3367	13.90 ±0.52	11.28 ±0.79	11.0 ±0.79	11.76 ±0.99	16.07 ±1.58	12.27 ±0.42	16.92 ±0.70	–
C23	16.07 ±0.52	–	–	–	–	14.49 ±1.28	15.65 ±0.52	–
P1152	12.69 ±1.34	–	–	–	–	11.00 ±0.79	14.55 ±0.98	–
P1163	16.67 ±1.20	–	–	–	–	12.52 ±0.81	13.96 ±1.27	–
P3382	16.07 ±0.52	–	–	–	–	13.11 ±1.04	13.62 ±1.12	–
P6708	11.59 ±0.70	–	–	–	–	–	16.67 ±1.43	–
P3418	16.92 ±2.11	–	–	–	–	–	13.71 ±1.06	–
PAL 7	17.27 ±1.27	14.13 ±0.80	14.81 ±1.50	13.54 ±1.08	17.51 ±1.40	15.23 ±0.79	14.45 ±1.27	–
PAL 41	14.38 ±0.79	12.27 ±1.23	13.54 ±0.85	11.84 ±1.72	13.54 ±1.27	13.54 ±1.27	13.96 ±0.85	–
PMO 52	16.07 ±0.52	13.11 ±0.79	13.96 ±0.52	15.65 ±1.30	15.48 ±1.30	13.11 ±1.23	13.54 ±1.08	–
P3435	14.81 ±1.50	14.21 ±0.94	15.65 ±0.52	13.37 ±0.68	12.94 ±0.83	–	–	–
PAL 2	–	–	–	13.11 ±0.79	15.51 ±0.70	–	–	–
P3582	15.23 ±0.79	–	–	–	–	–	–	–
P6698	12.69 ±0.00	–	–	14.38 ±1.69	–	–	–	–

In addition, the relationship between breadth and length was positive (i.e. oospores with large breadth also had large length measurements), for example, P3367 x P318, P3367 x P3078, P3382 x C6, PAL 41 x SON 35, and P1152 x PAL 45. This interaction between breadth and length was significant for oospore and oogonial measurements ($P<0.05$ and $P<0.001$ respectively) and for antheridial measurements ($P<0.01$). Furthermore, measurements of oospore and oogonial dimensions were strongly related, whereas the antherial dimensions had little relationship with either the oospore or oogonial dimensions.

7.3.3 Germination of Oospores

Statistical analysis of the data in Table 7.7 showed a significant increase in the number of germinated oospores at Day 42 compared to Day 7 ($P<0.001$). In addition, a significant interaction was found between mating combination and light treatment ($P<0.001$). For example, both blue and white light had a positive effect on germination when C6 was combined with PAL 7, but blue light was less effective when C6 was paired with PMO 52 and had no effect when C6 was paired with PAL 41. In addition, when PAL 41 was combined with P318, the dark treatment was more effective than the blue or white light treatments, however, when PAL 7 was combined with C6 the reverse was true. The oospores germinated by single or multiple germ tubes or by germ tubes terminating in sporangia (see Plate 7.3).

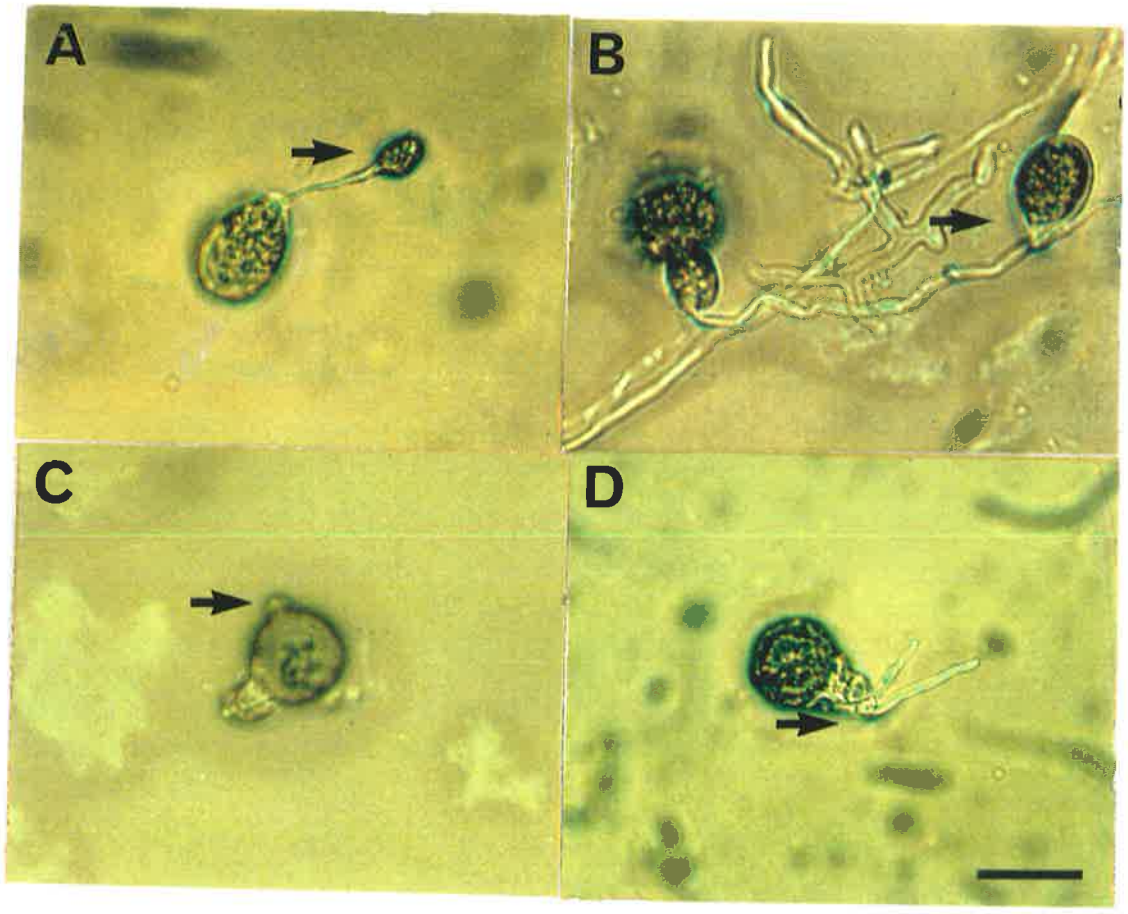
Table 7.7 Percentage of oospores of *P. citrophthora* isolates germinating under different light treatments at 7 and 42 days.

Oospores were isolated and plated onto S+L medium according to the method of Ann and Ko (1988, refer to Section 3.1.7) and incubated at 20 –21°C under dark (D), blue light (B) and white light (W) treatments. There were four replicates per treatment, each replicate containing an average of 50 oospores per plate. Plates were examined for germinated oospores at 7 and 42 days. Note that the dark treatment was omitted for some of the mating combinations because of insufficient oospores. a and b indicates significant differences between isolates for mating combination by light treatment (columns) at the $LSD_{0.05}$ level.

Mating Pairs	Treatment	%Germination	
		Day 7	Day 42
PAL 41 x P318	D	0.00[a]	7.40[b]
	B	0.00[a]	1.80[a]
	W	0.50[a]	1.50[a]
PMO 52 x C6	D	0.00[a]	0.00[a]
	B	0.00[a]	4.60[a]
	W	1.43[a]	8.60[ab]
PAL 7 x C6	D	1.06[a]	2.10[a]
	B	0.93[a]	13.1[b]
	W	0.46[a]	10.1[b]
P318 x P3367	B	0.00[a]	2.80[a]
	W	1.40[a]	6.80[ab]
PAL 41 x C6	B	0.00[a]	0.00[a]
	W	5.00[b]	10.0[b]
C6 X P3367	B	0.00[a]	6.30[ab]
	W	0.00[a]	9.10[b]

Plate 7.3. Germination of oospores of *P. citrophthora*.

Oospores were germinated according to the method by Ann and Ko (1989) as described in Section 3.1.7. **(A)** Sporangial germination (C6 x P3367) compared with **(B)** oospore germination by a germ tube terminating in a sporangium (PMO52 x C6, white light treatment). **(C)** and **(D)** show examples of oospore germination by germ tube formation (PAL 41 x P318, white light treatment and PAL 41 x C6, white light treatment, respectively). Bar represents 20 μm.


A
B
C
D

No germination of oospores, isolated from tissue culture, was observed after 56 days. However, only ten oospores were examined because the number of oospores produced in tissue culture of Citrus was very low (see Section 7.3.1).

7.4 DISCUSSION

This study has resulted in some important advances in our understanding of the sexual stage in *P. citrophthora*. Oospores were produced successfully between the majority of the *P. citrophthora* isolates tested in this study using both the carrot agar sandwich technique (developed by Kellam and Zentmyer, 1986a) and tissue culture techniques. Oospore germination was induced for the first time and a detailed morphological examination of *P. citrophthora* oospores carried out. In addition, no oospores were produced in matings where the same isolate was present on both discs, which supports the notion that there is no homothallism in *P. citrophthora* (Ribeiro, 1978).

Isolates P3367 and P3434, previously described as being the same isolate (P717, see Table 3.1) and supposedly the same mating type, were found to be A1 and A2 mating types, respectively. Change of mating type in cross-inducing species of *Phytophthora* may occur after long-term storage (Ko, 1981). However, it may be that these isolates were coded incorrectly, which highlights the need to check carefully cultures derived from other collections and to note inconsistencies for future reference.

The use of tissue culture techniques to induce the sexual stage was not as successful as the CA sandwich technique. The low frequency of oospores found on infected host tissue supports the results obtained by other workers. For example, after a minimum of 5 weeks only 1 to 20 oospores per cocoa pod were observed in culture (Kellam and Zentmyer, 1986). Obviously more work is needed to define optimal conditions for oospore production in tissue cultures of host species. An interesting observation from this study was that oospores formed only in rooted shoots. However, because of the limited amount of replicated plant material and low numbers of oospores, it was not possible to determine whether rooted shoots were significantly different from non–rooted shoots in terms of their effect on oospore production. These preliminary results indicate a useful area for further research.

There was considerable variation in the morphology of the oospores produced in matings of *P. citrophthora* isolates in this study. However, in spite of this, the oogonial measurements fell within the range found by Ribeiro (1978). Oogonial size has been used to distinguish between other *Phytophthora* species. For example, small oogonia are typically found in some species (< 23μm for breadth and length, e.g. *P. heveae*) in contrast to other species which are characterised by having large oogonial sizes (> 50 μm for breadth and length e.g. *P. cambivora*). Although the relationship found between oospore and oogonial breadth and length was consistent, the antheridial measurements were highly variable. Thus antheridial size is probably unsuitable for use as a quantitative trait for taxonomic purposes.

In this study, morphological characters varied depending on the particular mating combination chosen. Based on this observation the term "specific mating ability" was adopted to describe the variability associated with different mating combinations. For example, P3367 paired with P3078 or with C6 gave large (30.46 x 29.61) or small (24.47 x 23.87) oogonial dimensions, respectively. The abundance of oogonia on agar medium has been used to distinguish between species but in the case of *P. citrophthora* (Waterhouse, 1963) the results were too variable. Such variability could be again attributed to the effects of a "specific mating ability".

This effect of "specific mating ability" was also observed in the germination data. In general, low germination percentages were recorded (i.e. below 13%) and this concurs with 10 – 15% recorded for other *Phytophthora* species (see Ribeiro, 1983) with the exception of Ann and Ko who successfully germinated 90% of oospores of *P. parasitica* (1988). However, since different mating combinations were observed to have different requirements for germination, it was difficult to determine if one treatment for induction was generally more effective than another. Therefore, the predisposition of oospores from certain crosses to germinate may be an important factor in dormancy (see Section 2.4.3).

The actual function of oospores is somewhat unclear: oospores are thought to provide a means of survival under adverse conditions, yet *P. citrophthora* oospores have rarely been observed in nature or in culture. Oospores are also thought to provide a sexual mechanism for genetic recombination in *Phytophthora*, which is especially significant

for plant pathologists because it affects variation in pathogenicity and the ability to overcome host resistance. This is further discussed below.

All heterothallic species can potentially be crossed or be capable of recognising the opposite mating type which results in the production of oospores (Gallegly, 1968; Savage *et al.*, 1988). Single oospore-derived progeny produced in a single cross have been shown to differ phenotypically from each other and from the parent culture with respect to colony appearance, ability to form sporangia, size and shape of sporangia, and in pathogenicity (Boccas, 1981; Brasier and Sansome 1975; Galindo and Gallegly, 1960; Romero and Erwin, 1968; Satour and Butler, 1968; Ann and Ko, 1990). Thus, the hypothesis was proposed that sexual crossing of opposite mating types (A1 and A2) could be a possible mechanism leading to the production of new variants within a population (Shaw, 1983). However, in many cases there was no evidence that hybrids were formed. For example, Boccas (1981) examined the protein patterns of crosses of *P. capsici* x *P. palmivora*. It was found that only one of many progeny could have been a hybrid, with an intermediate protein pattern and colony morphology combined with the A1 compatibility type and pathogenicity level of the *P. capsici* parent, and the maximum growth temperature of the *P. palmivora* (A2) parent. More recent genetic evidence, from isozyme and RFLP analyses, suggests that sexual recombination can occur between A1 and A2 compatibility types of the same *Phytophthora* species (Förster and Coffey, 1990; Shattock *et al.*, 1986) but how frequently it occurs in the field remains unknown.

It may be that oospores in A1 x A2 matings result frequently from induced selfings (Ko *et al.*, 1986). If so, pathological variability within the population may be more stable and predictable. There is good evidence that the sexual stage in A1 and A2 matings is controlled by a diffusible substance also known as α hormone. Ko (1978) has shown that when an A1 culture is separated from an A2 culture by a porous polycarbonate membrane, diffusates from A1 pass through the membrane to induce selfing in A2; similarly, A2 diffusates induce selfing in A1. In this study, there was a possibility that many of the oospores produced in matings were due to a selfing phenomenon, particularly in view of RFLP data presented in the following chapter.

8. USE OF MOLECULAR MARKERS TO EXAMINE GENETIC VARIATION IN *P. CITROPHTHORA*

8.1 INTRODUCTION

Studies on variation in *Phytophthora* have been limited by the lack of genetic, as well as appropriate morphological and biochemical, markers. The use of naturally occurring physiological markers such as specific virulence genes, loci controlling mating type or vegetative incompatibility, has provided valuable information on the structure of fungal populations of several pathogens (Michelmore and Hulbert, 1987) but is limited to species which have been well characterised: this excludes *P. citrophthora*. Recently, molecular techniques have enabled the development of large numbers of phenotypically neutral genetic markers. In particular, Restriction Fragment Length Polymorphisms (RFLPs) of the nuclear and mitochondrial genomes have been employed to examine intraspecific variation in a number of fungi including *Phytophthora* (Goodwin *et al.*, 1989; 1991; Förster *et al.*,1988; 1990). The Polymerase Chain Reaction (PCR) has also been used to identify and map polymorphisms in eukaryotic organisms (e.g. Williams *et al.*,1990) however,there has been limited application of PCR to examine variation in fungal species. Recently, PCR amplified ribosomal DNA was used to discriminate between *Phytophthora* and other Oomycete genera (Lee and Taylor, 1991).

Little is known about the basic genetics (including genome size, number of chromosomes and ploidy level) of *Phytophthora* species. The analysis of genetic variation in *P.*

citrophthora has been further complicated by the difficulty of generating the sexual stage in this species.

The main focus of this study, was an examination of genetic variation in *P. citrophthora* isolates obtained from Citrus hosts. In particular, to examine if variation observed in other aspects of this study, such as morphological (Chapter 4) and pathological (Chapter 6) could be related to genetic variation. In addition, isolates derived from uninucleate zoospores were used to minimize the problem of genetic variability due to factors other than inheritance, such as heterokaryosis (Chapter 4). An additional aim was an attempt to determine chromosome number (and sizes) for *P. citrophthora* by pulsed-field gel electrophoresis.

8.2 EXPERIMENTAL PROCEDURES

8.2.1 RFLP Analysis

The main experiment in this study involved the use of RFLPs to detect variation between isolates of *P. citrophthora*. This involved the construction of a genomic library of *P. citrophthora* from which low copy number sequences would be chosen for use as DNA probes. RFLPs between parental and single zoospore-derived progeny were then identified. The isolates used in this study are listed in Table 8.1.

Table 8.1: Isolates of *P. citrophthora* used in RFLP analysis. Column 1 contains isolates from which single zoospore progeny were derived.

Isolates	
Column 1	**Column 2**
C23	PAL 7
C6	P3435
D64660	P0318
P1163	PAL 2
P6708	P3418
P3582	P3434
P3693	P3078
P6698	PAL 41
P3382	PAL 45
P1152	PMO 52

A. Construction of Genomic Library and Screening of DNA Probes

Methods for the construction of a genomic library of *P. citrophthora* isolate C23 using pTZ19R as a vector are described in Section 3.6. Dot Blot analyses (described in Section 3.7) was used to determine which clones would be suitable for use as DNA probes. Those clones which hybridised to mitochondrial (*P. citrophthora* mt DNA from Dr H. Förster), pMRI (maize ribosomal DNA from Dr. P. Langridge) or high copy number genomic DNA, were excluded. Clones which hybridised to inserts of low copy number genes were selected. Plasmid DNA was extracted as described in Section 3.3.6.

B. Identification of RFLPs

In total, 28 DNA probes, 26 constructed from a *P. citrophthora* (isolate C23) library (see above) and two (PCIT 4 and PCIT 15) from another *P. citrophthora* (isolate P1323) library (Goodwin *et al.*, 1990) were hybridised to Southern blots of *P. citrophthora* DNA digested with *Bam* HI, *Dra* 1 and *Hin*d III as described in Section 3.7. The 16 DNA probes which identified the most polymorphisms between isolates were selected for use in a more detailed analysis of *P. citrophthora* DNA digested with *Bam* HI and *Dra* I. In total, DNA from 20 parent *P. citrophthora* isolates were examined in this study. Three single spore-derived cultures were obtained from 10 of these parent isolates (as described in Section 4.2) and DNA from these was also compared (see Table 8.1).

The results were quantified by estimating the proportion of shared DNA fragments (F) between two isolates as determined by the following formula (Nei, 1987):

$$F = 2m_{xy}/(m_x + m_y)$$

where m_{xy} is the number of restriction fragments shared by the two isolates, and m_x and m_y are the numbers of restriction fragments in each isolate. F values were then expressed as percentage similarity (F x 100).

8.2.3 PCR Analysis

In this study, random primers specified in Section 3.9.1 were used to amplify DNA of parent and single zoospore-derived isolates of *P. citrophthora* (see column 1, Table 8.1). Polymorphisms were then identified in the amplified DNA. Different DNA preparations (see Section 3.5), either diluted with nanopure water (1:5) or undiluted, were compared.

The PCR reaction was performed as described in Section 3.9.2. The control reaction was run with only the reagents and the primers and no template DNA to ensure that there were no problems of contaminating DNA or self–priming.

8.2.4 Karyotype Analysis of *P. citrophthora*

Contour–clamped homogenous electric field electrophoresis (CHEF) was used in an attempt to determine the number and size of chromosomes in *P. citrophthora* isolate C23, as described in Section 3.8. Genome size was estimated by comparing the migration of chromosomes of *P. citrophthora* with that of chromosomes of known sizes (*S. cerevisiae* and *S. pombe*, see Section 3.8.3). Several methods were examined for preparing intact DNA and these are given in Section 3.8.

Megabase DNA was isolated on a 1% agarose gel in 0.5X TBE buffer electrophoresed for 24 hr at 200 V with a switching interval of 50 – 90 seconds.

8.3 RESULTS

8.3.1 RFLP Analysis

Genetic variation between parental and single–zoospore–derived isolates of *P. citrophthora* was determined using RFLP analysis.

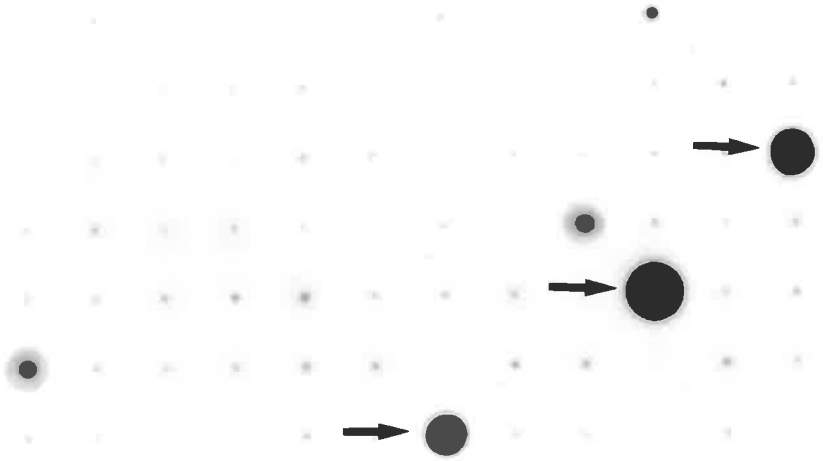
Table 8.2. Hybridisation Characteristics of Genomic DNA Probes to *P. citrophthora* DNA.

DNA probes were from a *P. citrophthora* (isolate C23) genomic library except for those marked with an * which were derived from a *P. citrophthora* (isolate P1323) genomic library (Goodwin *et al.*, 1990). Restriction endonucleases used to cut total DNA of *P. citrophthora* isolates were *Bam* HI, *Dra* I and *Hin*d III. The number of bands observed after probing DNA are given for A1 and A2 isolates. Intensity of hybridisation of each probe is indicated by the copy number as follows: H, high; I, intermediate; and L, low. Insert size is indicated in kilobase pairs (kb). – indicates missing values. Probes that were similar were separated into groupings.

	Probe	Insert Size (kb)	No. of Bands A1	A2	Copy No.	Grouping
1.	p39	1.2	20	24	H	
2.	p45	2.6	10	14	H	
3.	PCIT 4*	–	35	33	H	
4.	PCIT 15*	–	17	15	H	
5.	p40	6.3	20	18	H	Group p40
6.	p42	6.3	20	18	H	"
7.	p52	6.3	20	18	H	"
8.	p55	6.3	20	18	H	"
9.	p14	3.2	7	1	I	
10.	p15	1.6	7	6	I	
11.	p53	2.0	6	4	I	
12.	p57	4.5	6	7	I	
13.	p58	0.16	9	4	I	
14	p36	0.60	6	9	I	Group p36
15.	p44	0.50	6	9	I	"
16.	p32	1.4	2	3	L	
17.	p59	0.38	4	2	L	
18.	p63	0.44	5	4	L	
19.	p64	2.6	3	4	L	
20.	p89	0.79	4	8	L	
21.	p67	1.3	3	4	L	Group p67
22	p88	1.3	3	4	L	"
23.	p11	1.1	3	3	L	weakly-hybridizing
24.	p27	1.0	2	3	L	"
25.	p31	5.0	2	4	L	"
26.	p33	1.8	4	2	L	"
27.	p61	5.2	4	4	L	"
28.	p85	0.89	2	3	L	"

Figure 8.1. Hybridisation of random genomic probes to maize ribosomal DNA.

Dot blot analysis (see Section 3.7) of 83 clones from a *P. citrophthora* (isolate C23) genomic library, probed with rDNA (maize ribosomal DNA, pMR1). The arrows indicate where there was strong hybridisation of particular clones to pMRI which suggests that these inserts contain *P. citrophthora* rDNA sequences.



A. Construction and Screening of Genomic Library

A DNA library was constructed using genomic DNA from *P. citrophthora* isolate C23 (A1) and pTZ19R vector DNA (as described in Section 3.6). A total of 400 colonies were obtained following the transformation of *E.coli* (strain DH5a) with DNA from *P. citrophthora* isolate C23. Of these, 83 colonies which contained single inserts were chosen and screened to determine whether they contained high copy or low copy genomic, mitochondrial or ribosomal DNA. Clones which hybridised strongly to both ribosomal and genomic DNA were excluded from further experiments (see Figure 8.1). No significant hybridisation occurred with mt DNA (data not shown), indicating that the clones were not of mitochondrial origin.

B. Identification of RFLPs

Twenty-eight genomic DNA probes (see A above) were analysed for their suitability to detect RFLPs. Table 8.2 shows details of the hybridisation characteristics of these probes (which contained inserts between 0.2 and 6 kb in size) to *P. citrophthora* DNA. Although an attempt was made to screen out probes containing high copy number genomic DNA sequences, there were still a number of probes containing intermediate to high copy number genomic DNA sequences, representing dispersed repeats and/or tandem repeats. Some probes were found to cross-hybridise (i.e. p44, p52, p55 and p88), while others showed no clear banding pattern (p11, p27, p31, p33, and p61). These probes were therefore omitted from further experiments. Restriction enzymes differed in their ability to reveal polymorphisms. *Hin*d III did not completely digest *P.*

citrophthora DNA and was omitted from further experiments. Incomplete digestion was probably due to contaminants in the DNA preparation (these were not identified).

Sixteen probes were hybridised to southern blots of *Bam* HI– and *Dra* I–digested *P. citrophthora* DNA to identify RFLPs between parent and single zoospore–derived cultures. In general the results showed little genetic variation between parent isolates of *P. citrophthora.* Single zoospore–derived cultures had identical banding patterns to parent cultures (Figure 8.2). The banding patterns fell into two distinct groups which were not related to the geographical distribution of the isolates but were correlated with mating type (i.e. A1 or A2, refer to Table 7.2). This banding pattern was consistent for every probe, including PCIT 4 and PCIT 15. All A1 isolates (with the exception of P3418) had identical RFLP banding patterns and this also was found for the A2 isolates.

RFLPs were used to calculate percentage similarity (Fx100) between isolates and single zoospore–derived progeny (Table 8.3). A1 isolates were very dissimilar to A2 isolates. For example, four probes (p14, p32, p53 and p63) failed to give any common bands for *Dra*–I–digested A1 and A2 isolates, one probe (p14) failed to give any similarity for both *Bam* HI–and *Dra* I–digested isolates and seven other probes (p15, p39, p40, p45, p53, p63, PCIT 15) gave values ranging from 26 to 40% similarity for either or both *Bam* HI and *Dra* I–digested A1 and A2 isolates (see Table 8.3). In fact, p14 hybridised only with A1 isolates (Figure 8.3). The distinction between the A1 and the A2 isolates could also be seen when DNA samples of *P. citrophthora* isolates were separated on 1%

Figure 8.2. Autoradiograph of a Southern blot containing genomic DNA from parent and single zoospore-derived isolates of *P. citrophthora*.

DNA was digested with *Dra* I, electrophoresed on a 1% agarose gel, transferred to a Hybond-N^+ membrane and probed with ^{32}P labelled p45 DNA as described in Section 3.7. Parent isolates are denoted by a capital letter; single spore-derived cultures are denoted by small letters. Each letter represents a particular isolate group containing both the parent and single spore-derived cultures, as follows: **A**, C23; **B**, C6; **C**, D64660; **D**, P1163 ; **E**, P6708; **F**, P3582; **G**, P3693; **H**, P6698; **I**, P3382; **J**, P1152. The positions of a DNA size marker (Lambda *Hind* III) are given on the left in kilobases. Polymorphisms are indicated at 12.6, 6.7, 3.5, 2.8, and 1.9 kb.

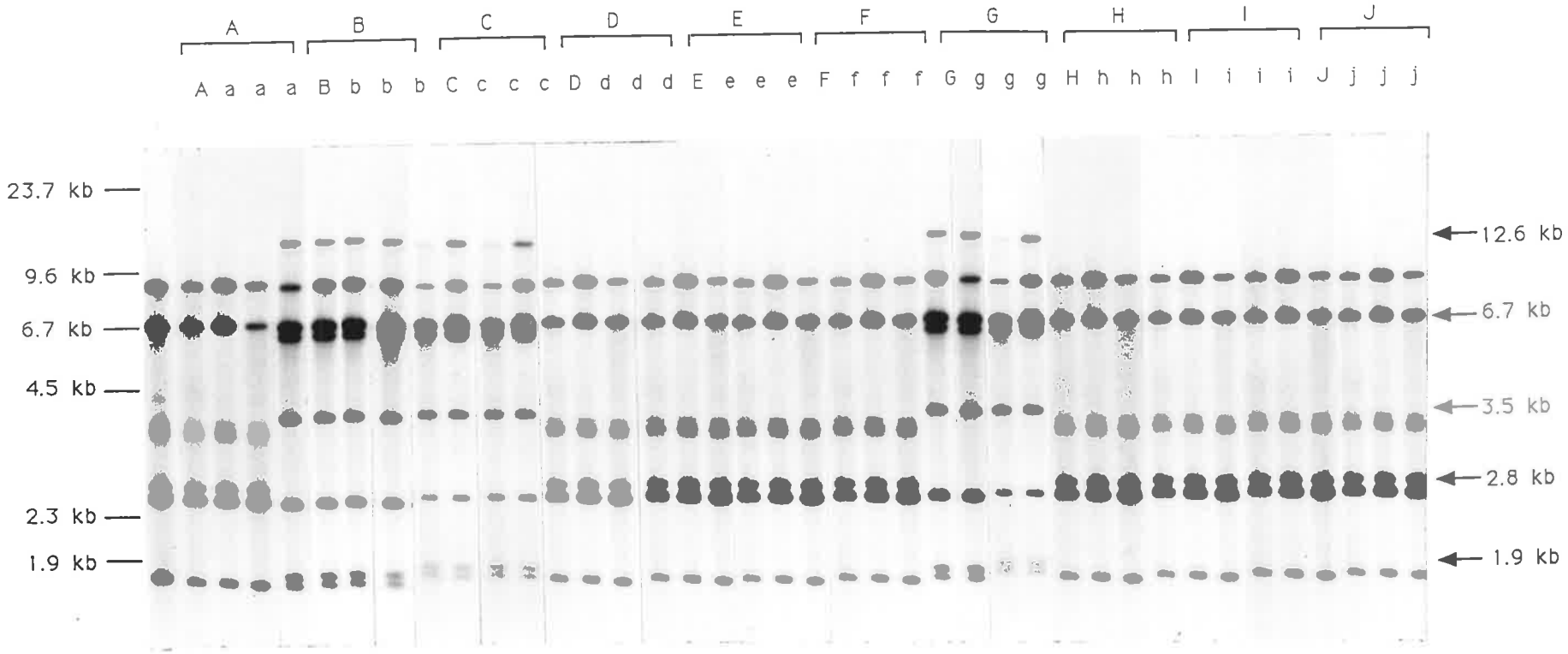

A
B
C
D
E
F
G
H
I
J
A a a a B b b b C c c c D d d d E e e e F f f f G g g g H h h h I i i i J j j j
23.7 kb
9.6 kb
6.7 kb
4.5 kb
2.3 kb
1.9 kb
12.6 kb
6.7 kb
3.5 kb
2.8 kb
1.9 kb

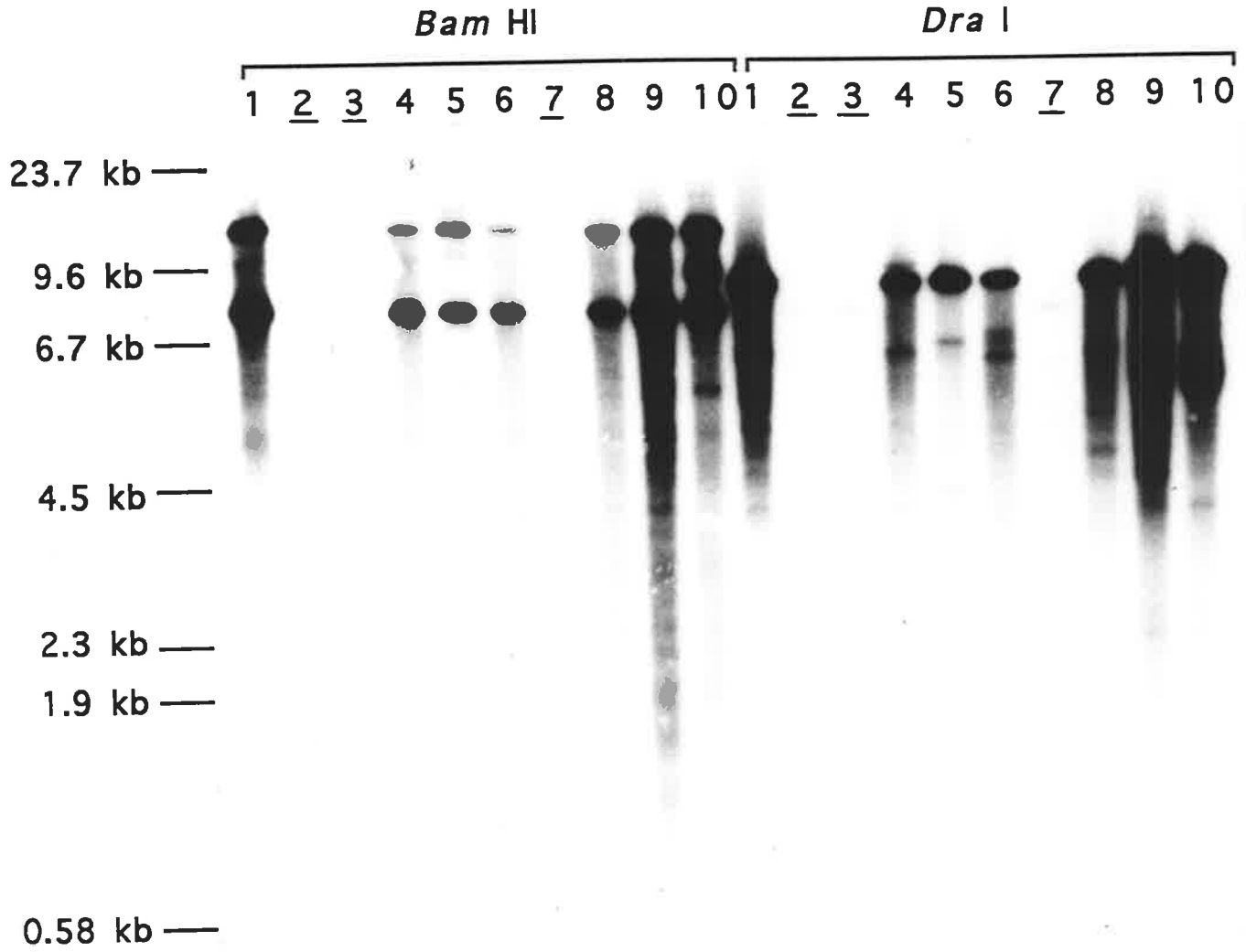
Table 8.3: Percentage Similarity (Fx100) between A1 and A2 Parent Isolates of *P. citrophthora*.

Percentage of similarity (Fx100) was calculated from RFLP banding patterns for both *Bam* HI– and *Dra* I–digested DNA of *P. citrophthora* isolates (A1 and A2) after hybridisation with ^{32}P labelled probes as described in Section 3.7.

Probe	Fx100 *Bam* HI	*Dra* I	*Bam* HI+ *Dra* I
p14	0	0	0
p15	40	75	57.5
p32	66	0	33
p36	50	66	58
p39	40	32	36
p40	32	43	37.5
p45	33	53	43
p53	33	0	16.5
p57	86	50	68
p58	50	73	61.5
p59	86	86	86
p63	40	0	20
p67	86	50	68
p89	50	80	65
PCIT 4	66	66	66
PCIT 15	26	36	31

Figure 8.3. Specific hybridisation of p14 with A1 isolates of *P. citrophthora*.

DNA was digested with *Bam* HI and *Dra* I, electrophoresed on a 1% agarose gel, transferred to a Hybond-N^+ membrane and probed with ^{32}P labelled p14 DNA as described in Section 3.7. Lanes as follows: 1, C23(A1); 2, C6(A2); 3, D64660(A2); 4, P1163(A1); 5, P6708(A1); 6, P3582(A1); 7, P3693(A2); 8, P6698(A1); 9, P3382(A1); 10, P1152(A1). A2 isolates are underlined in the figure: p14 did not hybridise with these isolates. The positions of a DNA size marker (Lambda *Hin*d III) are given on the left in kilobases.


Bam HI
Dra I
1 2 3 4 5 6 7 8 9 10 1 2 3 4 5 6 7 8 9 10
23.7 kb
9.6 kb
6.7 kb
4.5 kb
2.3 kb
1.9 kb
0.58 kb

agarose gels and stained with ethidium bromide (Figure 8.4). Here, only repeated sequences can be clearly distinguished, yet it is evident that these sequences differ for A1 compared to A2 isolates.

Examples of hybridisation to both low and high copy number sequences are seen in Figures 8.5 and 8.6 respectively. However, although there were unique bands associated with A1 and A2 isolates, there were also bands which were common to both groups. Some polymorphisms were detected within A1 isolates (Figure 8.7 A) or within A2 isolates (Figure 8.7 B). There were no examples of isolates with a mixture of both A1 and A2 banding patterns. Bands of A1 and A2 isolates had varying degrees of homology to different probes.

Isolate P3418 had a typical A1 banding pattern but an additional band was seen with some of the probes examined (e.g. Figure 8.8 A) and when probed with p36, one less band was revealed when compared to other A1 isolates (Figure 8.8 B). However, for those probes examined, the average percentage similarity between P3418 and other A1 isolates was 87% whereas P3418 showed only 25% similarity to the A2 isolates. In addition, P3418 hybridised to the A1–specific probe (p14); these data suggest that P3418 is related to the A1 isolates rather than representing a third group.

Figure 8.4. Ethidium bromide stained agarose gel containing restriction enzyme digests of DNA from *P. citrophthora* isolates.

Isolates of *P. citrophthora* were digested with *Bam* HI and *Dra* I and the samples were electrophoresed on a 1% agarose gel and stained with ethidium bromide. Lanes are as follows: M, marker (Lambda *Hin*d III); 1, C23(A1); 2, C6(A2); 3, D64660(A2); 4, P1163(A1); 5, P6708(A1); 6, P3582(A1); 7, P3693(A2); 8, P6698(A1); 9, P3382(A1); 10, P1152(A1). A2 isolates are underlined in the figure. The positions of a DNA size marker (Lambda *Hin*d III) are given on the left in kilobases.

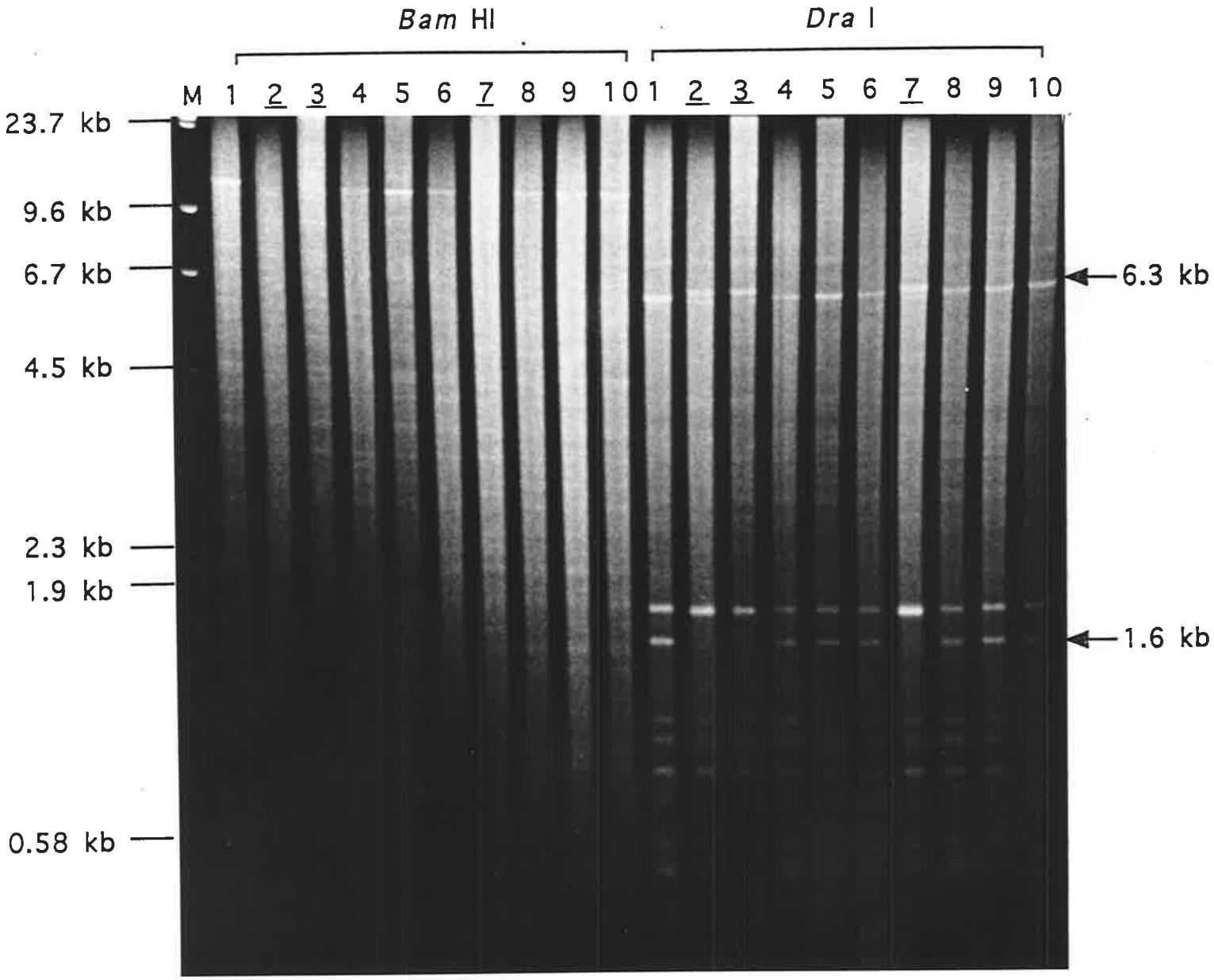
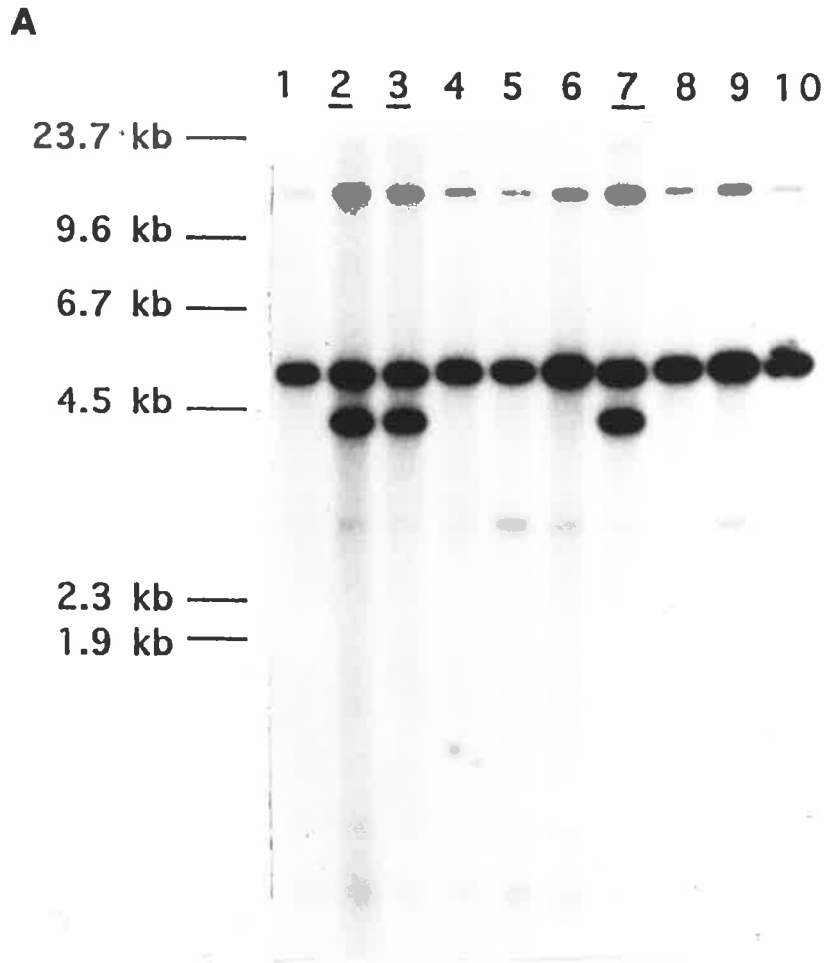

Bam HI
Dra I
M 1 2 3 4 5 6 7 8 9 10 1 2 3 4 5 6 7 8 9 10
23.7 kb
9.6 kb
6.7 kb
4.5 kb
2.3 kb
1.9 kb
0.58 kb
6.3 kb
1.6 kb

Figure 8.5. Hybridisation patterns seen after probing isolates of *P. citrophthora* with low copy number genomic DNA (probes p67 and p53).

DNA from *P. citrophthora* isolates was digested with *Dra* I **(A)** or *Bam* HI **(B)**, electrophoresed on a 1% agarose gel, transferred to a Hybond-N^+ membrane and probed with ^{32}P labelled p67 **(A)** or p53 **(B)** as described in Section 3.7. Lanes were marked as follows: 1, C23(A1); 2, C6(A2); 3, D64660(A2); 4, P1163(A1); 5, P6708(A1); 6, P3582(A1); 7, P3693(A2); 8, P6698(A1); 9, P3382(A1); 10, P1152(A1). A2 isolates are underlined in the figure. The positions of a DNA size marker (Lambda *Hind* III) are given on the left in kilobases. Note the additional bands for the A2 isolates in **(A)** and the distinct banding pattern of the A2 isolates in **(B)** compared to the A1 isolates.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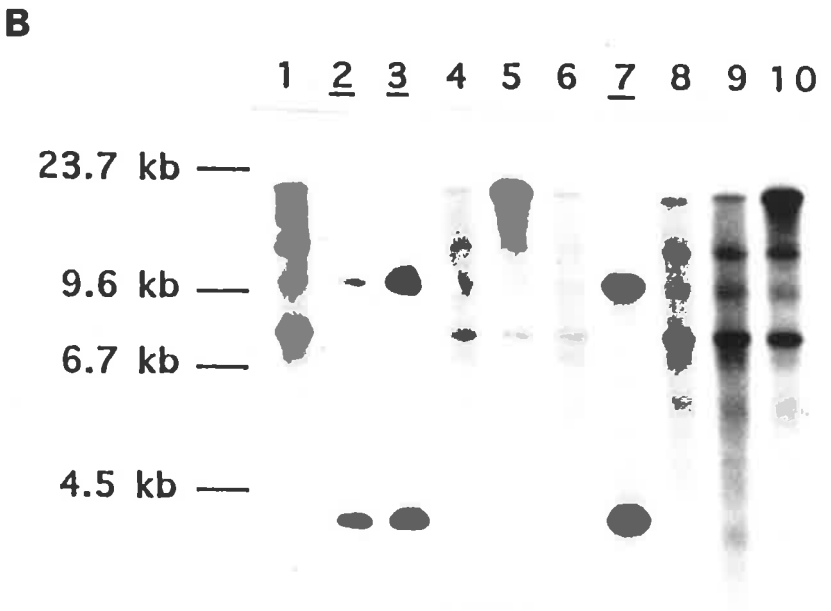
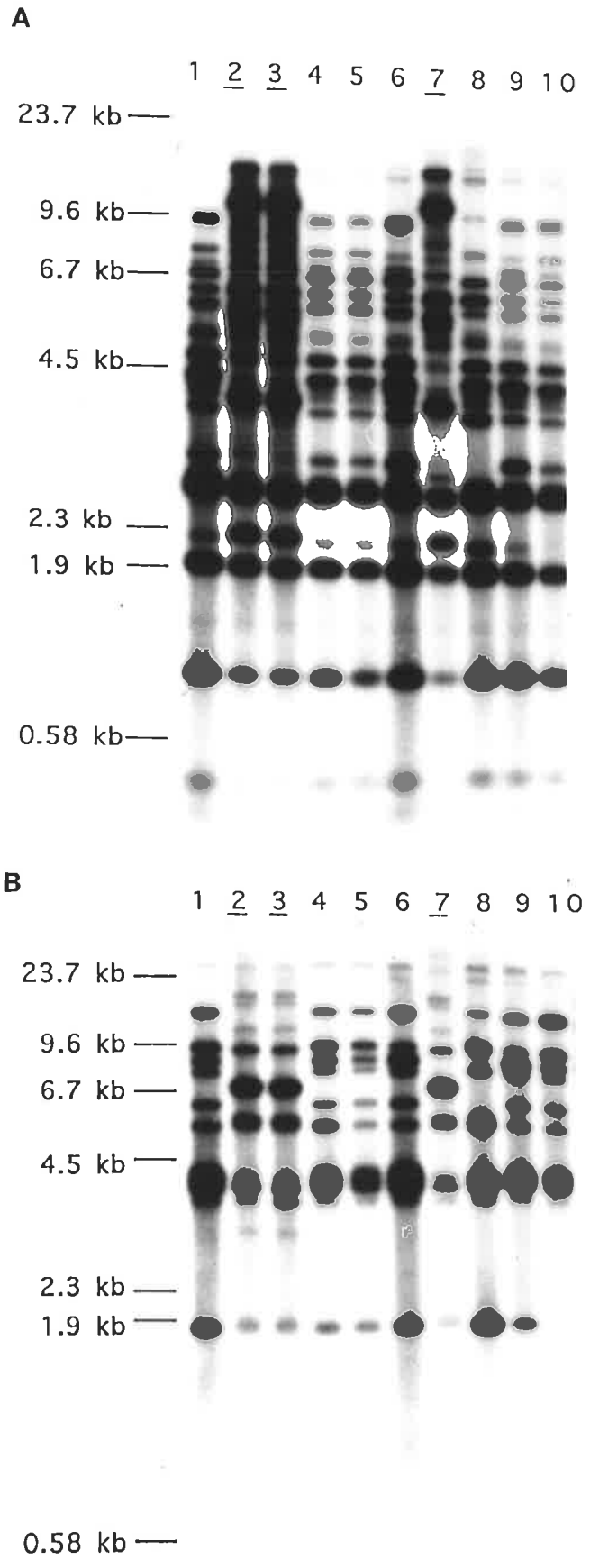

B
1 2 3 4 5 6 7 8 9 10
23.7 kb
9.6 kb
6.7 kb
4.5 kb

Figure 8.6. Hybridisation patterns seen after probing isolates of *P. citrophthora* with high copy number genomic DNA (probes p40 and PCIT 15).

DNA from *P. citrophthora* isolates was digested with *Bam* HI, electrophoresed on a 1% agarose gel, transferred to a Hybond–N^+ membrane and probed with either ^{32}P labelled p40 (**A**) or PCIT 15 DNA (**B**) as described in Section 3.7. Lanes are as follows: 1, C23(A1); 2, C6(A2); 3, D64660(A2); 4, P1163(A1); 5, P6708(A1); 6, P3582(A1); 7, P3693(A2); 8, P6698(A1); 9, P3382(A1); 10, P1152(A1). A2 isolates are underlined in the figure. The positions of a DNA size marker (Lambda *Hin*d III) are given on the left in kilobases.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B
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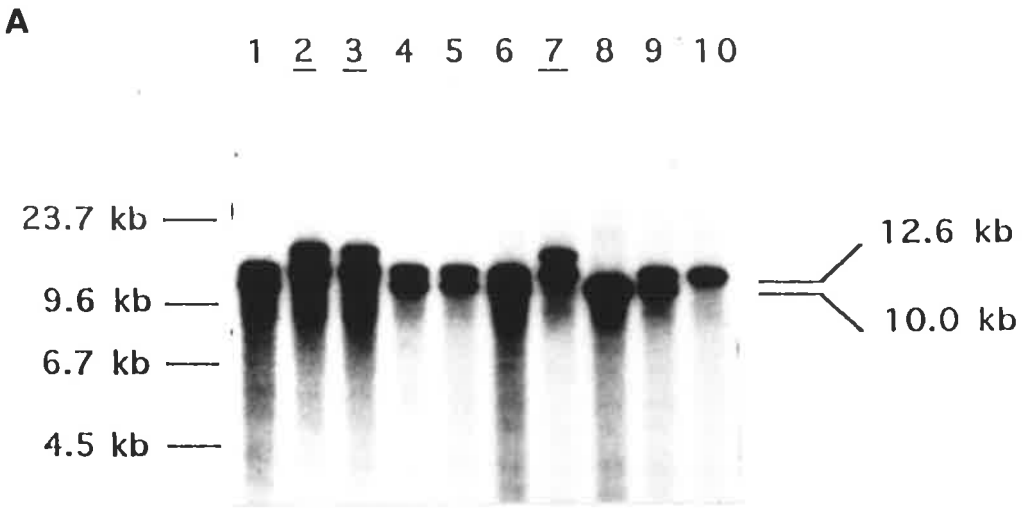
23.7 kb
9.6 kb
6.7 kb
4.5 kb
2.3 kb
1.9 kb
0.58 kb

Figure 8.7. Examples of polymorphisms within A1 and A2 isolates of *P. citrophthora*.

DNA from *P. citrophthora* isolates was digested with *Dra* I **(A)** or *Bam* HI **(B)**, electrophoresed on a 1% agarose gel, transferred to a Hybond–N^+ membrane and probed with ^{32}P labelled p63 **(A)** or p64 DNA **(B)** as described in Section 3.7. Lanes as follows: 1, C23(A1); 2, C6(A2); 3, D64660(A2); 4, P1163(A1); 5, P6708(A1); 6, P3582(A1); 7, P3693(A2); 8, P6698(A1); 9, P3382(A1); 10, P1152(A1). A2 isolates are underlined in the figure. The positions of a DNA size marker (Lambda *Hin*d III) are given on the left in kilobases.

(A) shows polymorphic patterns between A1 isolates. There is a single band for both isolates P6698 (Lane 8) and P1152 (Lane 10) 10.0 and 12.6 kb in size, respectively, compared to isolate P3382 (Lane 9) which has both bands. **(B)** shows an additional band in isolate D64660 (lane 3) 14.1 kb in size, compared to the other A2 isolates.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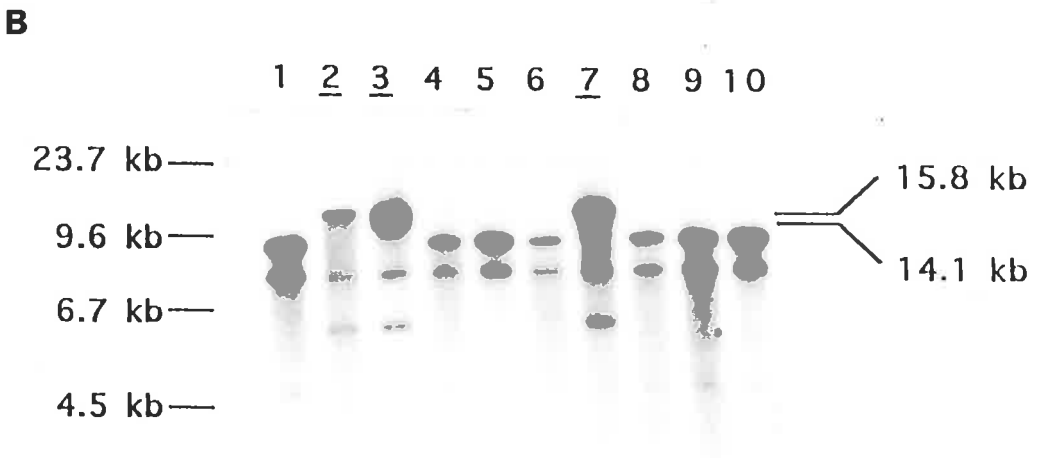
B
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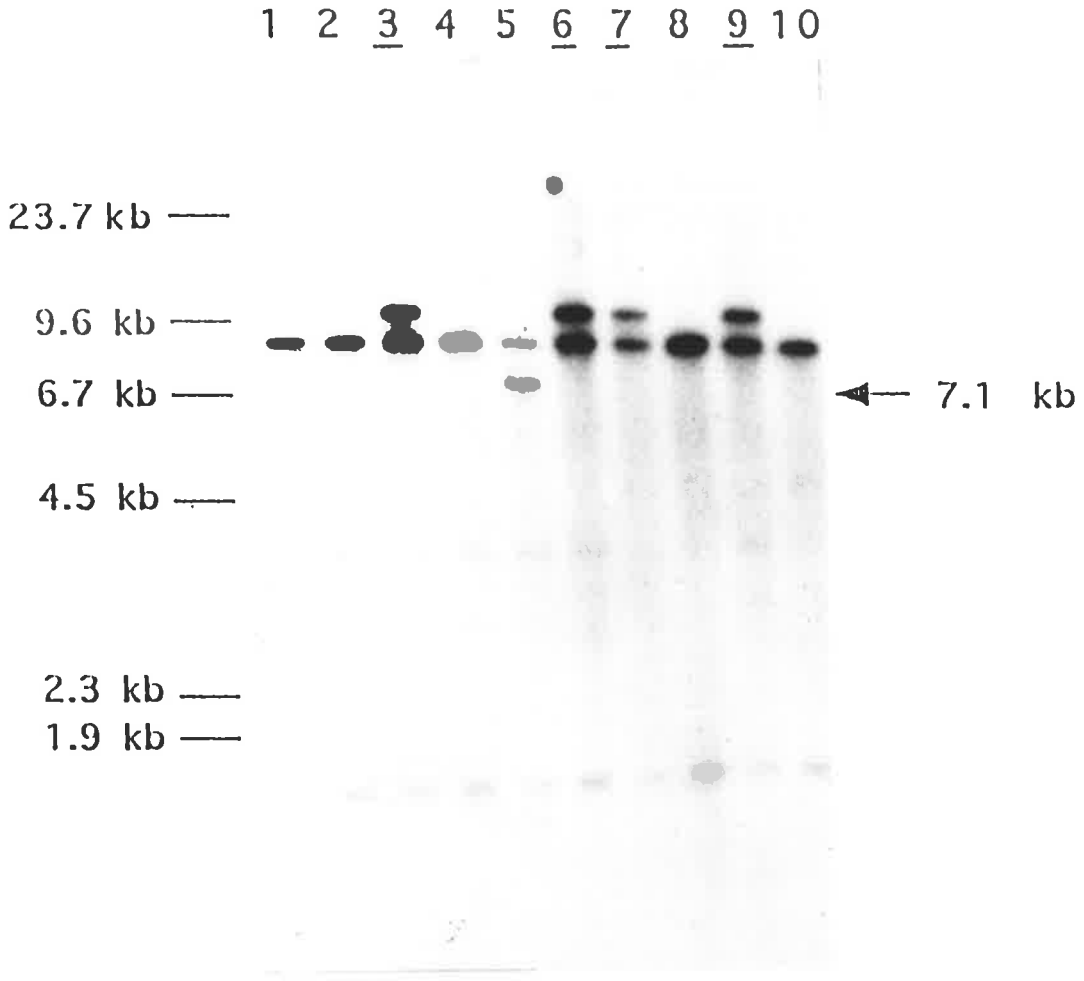
23.7 kb
9.6 kb
6.7 kb
4.5 kb
15.8 kb
14.1 kb

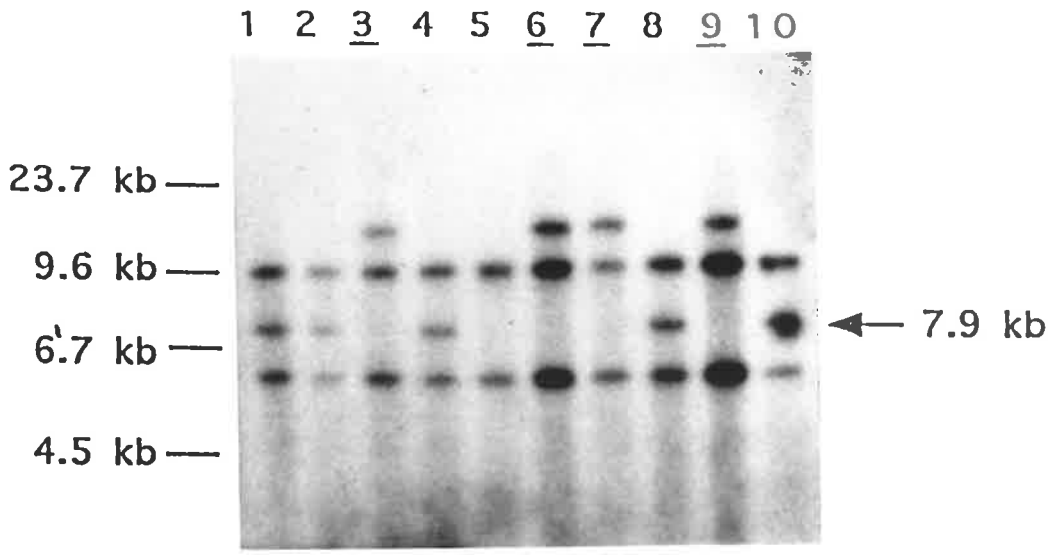
Figure 8.8. Polymorphisms detected between *P. citrophthora* isolates.

DNA from *P. citrophthora* isolates was digested with either *Bam* HI **(A)** or *Dra* I **(B)**, electrophoresed on a 1% agarose gel, transferred to a Hybond–N^+ membrane and probed with either ^{32}P labelled p67 **(A)** or p36 DNA **(B)** as described in Section 3.7. Lanes as follows: 1, PAl 7(A1); 2, P3435(A1); 3, P0318(A2); 4, PAL 2(A1); 5, P3418(A1); 6, P3434(A2); 7, P3078(A2); 8, PAL 41(A1); 9, PAL 45(A2); 10, PMO 52(A1). A2 isolates are underlined in the figure. The positions of a DNA size marker (Lambda *Hin*d III) are given on the left in kilobases. Note that isolate P3418 has an additional 7.1 kb band in **(A)** and no 7.9 kb band in **(B)** compared to the other A1 isolates (indicated by arrows).

A


1 2 3 4 5 6 7 8 9 10
23.7 kb
9.6 kb
6.7 kb
4.5 kb
2.3 kb
1.9 kb
7.1 kb

B


1 2 3 4 5 6 7 8 9 10
23.7 kb
9.6 kb
6.7 kb
4.5 kb
7.9 kb

8.3.3 PCR Analysis

The Polymerase Chain Reaction was used to amplify DNA sequences from the various *P. citrophthora* isolates as a further means of examining genetic variability. PCR products were formed with the primers R1 (see Figure 8.9), R2 and E4 (see Figure 8.10) but few amplication products were observed with C1 + C2 (data not shown). Between one and eight DNA segments, ranging in size from 0.1 kb to 2.8 kb were amplified depending on the isolate and primer combination. All amplifications were repeated three times with similar results despite the different DNA preparations (large–scale, small–scale, diluted or undiluted). Slight variation was observed in the relative amounts of amplified DNA. However, the results consistently support the RFLP data, in that size differences in amplified DNA segments were consistently observed between A1 and A2 isolates. No differences were observed between parent and single spore–derived cultures (data not shown).

8.3.4 Karyotype Analysis of *P. citrophthora*

Three different methods for the preparation of megabase DNA were tested. Method 1, involving the preparation of spheroplasts, was unsuccessful because the yields were too low. Method 2, in which zoospores were embedded directly in agarose prior to enzyme treatment, was also unsuccessful because the zoospore cell wall could not be digested *in situ*. Method 3, in which ground mycelium was directly embedded in agarose, yielded some promising results. As shown in Figure 8.11, two bands of 1.1 and 2.2 Mbp were separated. Therefore conditions were adjusted to give the maximal resolution of chromosomal DNAs greater than 2.2 Mbp. A modification of the conditions

Figure 8.9. Amplification of DNA from 10 isolates of *P. citrophthora* DNA using the R1 primer.

The method for PCR amplification is outlined in Section 3.9. Lanes were marked as follows: M, marker (Lambda *Hin*d III); 1, C23(A1); 2, C6(A2); 3, D64660(A2); 4, P1163(A1); 5, P6708(A1); 6, P3582(A1); 7, P3693(A2); 8, P6698(A1); 9, P3382(A1); 10, P1152(A1); C, control. A2 isolates are underlined in the figure. The control reaction was run with only the primer – template DNA. The positions of a DNA size marker (Lambda *Hin*d III) are given on the left in kilobases. Polymorphisms are indicated by arrows at 2.8 kb; 1.4kb; 1.1 kb; and 0.89 kb.

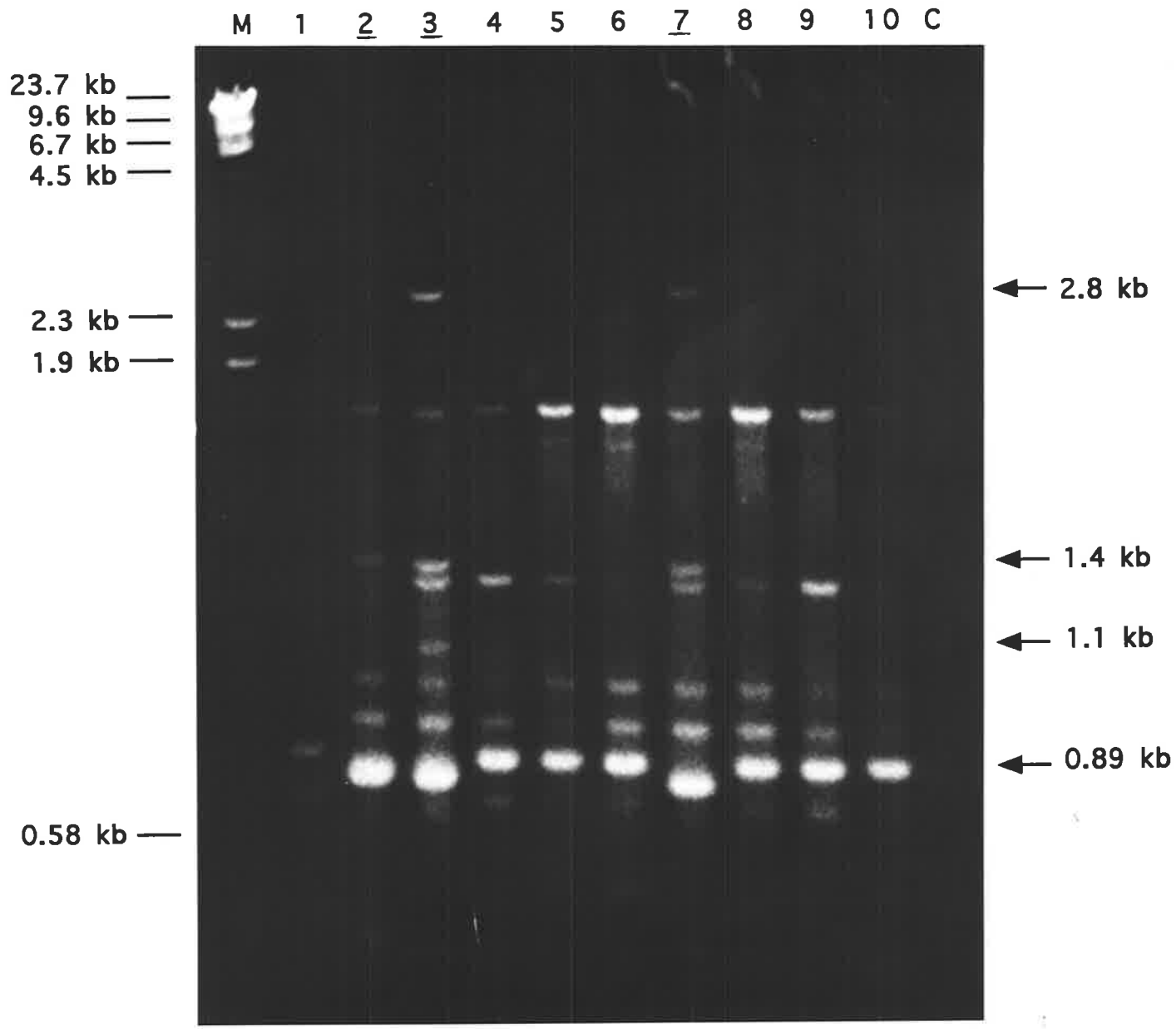

M 1 2 3 4 5 6 7 8 9 10 C
23.7 kb
9.6 kb
6.7 kb
4.5 kb
2.3 kb
1.9 kb
0.58 kb
2.8 kb
1.4 kb
1.1 kb
0.89 kb

Figure 8.10. Amplification of DNA from 10 isolates of *P. citrophthora* DNA using the R2 and E4 primers.

The method for PCR amplification is given in Section 3.9. The PCR products were derived from template DNA isolated by large–scale CsCl DNA preparation (see Section 3.5.2) and were fractionated on a 3% agarose gel. Lanes were marked as follows: 1 C23(A1); 2, C6(A2); 3, D64660(A2); 4, P1163(A1); 5, P6708(A1); 6, P3582(A1); 7, P3693(A2); 8, P6698(A1); 9, P3382(A1); 10, P1152(A1); C, control. A2 isolates are underlined in the figure. The control reaction was run with only the primer – template DNA. The positions of a DNA size marker (Lambda *Hin*d III) are given on the left in kilobases. Polymorphisms are indicated by arrows at 1.4 kb; 1.26 kb; 1.0 kb; 0.79 kb and 0.1 kb.

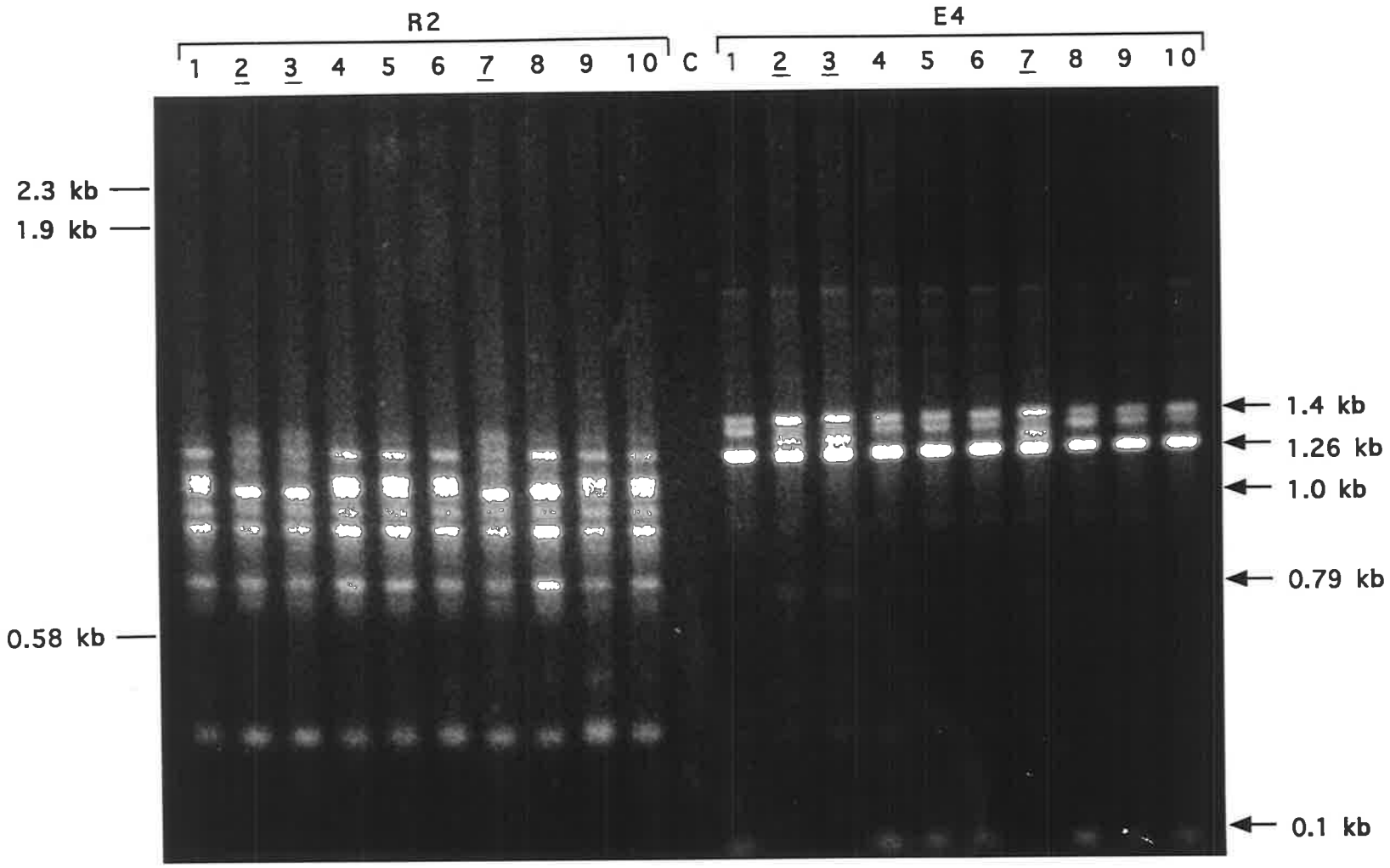
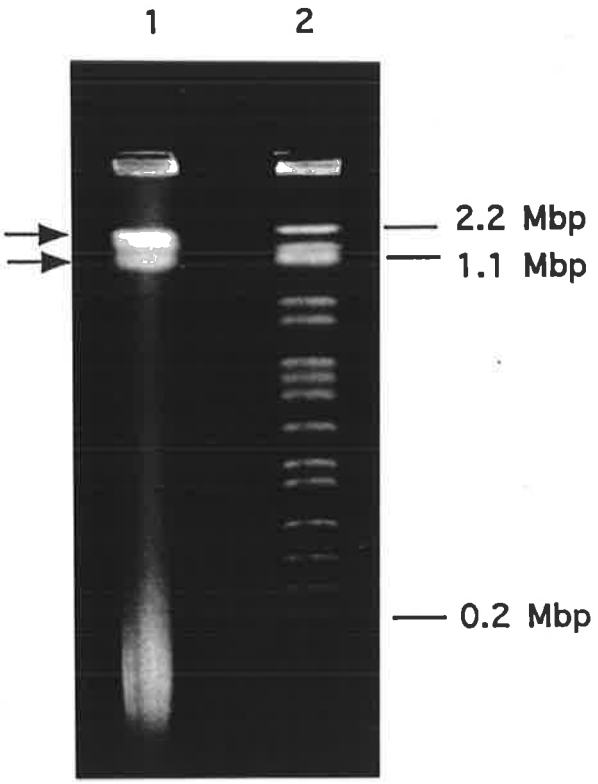

R2
1 2 3 4 5 6 7 8 9 10
C
E4
1 2 3 4 5 6 7 8 9 10
2.3 kb
1.9 kb
0.58 kb
1.4 kb
1.26 kb
1.0 kb
0.79 kb
0.1 kb

Figure 8.11. Separation of chromosomes of *P. citrophthora* (isolate C23) using contour-clamped homogenous electric field electrophoresis.

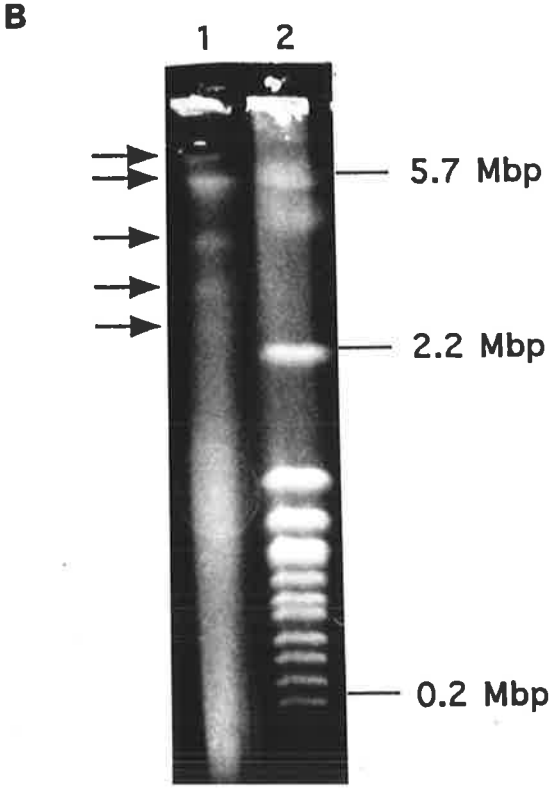
A. Comparison of *P. citrophthora* isolate C23 (Lane 1) and *S. cerevisiae* (Lane 2). Electrophoretic conditions: 1% agarose; 0.5X TBE; switching intervals, 50 – 90 sec; voltage, 200 V ; run time, 24 hr. The sizes of several chromosomal DNAs of *S. cerevisiae* are indicated. *P. citrophthora* DNA migrates as unresolved DNA of between 1.1 and 2.2 Mbp (indicated by arrows).

B. Comparison of *P. citrophthora* isolate C23 (Lane 1) and *S. cerevisiae* + *S. pombe* (Lane 2). Electrophoretic conditions: 0.8% agarose; 0.5X TBE; switching interval 200 – 400 sec; 125 V; run time, 72 hr. Resolution of four, and possibly five, bands in the size range of between 2.5 and 5.7 Mbp (indicated by arrows) The markers were placed in the same well. The size of both the *S. cerevisiae* and *S. pombe* chromosomal DNA is shown on the left. Some degraded DNA was present in the samples.

A



B



recommended by Bio–Rad for separating DNA between 1 and 5 Mbp was tested as follows: A 0.8% agarose gel in 0.5X TBE buffer was electrophoresed for 72hr at 125 V with a 200 – 400 sec switching interval. At least four, and possibly five, bands were resolved in the size range between 2.5 and 5.7 Mbp (see Figure 8.11 B). However, as can be seen in Figure 8.11 by comparing A and B, there was more DNA degradation and poorer resolution of bands than when shorter run times were used and this may have masked some smaller bands. Digestion of DNA with *Mlu* or *Not* I did not affect the number of bands. It may be that there were no restriction sites for these enzymes or that the enzymes did not cut under these conditions.

8.4 DISCUSSION

This study revealed that both parent and single–zoospore–derived isolates of *P. citrophthora* from Citrus clustered into two distinct groups using either RFLP or PCR analysis. These groups correlated with mating type (A1 or A2). Such a correlation has not been noted previously because of the difficulties of producing oospores in culture (refer to Chapter 7). For the twenty isolates within these two groups there was no genetic variability detected, with the exception of P3418. It is likely that this is a polymorphic A1 isolate because it hybridised with the A1–specific probe, p14.

Goodwin *et al.* (1990) found that of five Citrus isolates, only one isolate (P0318), showed distinct RFLPs. The genetic similarity of the isolates appeared to be correlated with geographic distribution, as P0318 was from Australia and the other isolates were

from California. In contrast, this study using a larger population of isolates, found no correlation between genetic similarity and geographic distribution, which has been supported by other workers (Förster *et al.*, 1990a). In addition, there was no correlation between differences in aggressiveness (Chapter 6) or morphology (Chapter 4) observed between isolates and RFLP patterns.

The A1 and A2 grouping of isolates found in this study may be attributable to either one of two hypotheses. It may be that the groups represent A1/A2 compatibility systems and the probes have detected a mating-type locus. Alternatively, it may be that the isolates are distinct species. Both hypotheses are discussed below.

The first hypothesis would infer that all the probes tested in this study are hybridising to the same DNA sequences associated with mating type. This is unlikely for several reasons; first, a random genomic library was used to generate the probes. Also many probes detected multiple restriction fragments which may be indicative of both repetitive and highly conserved DNA sequences. In addition, random PCR primers from cereals showed the same distinction between A1 and A2 mating type. To test this hypothesis further one could probe Southern blots of the CHEF gels, to see if all the probes show homology only to one particular chromosome.

Alternatively the A1 and A2 grouping is inappropriate and there are, in fact, two different species. There is some uncertainty in this study as to whether the A1 and A2 isolates actually mated or whether the oospores produced were due to selfing. Analysis

of isozyme markers of germinated oospores produced in laboratory matings between *P. megasperma* and *P. palmivora* was shown to be the result of selfing rather than interspecific hybridisation (Erselius and Shaw, 1982). Boccas (1981) suggested that only selfed oospores were viable in most interspecific crosses. Certainly, the dissimilarity in Southern hybridisation patterns between A1 and A2 isolates implies that no sexual recombination has taken place between these isolates. Analogous results have been reported with isozyme analysis where different patterns were observed between A1 and A2 isolates of *P. cinnamomi*. These findings also suggested that the mating types were genetically isolated and that sexual reproduction involving both mating types does not occur in the field (Weste and Marks, 1987).

The A1 and A2 isolates examined in this study do not appear to be unrelated species however, because both groups share the same highly repetitive sequences and some low copy DNA sequences. The hypothesis that the two mating types are related species is further supported by the results of Förster *et al.* (1990a). These workers used RFLPs from mtDNA to establish that Citrus isolates of *P. citrophthora*, including what are now known to be A1(P1163) and A2 (P318 and P3078) mating types, clustered separately from Cocoa isolates. Because mitochondrial DNA is usually maternally inherited it is considered a reliable method for investigating phylogenetic relationships between organisms (Crozier, 1990).

If the A1 and A2 isolates were initially the same species but have diverged, what could be the isolating mechanism? As noted in Chapter 7, *P. citrophthora* isolates require very

specific conditions for the production of oospores. Management practices, choice of host plant, local environment as well as microenvironment, will all influence this 'specific mating ability'. Thus oospore production may be a rare event in the field. If selfing is prevalent within *P. citrophthora* (see Section) then over time this mechanism may have resulted in differences in the genomes.

Several questions arise from this study: (1) how common is selfing in *P. citrophthora* and (2) do the A1 and A2 groupings of *P. citrophthora* isolates from Citrus hosts represent closely related but different species? These questions could be answered by growing single oospore–derived progeny and using RFLP analyses to examine the inheritance of marker loci (which was not undertaken in this study because germination procedures were still inadequate). In either case, one can expect the RFLP banding pattern of single oospore–derived progeny to be totally derived from one parent (indicating selfing) or to contain a mixture of markers from both parents (indicating sexual recombination between the parental genomes).

Recently, RFLPs were used as genetic markers to examine the extent of sexual recombination in another heterothallic *Phytophthora* species and pathogen of Citrus, *P. parasitica* (Förster and Coffey, 1990). The study showed that the majority of the 23 single oospore–derived progeny examined from each of two crosses carried both of the parental markers. However, except for work with *P. infestans* (Shattock *et al.*, 1987; Spielman *et al.*, 1990), where isozymes were used as genetic markers, no other direct evidence exists for genetic exchange within *Phytophthora* species.

If the A1 and A2 groupings found in this and other studies (i.e. Weste and Marks, 1987) are found to represent different species, it would be necessary to reassess the morphological criteria on which these isolates have been grouped. *P. citrophthora* isolates obtained from Citrus hosts may be distinguished from those obtained from other hosts by isozyme patterns, RFLP patterns and by DNA amplification by PCR (Förster *et al.*, 1990; Goodwin *et al*, 1990; Lee and Taylor, 1991) even though they were morphologically indistinguishable. However, isozymes and RFLPs do have different technical constraints. For example the isolation of sufficient good quality DNA for RFLP analysis is more difficult and time–consuming than the extraction of crude proteins required for isozyme analysis. However, when examining intraspecific variability, isozyme analysis may not always be suitable as it is dependent on the particular enzyme being assayed.

For a complex genus such as *Phytophthora*, polymorphisms detected by PCR may provide an alternative to RFLPs and isozyme analyses This study showed that certain primers allowed the identification of polymorphisms which could be used as standard genetic markers. Furthermore, the distinction between A1 and A2 isolates by PCR amplification with random primers developed for genome mapping in cereals supports the RFLP data and suggests that a specific mating–type locus is not involved.

In addition, chromosomal differences have been useful in allowing related species to be distinguished. For example, when *Kluyveromyces marxianus* var. *marxianus* and *K. marxianus* var. *lactis* were crossed, progeny readily formed. This supported the initial

hypothesis that these belonged to a single species. However, analysis using genetic markers and electrophoretic karyotyping showed that the progeny inherited the patterns of only one parent indicating that sexual recombination had not occurred. Therefore, the authors argued that *K. marxianus* var. *marxianus* and *K. marxianus* var. *lactis* should be reclassified as separate species (Steensma *et al.*, 1988). However, as found for *P. citrophthora*, the possibility also exists that the two were not compatible or that conditions were not conducive for a sexual cross.

This study successfully distinguished at least four (possibly five) chromosomes of *P. citrophthora*. In the literature, the chromosome number is usually found to be 9 to 10 for *Phytophthora* species, with some exceptions. For example, *P. megasperma* var. *megasperma* has between 22 and 27 chromosomes (Sansome and Brasier, 1974) in contrast to *P. megakarya* which has only five chromosomes (Sansome, 1980). However, before chromosomal differences between *P. citrophthora* isolates can be used as a taxonomic marker, conditions would need to be improved to overcome problems of DNA degradation and poor resolution of the bands which occurred as a consequence of the long electrophoretic run times.

Little is known about the molecular genetics of *Phytophthora* species. The genome of *Bremia lactucae*, which is closely related to *Phytophthora*, contains as much as 65% repetitive DNA distributed as short interspersed repeats with approximately one third of the repetitive DNA in blocks of 1000 or more copies (Prof. M. Michelmore, pers.

comm.). Therefore, the highly repetitive DNA sequences observed in *P. citrophthora* suggests the possibility that this genome may be similarly constructed.

Thus *P. citrophthora* was examined for genetic variability with the successful use of several molecular techniques. There were two groups of *P. citrophthora* isolates from Citrus hosts distinguished, which were correlated with mating type. The main question raised in this study is whether these two groups represent two mating types or two distinct species. This question and its implications will be examined further in the following general discussion.

We shall not cease from exploration

And the end of all our exploring

Will be to arrive where we started

And know the place for the first time

T.S. ELIOT

1888 – 1965

9. GENERAL DISCUSSION

This study investigated *P. citrophthora* isolates on Citrus hosts. The most significant findings were as follows: The genetic analysis clearly distinguished two groups of isolates of *P. citrophthora*. These two groups correlated with mating type. Both groups were equally variable with respect to morphological and pathological criteria and so could not be separated on these bases. The sexual stage of *P. citrophthora* was generated in culture and oospores germinated for the first time. In addition, both *in vivo* and *in vitro* rapid screening techniques were developed to distinguish resistant from susceptible cultivars to infection by *P. citrophthora*. All of these aspects are discussed below in relation to the current literature and how they may be extended in future studies.

The current screening of Citrus for disease resistance has used clonal material from 6 months to several years–old because it was assumed that juvenility may mask the resistant response in younger seedling material (Prasad and Raghavendra Rao, 1985). It was shown in Chapter 5, that 2 to 3–month old rootstocks of known susceptibilities to *P. citrophthora* could be successfully distinguished using both root and collar inoculation techniques. Each of these techniques was assessed differently. In root inoculation, the severity of infection on the roots was assessed; tap roots of resistant cultivars were found to be significantly less infected than those of susceptible cultivars, whereas feeder roots were equally susceptible for all cultivars. In collar inoculations, lesion length was used as an indicator of susceptibility: susceptible

cultivars had more extensive lesions than resistant cultivars. For both techniques, the degree of the resistant response correlated with measured plant growth.

Susceptibility ratings using the root-dipping technique were in general accordance with results obtained using collar inoculation, unlike in the literature where moderately resistant cultivars showed various levels of susceptibility to collar versus root inoculation (Broadbent *et al.*, 1971). Such discrepancies may be attributed to the manner of root assessment. Assessment has been shown to be more reliable where tap root and feeder root systems were quantitatively assessed separately for infection (Grimm and Hutchison, 1973).

The mechanism which may account for the tap root resistant response is unclear. Smith *et al* (1991) using video image analysis of plant root growth and lesion development of *P. megasperma* on soybean tap roots, showed that in resistant cultivars the rate of pathogen invasion in the tap root was limited but failed to suggest a possible mechanism (Smith *et al.*, 1991). In other studies it has been found that the Citranges produced new tap roots which were for some time seemingly free of infection but eventually showed symptoms (Menge, unpublished data). Apparently, Menge later found these "healthy roots" to be infected, although they showed no symptoms at the time of initial sampling.

In Citrus tap roots the hypodermis may be a more important barrier to infection than the epidermis. Suberisation of the hypodermis may be an important feature of the plant response, firstly as a direct barrier to invasion by fungal hyphae and also to limit the rate of spread of the pathogen (Shishkoff, 1987). Such a process could possibly account for the above observations by Menge. For example, it is possible that invading fungal hyphae pass through the hypodermal cells in the very early stages of root differentiation before any suberin is laid down (Smith *et al.*, 1988). Suberisation occurring in cells already colonised by the fungi would give rise to the observed anatomy of infection. If the hyphae in the hypodermal cells survived after suberisation then hyphal transport to the cortex would be still maintained and infection would progress.

To further examine this hypothesis, microscopic and biochemical studies of the infection process of *P. citrophthora* on Citrus would be valuable. Basic questions need to be answered about the infection process of these fungi and the host response.

Generally, root inoculation techniques (using zoospores) have been used in preference over collar inoculation techniques (using mycelial discs) because: 1) The inoculum density of mycelium was considered to be not so readily quantifiable (as when using zoospores) and is probably higher than that present under field conditions. Thus, selection would be for rootstocks with a high level of resistance to *Phytophthora* (particularly with young plant material) whereas those with intermediate resistance may be eliminated, although they may have other positive traits, such as

salt–tolerance (Smith *et al.*, 1991a). 2) Because roots are less subject to environmental and physiological factors that influence resistance root assessment has been considered to be a more reliable means of determining resistance (Wagner, 1989). However, the observations in Chapter 5, question the validity of such assumptions made in the literature: In comparison with root inoculation techniques, collar inoculation techniques were easier to execute and produced very clear distinctions between susceptible and resistant cultivars in spite of the inherent variability found within a cultivar. In addition, using collar inoculation techniques, qualitative differences were observed between susceptible cultivars that were not observed with the root inoculation techniques.

There are some further considerations when using *in vivo* assays to screen rootstocks for disease resistance. For example, the effect of environmental factors on both pathogen activity and host resistance has previously been noted (refer to Section 2.6.1) and must be considered. The experiments in this study should be repeated under conditions simulating those found in the field but for preliminary purposes this is less important. Under South Australian conditions, root rot due to *P. citrophthora* occurs during winter when rainfall is abundant and temperatures are cool and pathogen activity is greatest. It is clear from the literature that environmental conditions have not always been fully considered in screening experiments. This may account for the observed discrepancies in a cultivar such as Carrizo Citrange, that was considered to be either susceptible or moderately resistant depending on the set of conditions under which screening was conducted (Hough, in press).

Screening for resistance in tissue culture using a modified excised shoot assay produced results that were analogous to the effects of collar inoculation in the growth cabinet (Chapter 6). Normally nucellar seedlings are raised by vegetative propagation which may take months even years before material can be incorporated into conventional screening programmes. Micropropagated clonal material may be produced from a single shoot explant in six weeks allowing for a more rapid and efficient screening of Citrus than if seeds had to be produced and grown up in a field test. In addition, following initial selection, new shoots may be rooted within six weeks, and these can be established in soil for further testing *in vivo*.

The modified excised shoot assay has also been used successfully to screen almond rootstocks for resistance to *P. cambivora* (Scott *et al.,* 1992). Perhaps the technique could be further extended to the assessment of other woody plant species to *Phytophthor*a infection. In addition, the tissue culture system has other advantages for increasing our understanding of host/pathogen interactions in a sterile and controlled environment as evidenced by the chemotropic and oospore work conducted in this study.

Variability of *P. citrophthora* was examined at the pathological morphological and genetic levels.

The tissue culture technique allowed the assessment of differences in aggressiveness between isolates of *P. citrophthora*. Considering the range of pathogenic variability

of Citrus isolates of *P. citrophthora* now known to exist at a single site (C6 compared to C23 isolates, from Loxton, S.A.), it is possible that much more variation exists in the field. It is not clear whether such variability in the population has arisen via natural divergence or whether, in fact, these isolates were introduced from rootstock material obtained from different sources.

The isolates derived from uninucleate single zoospores, in this study showed no variation in pathogenicity or RFLPs, however, as the number of isolates examined were low, no final conclusions should be drawn. This observation may be related to the possibility that sexual exchange does not occur readily within *P. citrophthora*. In contrast to the similarity observed between single zoospore–derived progeny in this study, recent work with *P. fragariae* found variability in pathogenicity in single zoospore–derived progeny before and after subculturing (Kennedy, pers. comm.). The number of nuclei within the zoospores from which these progeny were derived was not determined but may be important, as isolates derived from zoospores which contain more than a single nucleus have been shown to be variable (Caten and Jinks, 1968). Therefore, multinucleate zoospores may be a major source of variation via heterokaryosis. This has serious implications for management of disease because new strains may arise which could be less affected by particular fungicides or which could overcome host resistance.

The variability of morphological characters (including both sexual and asexual characters) found in this study between *P. citrophthora* isolates taken specifically

from Citrus hosts (Chapter 4 and 7) illustrates some of the current difficulties in *Phytophthora* taxonomy. A clearer consensus on what constitutes the species "*P. citrophthora*" needs to emerge. Recently, Hall (1991) commented on the wide variation that has been reported in the literature to occur within *Phytophthora* species. It was noted that although isolates may differ from a 'type' culture this does not mean that they are not within the range of that species, merely that the 'type' culture helps determine the name to be applied. The results in this study suggest that classification based on the concept of a 'type' species should be modified to include the full range of variability within a species by examining large numbers of isolates. Furthermore, taxonomic characters, such as sporangial size or form, which show continuous rather than discrete variation, should be treated with caution.

In *P. citrophthora*, the use of morphological characters to define species range presents problems. The Waterhouse keys (1963; 1970), following from that of Tucker (1931), were useful at a time when much confusion existed about the genus *Phytophthora* and these were the only keys available. However, only morphological classification was available. In spite of this, some of the features of *P. citrophthora* described in the Waterhouse keys are not consistent with subsequent descriptions of this species given by other workers (Ferguson, 1976; Ribeiro, 1978). Many isolates considered to be *P. citrophthora* have later been found to be incorrectly classified as more information was accumulated (Erwin *et al.*, 1983). For example, one isolate considered to be non-pathogenic to Citrus (Mitchell *et al.*, 1977) has now been reclassified as *P. palmivora*. (Mitchell, pers. comm.). Unfortunately, even the recent

updated keys are incomplete (Stamps *et al.*, 1990). Some of the observed discrepancies may be accounted for by the fact that morphological characters can be affected by environmental factors, particularly if cultural conditions are not standardised. This is not always possible because some isolates have as yet unknown specific cultural requirements, particularly if sexual characters are used, as was found to be the case in this study (Chapter 7).

Ideally, genetic, physiological and morphological limits of a species should all be used to improve on our current definition of a species. The degree to which genes are conserved versus the diversity exhibited can be used as a measure by which a molecular marker such as RFLPs will be useful at the taxonomic level. Isozymes may be less useful as they may be either too variable to allow isolates to be grouped together or too similar to allow discrimination, depending on the enzyme analysed. For example, isozyme studies failed to detect differences between some A1 and A2 isolates used in this study (Förster *et al.*, 1990a). PCR is an alternative technique which was successfully employed in this study for examining genetic variation between isolates and supported the results from RFLP analysis. Recently, PCR analysis of the internal spacer regions between rDNA sequences indicated a close relationship between Cocoa isolates of *P. citrophthora* and *P. capsici* (Lee and Taylor, 1991) whilst still distinguishing between the two species.

Another characteristic which can also be used for analysing variation within a species is chromosome number (Chapter 8). Chromosome counts using cytological techniques have been used in some cases for species characterisation (Sansome, 1980) and may become more prevalent with the recent development of CHEF techniques which allow for the successful karyotyping of chromosomes, as found in this study (Chapter 8).

It is apparent from the DNA analysis in this study that defining what constitutes the species *P. citrophthora* is complex. Two groupings of *P. citrophthora* isolates were identified using PCR and RFLP analysis (Chapter 8) which correlated with mating type whereas the groups were morphologically indistinguishable (Chapter 4). This has been observed in other studies. For example, *P. citrophthora* isolates taken from Cocoa and Citrus appeared morphologically similar but were shown by RFLP analysis to be genetically distinct (Förster *et al.*, 1990a). One problem highlighted by the RFLP analysis is that of species definition; that often there is no certainty that one is working with a single species or across several species. This has obvious consequences when studying the genetics of an organism. The problem has been further compounded because a given isolate may have been re-coded several times which presents problems when following up another author's work. For example, Goodwin *et al.* (1990) using RFLP analysis found two major groups within *P. citrophthora* but because the isolates had been re-coded it was not possible to compare their results with those found here.

In this study, analysis of the genetic data (particularly RFLPs) further infers that even *P. citrophthora* isolates specifically taken from the same host (Citrus), may in fact be different species. An alternative hypothesis is that the DNA probes are hybridising to a mating-type locus although this would appear to be less likely because of the consistent banding patterns given by a large number of different probes used in the RFLP analysis and because of the homology found using random plant primers in the PCR analysis. However, both hypotheses would need to be tested further via either sexual recombination experiments or by testing for localisation of probes to chromosomes, using the oospore germination and CHEF techniques developed for *P. citrophthora* in this study. If the A1 and A2 groups were initially the same species, the question arises regarding the mechanisms responsible for this divergence. To examine this question a more detailed knowledge of sexual reproduction in *Phytophthora* spp. is needed. This should be now possible, following the development of techniques for inducing oospore formation described in Chapter 7.

The sexual stage, as illustrated in this study, is one of the most complex areas of *Phytophthora* biology because the genus is divided into homothallic and heterothallic species. Homothallic species produce oospores promptly and abundantly in normal single isolate culture without special external stimuli. In contrast, in heterothallic species, single isolates exist as either A1 or A2 compatibility types and the pairing of A1 and A2 types in culture is necessary for the induction of oospore formation although, in some cases it has been shown that single isolates of either compatibility type may be induced to self in response to certain external stimuli (Ko, 1978). One

question raised in this study is whether selfing can occur within heterothallic *P. citrophthora*. If so, reproductive isolation through selfing may have resulted in the A1 and A2 groups observed in this study.

Although the sexual stage has been successfully produced *in vitro,* the occurrence of the sexual stage in nature is unknown and its importance, therefore, difficult to assess. The potential for gene flow would be difficult to determine via normal Mendelian genetics because of the "specific mating ability" of isolates observed in this study which may also be responsible in part for the difficulties in producing and germinating oospores. In this sense, molecular studies may be invaluable to our knowledge of *P. citrophthora.* For example, the degree of linkage disequilibrium between polymorphic markers can indicate the significance of sexual reproduction; if all the polymorphisms in a population exist at a few loci then outcrossing and recombination at the loci are probably not occurring (Michelmore and Hulbert, 1987). The few polymorphisms observed in this study between isolates of *P. citrophthora* would therefore imply that there is very little, if any, genetic exchange of material via sexual recombination.

If this is the case, then the variation in cultural morphology, growth rates and pathogenicity observed in this and other studies is difficult to explain (Boccas, 1981; Caten and Jinks, 1968). It may be that the variation observed derives from asexual mechanisms. Distinguishing between nuclear exchange, chromosome exchange or

nondisjunction and mitotic crossing–over requires an analysis of co–dominant markers such as isozymes or RFLPs, as well the use of drug resistant mutants to denote new variants since morphological characters are ambiguous. The role of these possible asexual mechanisms for generating new pathotypes in nature is unclear here. Changes in morphology and growth rates of single zoospore–derived progeny have been attributed to cytoplasmic inheritance of characters from the mother cell (Caten and Jinks, 1968).

However, it may be that too much emphasis is placed on genetic interpretation to explain changes in colony morphology and growth rates. In this study it was evident that the variability in morphology found for single zoospore–derived progeny was transient (Chapter 4). In addition, the differences observed between the morphology of parent isolates and single zoospore–derived progeny did not correlate with the RFLP banding patterns. Although it is true that the genome (nuclear plus organellar) plays a primary role in the inheritance of form, it is an ancillary one. Morphology is really the expression of cellular organization as influenced by environmental factors (such as nutrition, moisture, temperature). Thus it is difficult to determine genotypic characteristics from phenotypic data. The importance of this point has led to a recent review on the subject by Harold (1990).

Further studies would need to focus on what are the mechanisms responsible for variability within *P. citrophthora* and should aim to elucidate our understanding of sexual reproduction within *Phytophthora* and in particular, the sexual mechanism in heterothallic species, which has led to problems with the production and germination

of oospores in culture and which is further confounded by the selfing phenomenon. Clearly the next avenue of research would be to examine whether genetic exchange via sexual recombination can occur between the two groups of *P. citrophthora* isolates examined in this study.

In conclusion, this study has highlighted the complexity which exists within *P. citrophthora* to a previously, unrecognised extent. This type of complexity has been noted for other *Phytophthora* species (Chapter 2). The introduction of other classification methods, such as molecular analyses, electrophoretic karyotyping, and a better understanding of the sexual and asexual stages, may help resolve the question of what characteristics designate *P. citrophthora* as a species. Overall, the information obtained in this study, with respect to taxonomy, pathogenicity and host/pathogen relationships, has contributed to our understanding of this important pathogen as well as opening up other avenues of potentially exciting research.

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