Project title:	Identification of sources of inoculum and the development of rapid diagnostic tests for *Pythium* infestation of ornamentals on nurseries
Report:	Final (31 March 1998)
Project number:	PC 97
Project leader:	Dr J G White
Researchers:	Ms A J Wakeham, Ms S R Kenny
Location:	HRI Wellesbourne
Project co-ordinator:	Mr Stuart Coutts
Date commenced:	1 January 1995
Date completed:	31 December 1997
Keywords:	*Pythium*, infestation of nurseries, ornamentals, serological detection, fungicide resistance, surface sterilants

Whilst reports issued under the auspices of the HDC are prepared from the best available information, neither the authors or the HDC can accept any responsibility for inaccuracy or liability for loss, damage or injury from the application of any concept or procedure discussed.

©1998 Horticultural Development Council
No part of this publication may be reproduced in any form or by any means without prior permission from the HDC.

CONTENTS

		Page No.
PRACTICAL SECTION FOR GROWERS		1
A.	GENERAL INTRODUCTION	5
B.	PART 1 - ON-NURSERY MONITORING OF *PYTHIUM* INFESTATION	
	Introduction	6
	Materials and Methods	6
	Results	7
	Discussion	16
	Conclusions	18
B.	PART 2 - SEROLOGICAL DIAGNOSTICS STUDIES	
	Introduction	20
	Materials and Methods	21
	Results	21
	Discussion	29
	Conclusions	29
B.	PART 3 - FUNGICIDE RESISTANCE AND REDUCED SENSITIVITY	
	Introduction	31
	Materials and Methods	31
	Results	32
	Discussion	40
	Conclusions	41
B.	PART 4 - SURFACE STERILANT CHEMICALS	
	Introduction	42
	Materials and Methods	42
	Results	44
	Discussion	44
	Conclusions	46
C.	OVERALL DISCUSSION	47
D.	OVERALL CONCLUSIONS	48
E.	ACKNOWLEDGMENTS	49
GLOSSARY		50

PRACTICAL SECTION FOR GROWERS

Pythium is a complex group of fungi, numbering 85 different species and groups of isolates which cannot be named, also having members which are fast- or slow-growing, or are adapted to hot or cold temperatures. Many are pathogens of higher plants. Some species do not attack crop plants, but just live around their root systems, whilst one group of *Pythium* species are pathogenic on both *Pythium* and other species of fungi. *Pythium* also has the ability to rapidly develop resistance (or reduced sensitivity) to some fungicides, and as fewer fungicides become available for the ornamentals sector, the pressure on them is very great. These considerations make it very difficult to work sensibly with *Pythium*, and the purpose of this project was to gather information on the state of infestation of nurseries, which would in turn enable us to provide improved advice on avoidance of *Pythium* problems.

The project was originally collaborative with five plant propagators and growers-on, and in the second and third years three specialist pot and cut plant producers became collaborators in the work. Over the period of the project, four areas of work were considered:

1. What is there? Described in the Report as 'on-nursery monitoring'.
2. Considerations for detection of *Pythium* with serological diagnostic tests.
3. The current status of fungicide resistance (or reduced sensitivity).
4. The use of surface sterilant products for reducing levels of *Pythium* inoculum in nurseries.

1. What is there?

This aspect was most heavily supported by the grower collaborators, and this reflects their serious concern that it is not possible to deal with a problem until you know the true scale of it. The project showed that the nurseries have *Pythium* almost everywhere, and that practical ways of working round it would be needed because it is unlikely that it could be eradicated and then kept out. Water supply was not a source of *Pythium* when taken into the nurseries, but once in a variety of forms of storage, or in pipework the fungus could readily be detected. Peat based compost was generally suspected of bringing

© 1998 Horticultural Development Council

in *Pythium*, but in this, and other work we have done, significant isolation from peat composts has not occurred. With the loading of the fungus already on nurseries it is unlikely that *Pythium* from such a source would be commercially significant. It is well known to science that black peat, which is the main constituent of common peat-based composts, has no suppressive ability for *Pythium*, while white peat, taken from upper horizons in the bog, is almost completely suppressive. A combination of economics and availability pushes usage towards the black peats.

While *Pythium* on floors and in corridors on nurseries might not seem very important, the presence of the fungus on key machinery such as seeders and compost shredders is obviously a different matter. Such contamination inevitably means that the grower will have to use fungicide on trays of seedlings, and the fungicides available will only hold back the infection, not cure it. So, plants grown-on in pots or gardens will carry their own *Pythium* with them, and have the uncertain future, which prompted this project. The work also highlighted the importance of benches for gapping-up and for standing trays or pots. By their very nature, benches used for gapping-up are likely to have trays carrying infection placed on them. That infection will inevitably transfer to the bench, and in turn to other trays, creating an efficient way of moving disease within the nursery. Simply sterilising those benches at the end of the working day would considerably improve on present practice. Alternatively, to use sheets of polythene to cover the bench and replace at regular intervals would be beneficial. For benches used for standing trays and pots, minimum hygiene requirements should be to treat with an effective surface sterilant before putting new batches in place.

We return to the *Pythium* on floors and in corridors, because workers also use those when moving between work areas. That we were freely able to isolate *Pythium* from footwear is a reminder that whatever is done to control the fungus may be undone by workers re-introducing it in the course of their work. In some areas of horticulture the use of footdips to prevent ingress of fungi into growing areas is commonplace, and it could be of value in this context.

2. Serological detection of *Pythium*

In an ideal world, science would provide growers with a cheap, rapid test for *Pythium* which they could

© 1998 Horticultural Development Council

use in the nursery and which would identify the species present. Because the genus is so complicated, it is unlikely that we will satisfy all the criteria. In the course of the present project it became obvious that simple answers would not be possible. Because members of the genus *Pythium* clearly have at least two separate origins, in addition to the different functions outlined above, no single antibody probe would satisfy all practical needs. New technology identified in the course of the project also showed how we might in time answer the needs of the grower. For less demanding areas, there are ways round the problems of cross-reactivity, and the zoospore trapping immunoassay developed as a spin-off from this project is one of those.

3. Fungicide resistance (or reduced sensitivity)

It is well known that the fungicides based on the acylalanine molecule have often led to the rapid development of resistance in *Pythium*. The resistance is a single gene switch, and appears not to be reversible. In the context of this project, isolates of *Pythium* with the ability to grow well in the presence of furalaxyl (Fongarid) were common. This is not to say that there are not situations where the fungicide would be useful. Of the collaborating growers, several had deliberately not used the fungicide for some years in an attempt to create a situation where it would once again work. To combine this approach with cleansing with surface sterilants those areas contaminated with furalaxyl resistant *Pythium* would predictably be more effective.

Data on propamocarb (Filex) and etridiazole (Aaterra) showed some species of *Pythium* with very high ED50's* compared to base levels for both fungicides of <10 μg/ml. Not enough is known about these fungicides to say whether this shows the development of resistance as with furalaxyl, or a gradual eroding of the ability of the fungicides to control those species. However, for both fungicides the current application rates are relatively high, and there would appear to be a buffer between the amount of fungicide applied, and the levels which these species are able to tolerate. This aspect will be monitored in the second phase of the project.

For fosetyl-Al (Alliette) there is no test by which ED50's may be measured. The fungicide is thought to act by being taken up into the plant and then converts into the active ingredient. Because of the frailty

© 1998 Horticultural Development Council

of cover, which the growers have, this aspect will now be investigated in detail.

4. Surface sterilant chemicals

At the time of this survey, only four of the collaborative growers used any surface sterilant, and each one used a different product. All the growers were unconvinced that current surface sterilants actually work, but accepted that they could improve their hygiene with a product at known effective rates. The tests in this project were designed to severely assess any chemical, and therefore identify those which are very effective, and which should be excellent in less stringent conditions. Two peroxide based sterilants (Jet 5 from Hortichem, and Reciclean from Kemira) were most effective, whilst contact bleach was shown to be very good at relatively high concentration. For large expanses of hard standing or benching the former would be most useful, while for discrete areas such as gapping-up benches the ease of making a regular, simple dilution of bleach for routine use would be appropriate.

Action Points

1. To develop an awareness of where *Pythium* is likely to contaminate surfaces and to encourage an attitude of cleanliness in workers responsible for machinery such as seeders.
2. To be aware that hygiene at the start of the processes may save considerable fungicide usage at a later date.
3. To note the most effective surface sterilants and to use them systematically to reduce levels of *Pythium* inoculum.

* ED50 is the concentration of fungicide in µg/ml which reduces growth of a fungus to half that of the untreated control.

© 1998 Horticultural Development Council

A. GENERAL INTRODUCTION

Pythium affects all aspects of UK horticulture, and although it is generally viewed as a damping-off pathogen, this is far from the whole truth. In the context of UK ornamentals production, *Pythium* is never out of the conversation for long, and for every situation which seems to have been answered, a new one arises. In meetings and at demonstrations around the country, growers have always been keen to discuss their own problems with this damaging group of fungi.

It is obvious that progress in horticulture has taken directions which help, not hinder, *Pythium*. Large propagation units provide high pressure, high intensity growing conditions in which the fungus thrives. Growing systems which rely on regular addition of large volumes of water, often stored somewhere between source and end-use, suffer from time to time. Growing systems where large volumes of water, or more correctly nutrient solution are introduced and withdrawn fit well with the stimuli needed for zoosporic species of *Pythium* to regularly liberate millions of swimming units of infection (zoospores). Transport systems move both the fungus, and any sources of resistance to fungicide it may have developed, between nurseries.

In spite of this, large sectors of the ornamentals industry appear to be in good health. While some aspects of this project work could benefit some growers considerably, it is our feeling that for many, the work might just act as a guide to how they might tighten up some aspects of production... should there be time in the working day.

© 1998 Horticultural Development Council

B. PART I - ON-NURSERY MONITORING OF INFESTATION

Introduction

The scientific literature does not contain comprehensive information on the *Pythium* species, which are most prevalent in UK ornamentals. On a world basis, the literature helps us less, because workers generally focus on one or two species, to the exclusion of the bulk of *Pythium* activity in this complex area. A major component of this project was to survey the nurseries of the eight collaborating growers, to determine the concerns of those growers about the effects of *Pythium* on their operations and to highlight 'real' problem areas. The work started with a discussion with each grower, followed by a site walk and extensive sampling of structures, machinery and other parts of production. All nurseries had one visit in spring of the first or second year of the project, some several visits. The findings are described below.

Materials and Methods

Survey methods for *Pythium* are not complicated, relying only on a swab of sterile filter paper which can be exposed to any area suspected of being contaminated, and then placed on a selective growth medium. Throughout the work, cornmeal agar (Difco) amended with the antifungal agent pimaricin (100 mg litre^{-1}) and the anti-bacterial agent rifamycin (30 mg litre^{-1}). Incubations were routinely at room temperature (17 - 23°C), with sub-culture of any *Pythium* isolates onto V8 Juice agar (3 g agar, 0.4 g calcium carbonate, 20 ml V8 vegetable juice [Campbell's Soups, King's Lynn, Norfolk, UK] 180 ml distilled water), with or without rifamycin. Taxonomic identification was done using the key of Plaats-Niterink (1981) and for most isolates this was sufficient. In the course of the project a form of identification based on molecular methods was developed, and this was used for some of the HS group isolates.

Following identification, isolates were put into a culture collection for storage on slopes of cornmeal agar. This enabled selected species to be retained for use in fungicide sensitivity tests and in work with surface sterilant chemicals.

© 1998 Horticultural Development Council

Results

For reasons of commercial confidentiality the collaborating growers are identified only as numbers, with no information on their crops or geographical location.

Discussions on current practice

None of the growers were content that either fungicides, or surface sterilant chemicals could be relied on to give good disease control. To some extent this was compounded by commercial pressures which, they saw, left them often using products without full knowledge of the way to give the materials the best chance to work. Also, that perceived need to use materials often came after an outbreak of disease which inevitably meant that they could do little to improve on a bad situation. Few had looked at currently available diagnostic products for early disease diagnosis, although they acknowledged the logic of using such tools if they were available. The growers that had used current commercial diagnostic tests found them too expensive, and too prone to giving the wrong answer because of cross-reactivity problems of the constituent antibodies which are well known in the scientific literature.

Growers in general did not see *Pythium* problems coming from their water supply, and from limited work it would appear likely that this is true. Five growers used mains water supply, three used borehole water, two supplemented supply with rainwater or well water. They were all aware of problems arising once the water was in storage facilities and/or pipework on the nursery, and this did appear to be an area where significant progress in control of *Pythium* could be made. No grower found it necessary to apply any control measure for *Pythium* to incoming water.

Early in the project a study was made with one of the major suppliers of peat-based composts to the UK ornamentals industry of peat sources from which composts result. In a visit to a series of peat bogs, peat was collected using sterile technique, and put through the standard isolation method. *Pythium* was not detected in 104 samples taken from four different bogs, or in 22 samples taken from the factory. With

© 1998 Horticultural Development Council

this result we can suggest that although growers clearly had a tacit assumption that peat does bring *Pythium* onto the nursery, this work suggests that there is probably more fungus already there than is brought in. This aspect is a considerable problem for the researcher because it simply is not possible to know whether *Pythium* appearing in compost at any stage of production of plants came in with the compost or was picked up from any of the large number of surfaces met by that compost during commercial processes. This point is illustrated by the proven instance of clean trays being put onto a seeder only to come off that machine already contaminated with *Pythium*. Given the enormous cost of some ornamentals seed, and the fact that *Pythium* can invade germinating seed in hours, this is clearly not an occurrence to be encouraged. Indeed, that it was relatively easy to demonstrate suggests that more focus on the degree of contamination of machinery is essential.

The use of fungicides was clearly limited to those materials with clearance for ornamentals, with most growers acknowledging a role for Fongarid (furalaxyl) where resistance in *Pythium* to that fungicide is not present, general use of Filex (propamocarb) and Aaterra (etridiazole), with some doubt about their value, and no use of Alliette (fosetyl-Al) for *Pythium* because of expressed doubt about its efficacy. This is a very narrow range of fungicides for an industry as diverse as the one under consideration, and gives pointers to the need for new, effective materials, and also efforts by the growers to minimise the need to use fungicides by cleaning up with effective surface sterilant products.

Methyl bromide was used by some growers to sterilise hard standing and re-cycled plasticware, but this will be lost in the year 2002, and should not be considered a prospect for helping the industry long-term. Of the eight collaborative growers, only four used surface sterilant chemicals, with one grower using each of Panacide, Panaclean, Menna-Ter-Spezial and commercial bleach. With the results below, this is clearly an area of weakness in the industry, and one way that growers have of making radical reductions in the loading of *Pythium* in their nurseries, is to reduce the inoculum which reaches their production sometimes as early as the seeder.

© 1998 Horticultural Development Council

CASE STUDIES

Grower 1

Number of attempts at isolation of *Pythium*	30	
Number of attempts positive	14	
Sites from which *Pythium* isolated		Floors
		Matting
		Compost shredder
		Work surfaces
Species of *Pythium* isolated		*P. paroecandrum* (x 6)
		P. ultimum var *sporangiferum* (x 2)
		P. ultimum var *ultimum* (x 2)
		Pythium Group F (x 2)
		Pythium HS Group (x 1)
		Species unknown (x 1)

Grower 2

Number of attempts at isolation of *Pythium*	59	
Number of attempts positive	33	
Sites from which *Pythium* isolated		Floors
		Benching
		Compost preparation area
		Work surfaces
		Standing water
		Mypex over hard standing
		Antirrhinum roots

© 1998 Horticultural Development Council

Species of *Pythium* isolated

P. intermedium (x 2)
P. irregulare (x 2)
P. paroecandrum (x 11)
P. torulosum (x 1)
P. ultimum var *ultimum* (x 3)
Pythium Group F (x 9)
Pythium HS Group (x 1)
Species unknown (x 4)

Grower 3

Number of attempts at isolation of *Pythium*	87	
Number of attempts positive	37	
Sites from which *Pythium* isolated		Floors Benching Under-benching gravel Capillary matting Pipework Ebb and flow system Water storage tank Danish trolleys

Species of *Pythium* isolated

P. intermedium (x 1)
P. irregulare (x 1)
P. paroecandrum (x 9)
P. salpingophorum (x 2)
P. torulosum (x 1)
P. ultimum var *ultimum* (x 1)
Pythium Group F (x 17)
Species unknown (x 5)

© 1998 Horticultural Development Council

Grower 4

Number of attempts at isolation of *Pythium*	58	
Number of attempts positive	37	
Sites from which *Pythium* isolated		Floors
		Seeders
		Benches
		Used plasticware
		Danish trolleys
		Mypex covering
		Chrysanthemum roots
Species of *Pythium* isolated		*P. intermedium* (x 3)
		P. paroecandrum (x 5)
		P. polymastum (x 3)
		P. salpingophorum (x 2)
		P. sylvaticum (x 4)
		P. ultimum var *sporangiferum* (x 1)
		P. ultimum var *ultimum* (x 5)
		Pythium HS Group (x 8)
		Species unknown (x 6)

Grower 5

Number of attempts at isolation of *Pythium*	50	
Number of attempts positive	30	
Sites from which *Pythium* isolated		Assorted machinery
		Floors

© 1998 Horticultural Development Council

Benching
Mypex covering
Capillary matting
Pipework and walls

Species of *Pythium* isolated

P. intermedium (x 1)
P. paroecandrum (x 6)
P. sylvaticum (x 3)
Pythium Group F (x 7)
Pythium HS Group (x 4)
Species unknown (x 9)

Grower 6

Number of attempts at isolation of *Pythium* 114
Number of attempts positive 58
Sites from which *Pythium* isolated

Floors
Prepared compost
Danish trolleys
Mypex covering
Capillary matting
Gravel storage
Sorting bench
Begonia cuttings
Viola plants

Species of *Pythium* isolated

P. sylvaticum (x 11)
Pythium Group F (x 4)
Pythium HS Group (x 15)
Pythium Group T (x 5)

© 1998 Horticultural Development Council

Species unknown (x 23)

Grower 7

Number of attempts at isolation of *Pythium*	94	
Number of attempts positive	53	
Sites from which *Pythium* isolated		Compost bin
		Compost shredder
		Floors
		Recycled trays
		Mypex coverings
		Danish trolleys
		Potting machine
Species of *Pythium* isolated		*P. rostratum* (x 7)
		P. sylvaticum (x 4)
		Pythium Group F (x 7)
		Pythium HS Group (x 4)
		Pythium Group T (x 1)
		Species unknown (x 30)

Grower 8

Number of attempts at isolation of *Pythium*	90	
Number of attempts positive	51	
Sites from which *Pythium* isolated		Floors
		Compost shredder
		Benches
		Danish trolleys
		Workers boots
		Chrysanthemum roots

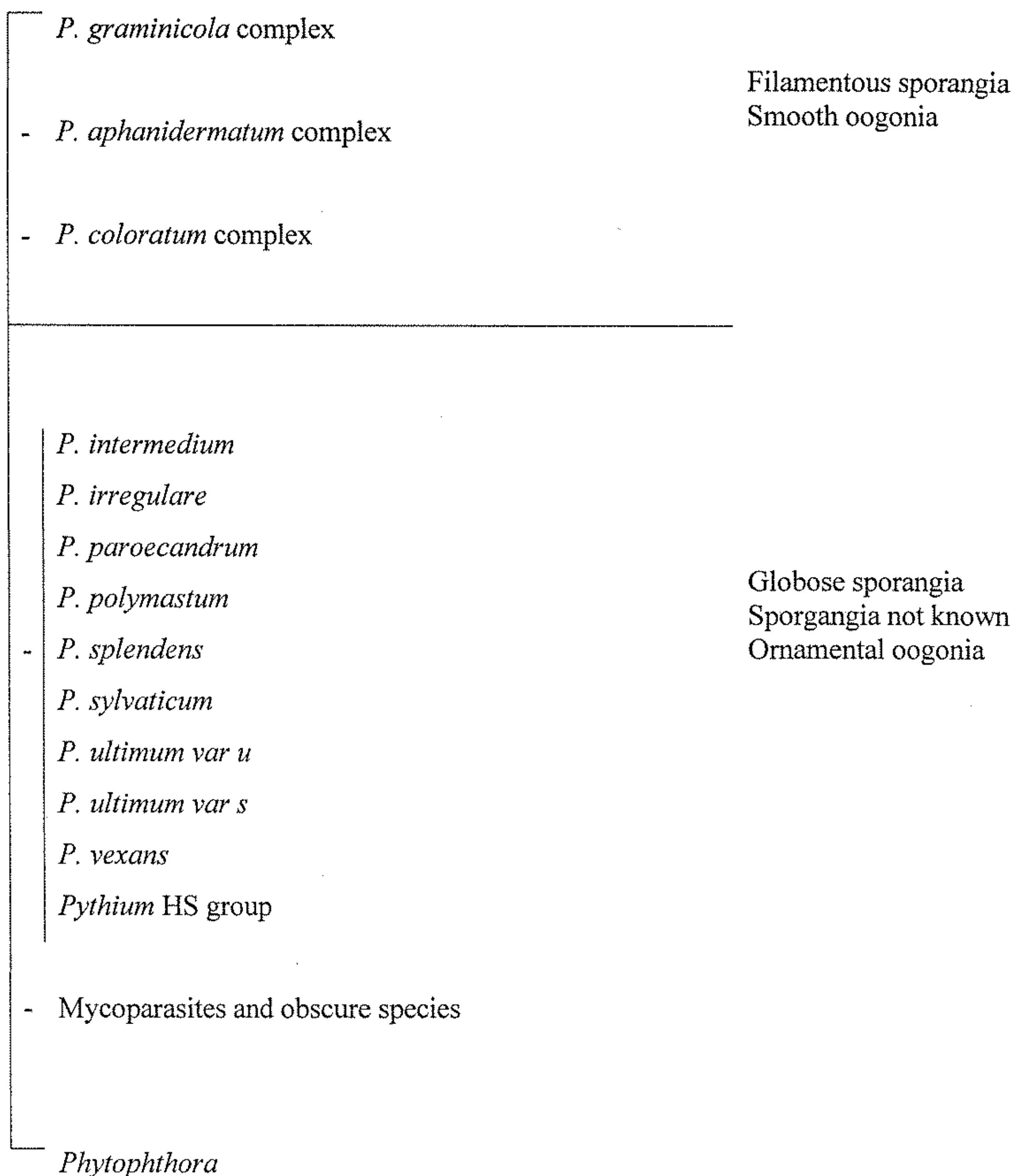
© 1998 Horticultural Development Council

	Lily roots
Species of *Pythium* isolated	*P. mastophorum* (x 1)
	P. oligandrum (x 3)
	P. rostratum (x 4)
	P. spinosum (x 1)
	P. sylvaticum (x 19)
	Pythium HS Group (x 7)
	Species unknown (x 16)

The above data illustrate several general points, the first being that with relatively un-sophisticated methods it was generally possible to find *Pythium* in over 50 % of isolation attempts. This only matters in the present context if the *Pythium* species are pathogens of ornamentals, and as a general screen it was decided to check isolates against antirrhinum and geranium in an agar plate test. Because we already know that *Pythium* species with different temperature optima are likely to exist in the industry, tests were made at 15, 20 and 25°C. Isolates of the species on water agar were baited with seedlings grown from surface sterilised antirrhinum and geranium seed, and the plates were incubated at room temperature for 3 days. Counts were made of seedlings disabled by the fungus. Of recognised species, *P. intermedium*, *P. irregulare*, *P. sylvaticum*, both forms of *P. ultimum* and members of the *Pythium* HS group were aggressive pathogens across the temperature range and almost all seedlings were reduced to gelatinous masses. Using molecular identification methods from MAFF-funded work, the two most common HS Group types were shown to be identical to *P. ultimum* var *ultimum* and *P. splendens*. It is thought that these isolates have either lost the ability to produce sexual reproductive organs by which they could be identified, or that they represent a primitive form of the two species. Either way, they slot firmly into the group of common fast-growing pathogens described above. During the course of this project, MAFF work on molecular aspects of *Pythium* clarified the relationships between some species, and to guide the reader, Fig 1 is reproduced from that work.

© 1998 Horticultural Development Council

Fig. 1. Divide of the *Pythium* species



Data ex. the work of Dr Pi-Han Wang shows the molecular relationship of the common fast-growing plant pathogenic species of *Pythium* on one tight group within the genus. Other important species are in the other half of the genus in the *P. aphanidermatum* complex.

© 1998 Horticultural Development Council

P. paroecandrum was isolated with great regularity, and while it did cause some damage at all temperatures, this was in general slight. *Pythium* Group F (only mycelium and filamentous sporangia are formed, and very little is known about this group) isolates which were common at some sites illustrated the importance of temperature in infection, causing relatively little damage at 15 or 20°C but severe damage at 25°C. The same pattern was seen with *P. polymastum* and *P. salpingophorum*. *P. torulosum* caused damage only on antirrhinum. Of the unknown species which were pathogenicity tested, all were aggressive pathogens across the temperature range, and in the next phase of the project it will be essential to characterise these to the species level and determine where they fit into the genus. A small number of slow-growing species such as *P. rostratum* were isolated, and were shown to be pathogens. Because in such work the fast-growing species tend to dominate isolation work, it is difficult to determine just how frequent in occurrence the slow-growers are, or their importance to the industry.

Most unusually, the major pathogen expected from indications from past work, *P. aphanidermatum* was not isolated once, and the only representative of its molecular group, *P. torulosum*, was isolated only twice. There is no obvious explanation for this observation, but as the former is the species most commonly isolated worldwide it is unlikely to be absent from the industry in general.

Discussion

The major pathogenic *Pythium* species in ornamentals nurseries were shown to be the common fast-growing species which are now known to come from one tight-knit group in the *Pythium* genus. There was considerable variation in the frequency of these from nursery to nursery, and swings in population depending on the type of culture used eg where ebb and flow systems predominated, the Group F *Pythium* was very common, and being a prolific producer of zoospores, this would appear logical. Isolates which could not be keyed down to species level were of a small number which produced oogonia in single culture, but which did not fit any of the recognised descriptions in the work of Plaats-Niterink. These problems should be rectified in the second phase of the project.

It was clear from the early discussions that all the growers were conscious of *Pythium* as a problem over which they exerted relatively little control. There was some feeling that *Pythium* was brought in on

© 1998 Horticultural Development Council

compost or peat, but with little hard evidence. From our one visit to the peat bogs of a major producer, we find it hard to visualise peat as a major contributor to the inoculum. For most of these nurseries it would be accurate to say that pathogenic fast-growing *Pythium* species were endemic, and that it was common in extremely sensitive parts of the nurseries. Mentioned above is an instance of *Pythium* being spread onto trays at seeding. This reflected a lack of attention to detail which is probably not unreasonable in a dynamic commercial world where there is never enough time for a brief pause to take stock. As this, and similar instances inevitably precipitate the need for fungicides in the life of those seeded trays, it would appear essential to deal with it, and to keep the seeder *Pythium*-free.

Infestation on working benches was commonplace, with those benches used for 'gapping-up' of trays of seedlings being sites of regular isolation. In the review meeting in spring 1997, this was focussed on as one area which no-one thinks to sterilise, but which by definition of its function means that infected material must pass over on a daily basis. The tools used for gapping up would similarly be contaminated, and something which could be disposed of, or sterilised at regular intervals.

Standing benches and areas such as those covered with Mypex were again a common source of *Pythium*. Here, there is the ongoing problem of how to keep them clean, because trays with *Pythium* could result in immediate contamination of sterilised benching or Mypex. Worse, because workers footwear yielded pathogenic *Pythium*, the act of placing trays on clean Mypex could effectively inoculate the surface.

Danish trolleys, by which plants are moved around the UK and Europe were one of the most consistent sources of *Pythium*, not only common species, but also of the unknowns from two nurseries. It was clear that they are an accepted 'part of the furniture' which no-one gave too much thought to. However, as an excellent means of moving *Pythium* over long distances, and potentially of moving fungicide resistance, Danish trolleys have an unrivalled position. While growers may be reluctant to spend too much time working on the trolleys themselves (human nature), for self protection one would encourage them to insist that any trolleys incoming should have evidence from source that they have been sprayed with an effective surface sterilant before loading.

© 1998 Horticultural Development Council

Re-used plasticware was relatively uncommon, and although *Pythium* was detected on some re-cycled containers, this must be relatively minor in importance. One grower who did regularly re-use plasticware took the precaution of annual sterilisation with methyl bromide.

Water supply, once it was outside source, was considered vulnerable to contamination by *Pythium*. Uncovered storage of water is particularly vulnerable, and once there is organic matter in the water, the zoosporic species of *Pythium* may multiply rapidly. This was one reason for the initiative to produce an assay for viable zoospore inoculum in water, because it was demonstrated that one grower was taking water from storage inside a glasshouse which was effectively a suspension of *Pythium* zoospores. To be able to rectify that situation it is essential to be able to monitor the populations of zoospores particularly where there are very few, and to have effective treatments with which to reduce the population to zero.

In this study, it is clear that one of the original concepts of the project, that the industry is selling plants with infection of pathogenic *Pythium*, with all that this entailed, is correct. Since the curative properties of furalaxyl are generally not available because of resistance to the fungicide, we must assume that fungicides which are used merely suppress disease for long enough to clear the plants. We therefore must look at ways of improving on this situation without reducing the productivity of nurseries. The possibilities have been discussed with the collaborating growers, and their views formed the basis of the new phase of the project (PC 97a Ornamentals: sources of *Pythium* inoculum, fungicide resistance, and efficacy of surface sterilants). Some relatively simple devices may be used to improve on what we have, and they will be explored in the overall discussion.

Conclusions

1. The common fast-growing *Pythium* species were found to be endemic in the nurseries surveyed. It was unlikely that inoculum in peat-based compost would contribute significant inoculum over and above what was already there.

2. Growers lacked confidence in both fungicides and surface sterilants. They used three of the four available fungicides, but only four growers significantly made use of surface sterilants to reduce known contamination.

© 1998 Horticultural Development Council

3. In the very nature of nursery operations it would be very difficult to make a *Pythium*-free environment, but attention to the hygiene of some key equipment and hardware such as Danish trolleys would considerably reduce problems downstream.

References

PLAATS-NITERINK,A J van der (1981). Monograph of the genus *Pythium*. *Studies in Mycology* **21**, 244pp.

© 1998 Horticultural Development Council

B. PART 2 - SEROLOGICAL DIAGNOSTIC STUDIES

Introduction

Part 1 of the report gives in graphic detail the potential scale of the problem with *Pythium* in the ornamentals industry. Because early detection and diagnosis of disease are essential to the minimisation of disease, serological assays are an obvious way to achieve this objective. To date, commercial assays have made little impact on the industry because they are expensive, and have quickly been found not to be reliable. Part of the latter problem has been that currently available kits were produced in the USA using their species of *Pythium*, which could not be expected to match those in UK ornamentals production.

From this laboratory, we had some 10 years ago, the production of an assay for cavity spot pathogens in potential carrot fields (White, Lyons & Petch, 1996). This assay was based on relatively simple reagents, and worked because the object of detection was a *Pythium* which grew in soil in the winter months, and could be measured by virtue of the enzymes it produced and released into soil. The present situation is far too complex for such a simple solution, but as part of the project it was necessary to investigate the potential for antibody-based tests which the industry might use. Major constraints were that the assay should detect pathogenic species of *Pythium*, but should not detect non-pathogenic species or the beneficial species. Central to this work was the concept that any test should be easily available to adviser or grower, also rapid and cheap. Developments in our knowledge during the course of this project significantly affected our objectives and resulted in acceleration of part of the work to produce an immediate test for zoospores in irrigation water, with setting aside of some work which would probably have produced a laboratory-based ELISA test. The work described below is preparative work which enabled one test to go ahead (Appendix 1) and which will feed into a system of lateral-flow dipstick technology developed by Dr N F Lyons during the period 1997-98.

© 1998 Horticultural Development Council

Materials and Methods

It was decided to produce a polyclonal antibody (PAb) to pooled antigen of five members of the common, fast-growing pathogenic *Pythium*'s, namely *P. intermedium*, *P. irregulare*, *P. sylvaticum*, *P ultimum* var. *ultimum* and *Pythium* HS group. Isolates were chosen from those taken from nurseries early in this project. Full details of this are given in Appendix 1. The PAb (coded 95\10\2) was shown to have high avidity for its homologous antigen with good recognition to a dilution of 1:25600.

The PAb was characterised by the standard protease and periodate tests, using antigen of *P. sylvaticum* and *Pythium* HS group. Further tests were the DIG glycan differentiation to determine the presence of glycoprotein epitopes and boiling of antigen to determine heat stability.

Further characterisation was for cross-reactivity within the genus *Pythium*, with isolates from this project, and with non-*Pythium* fungi .

A monoclonal antibody (Mab) raised at Birmingham University to *P. aphanidermatum* was assessed in MAFF work for cross-reactivity within the genus.

Results

The PAb was of high sensitivity to the antigen preparation, with a dilution end point of 1:102 400. Ability of the PAb to bind to both *Pythium* spp. was reduced after treatment with protease, but more so after treatment with periodate. Heat treatment of antigen had minor effects, but the DIG glycan kit showed recognition of glycoprotein epitopes. The practical implication of this is that although the PAb is very good for what it was raised to, it will also recognise many other species of *Pythium* which contain exactly the same components.

Fig. 2 shows the cross-reactivity pattern with 16 species of *Pythium* taken from the HRI culture collection. Eleven of those were from the molecular grouping of the species to which the antibody was

© 1998 Horticultural Development Council

raised, and most of those were selectively recognised. Species not recognised were *P. aphanidermatum* and *P. dissotocum* from the other half of the genus (Fig. 1), and the mycoparasite *P. oligandrum*. This is good for recognition of what the antibody was raised to, and the failure to recognise beneficial or unimportant species. However, failure to recognise the world's most common pathogenic *Pythium* is not helpful.

Fig. 3 shows similar data for a range of *Pythium* spp. from the nurseries, with the pathogenicity guide given in the key. Again, the antibody largely recognised pathogens from the survey with the exceptions of one isolate of *P. irregulare*, although signal with the species at 13 and 14 was weak. *P. torulosum*, from the same group as *P. aphanidermatum*, was not recognised. Fig. 4 shows similar data for species and isolates taken from Ornamentals Advice Centre samples. Most were not identified to species level, but a natural assumption since they came from diseased plants would be that they were all pathogens. A key observation is that *P. torulosum* was again not recognised, as was the case for four other isolates and the non-*Pythium* control. Similar results were obtained when the antibody was assessed with one isolate of each of the species to which it was raised, plus 13 non-pythiaceous fungi. The former were recognised and the latter were not. This data is not presented.

A series of experiments were carried out using Pab 95/10/2 *Pythium* in compost with variations on ELISA. In all, a major problem was high background with *Pythium*-free compost. There was not an obvious way to overcome this, and the work was stopped and is not presented.

© 1998 Horticultural Development Council

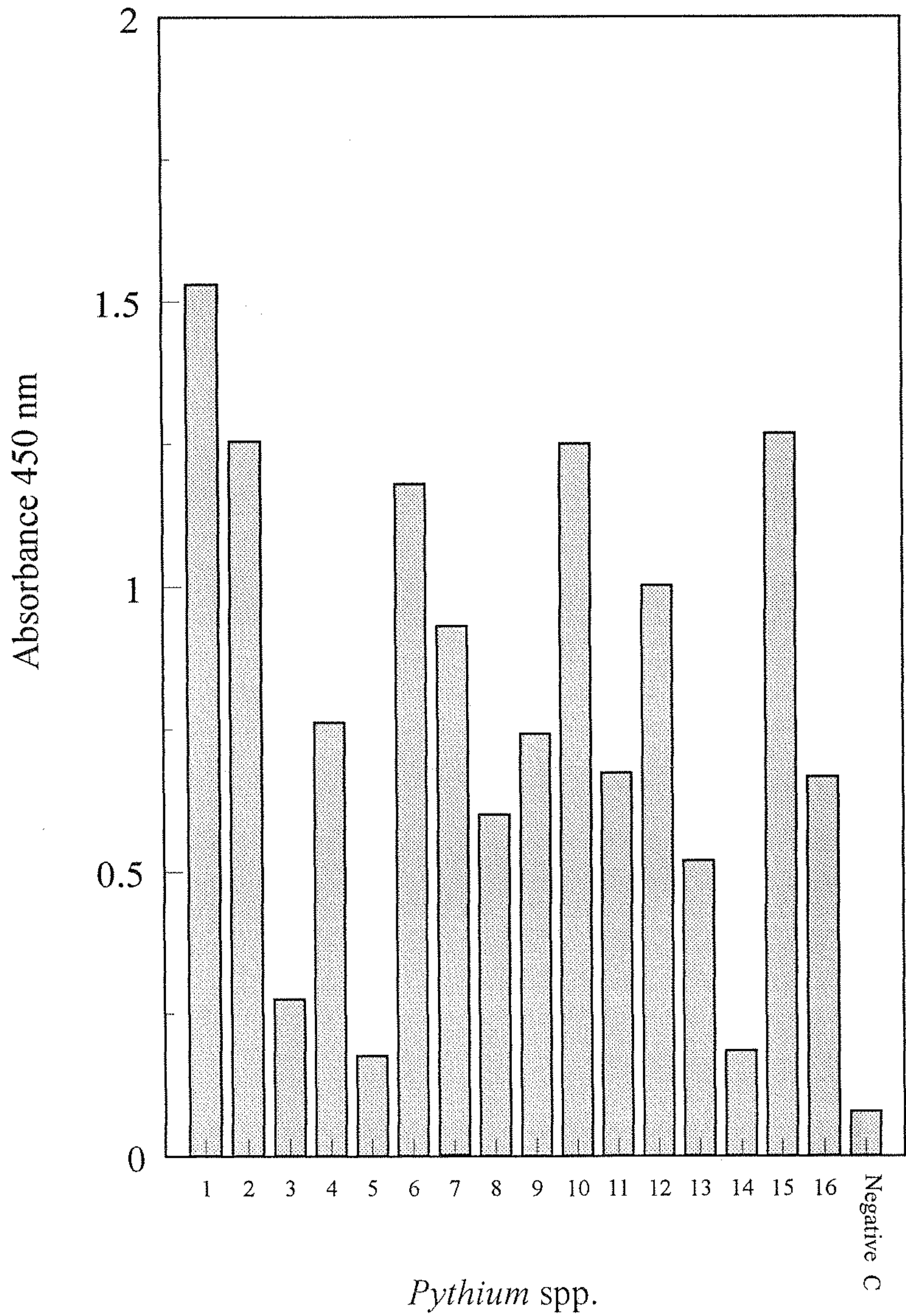
Fig. 2. Recognition by Pab 95/10/2 to verified isolates of *Pythium* from the HRI Culture Collection.

Key

Number	Culture Collection Code	*Pythium* Species
1	E	*P. ultimum*
2	Com 3	*P. intermedium*
3	PA1	*P. aphanidermatum*
4	40 W	*P.* hyphal swelling group
5	P. olig	*P. oligandrum*
6	P. inter	*P. intermedium*
7	OI	*P. sylvaticum*
8	N2	*P. irregulare*
9	PU	*P. ultimum*
10	PV8	*P. violae*
11	P. sykv	*P. sylvaticum*
12	K	*P. irregulare*
13	P. irreg	*P. irregulare*
14	A	*P. dissotocum*
15	P. spino	*P. spinosum*
16	P. paro	*P. paroecandrum*
17	Negative control: 3% TCB	

© 1998 Horticultural Development Council

Fig. 2



© 1998 Horticultural Development Council

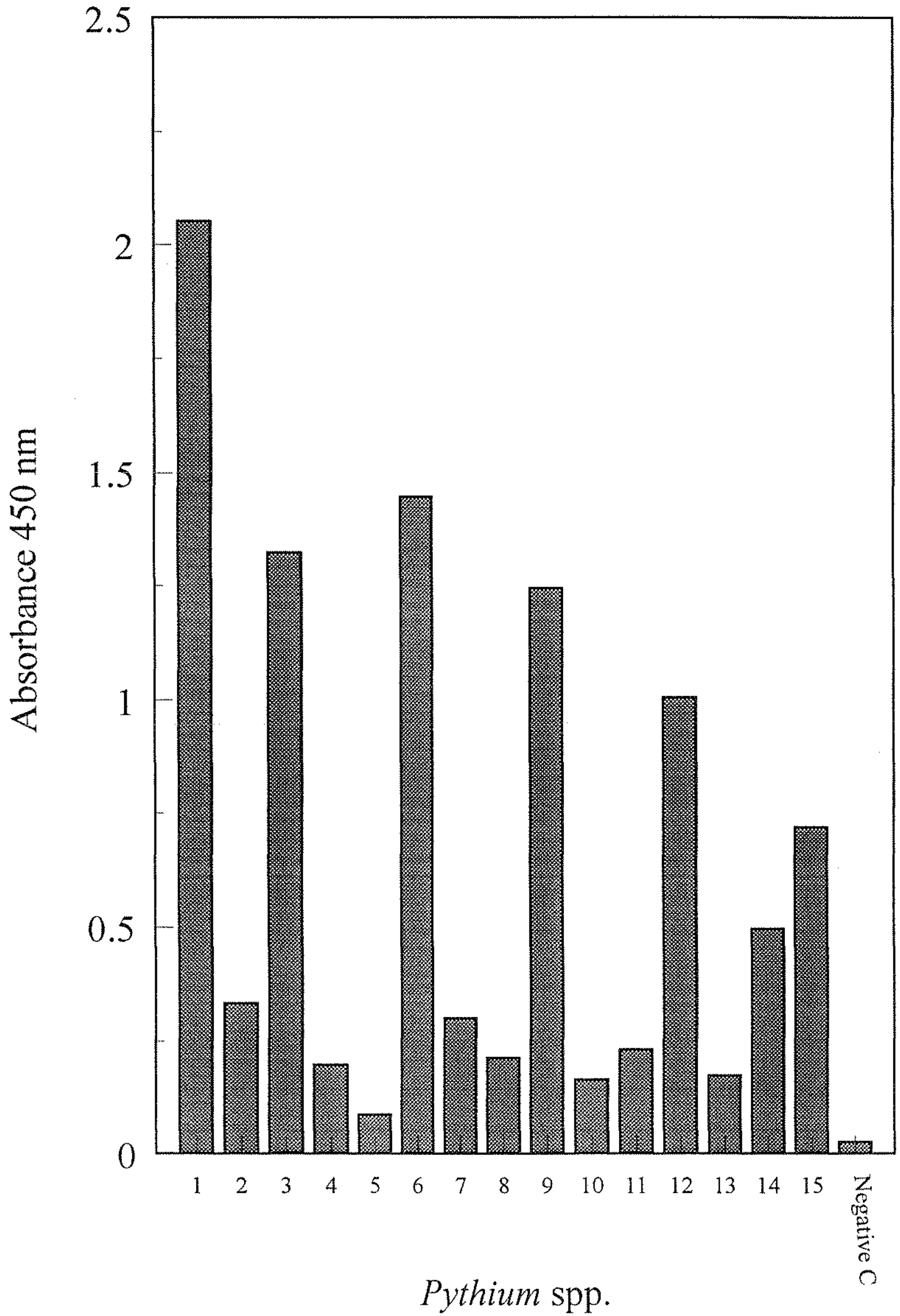
Fig. 3. Recognition by Pab 95/10/2 of a range of *Pythium* spp. and isolates from nurseries of collaborative growers.

Key

Number	*Pythium* species	Status of Pathogenicity
1	*P. sylvaticum*	Pathogen
2	*P. polymastum*	Weak/Non
3	*P. intermedium*	Pathogen
4	*P. torulsoum*	Weak/Non
5	*P. torulosum*	Weak/Non
6	Hyphal swelling group	Pathogen
7	Group F	Weak/Non
8	Group F	Pathogen/Weak
9	*P. ultiumum*	Pathogen
10	Group F	Non
11	*P. paroecandrum*	Weak/Non
12	*P. irregulare*	Pathogen
13	*P. irregulare*	Pathogen
14	*P. ultimum* var *sporangiferum*	Pathogen
15	*P. salpingophorum*	Pathogen/Weak

© 1998 Horticultural Development Council

Fig. 3



© 1998 Horticultural Development Council

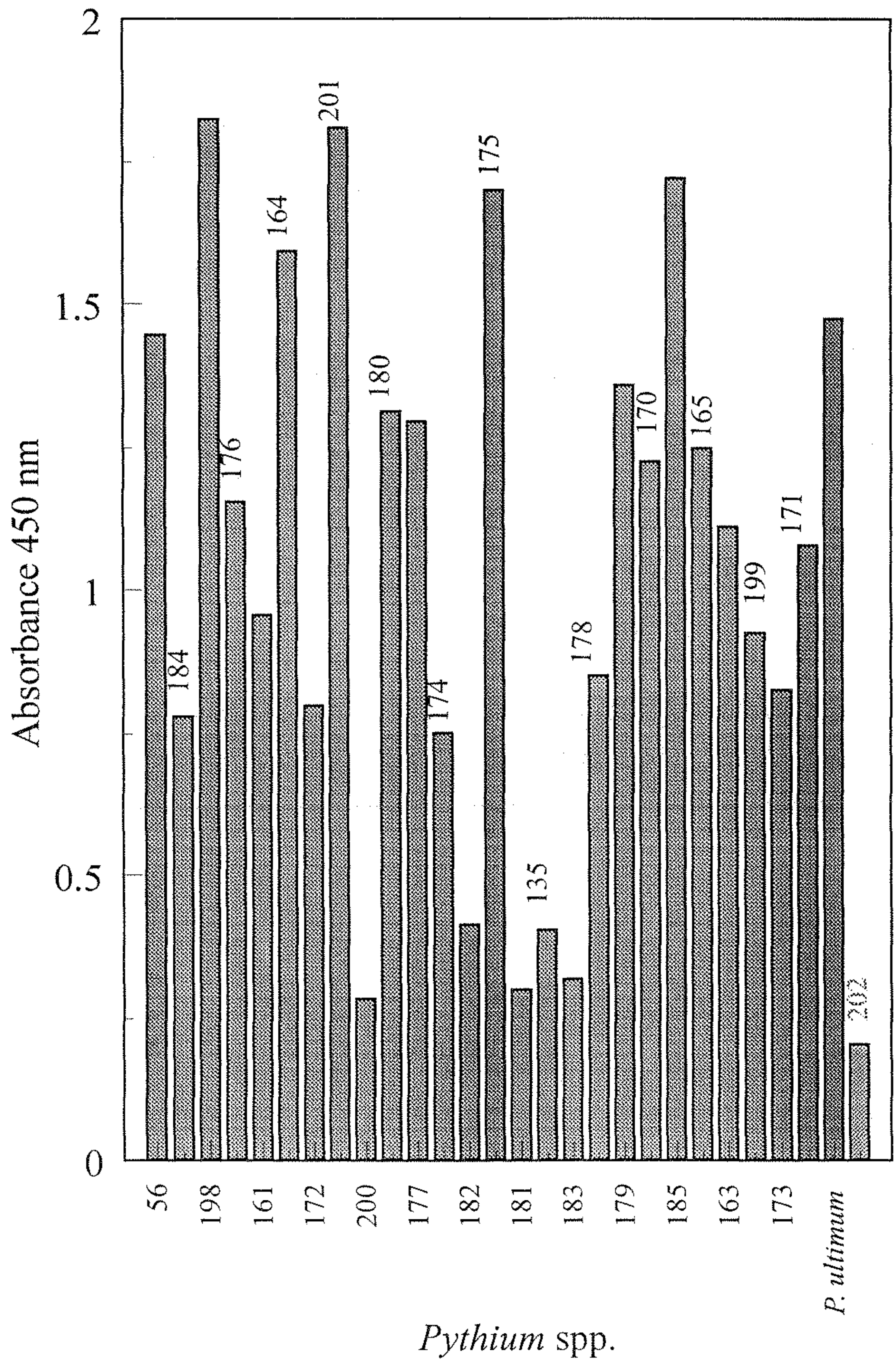
Fig. 4. Recognition by Pab 95/10/2 of *Pythium* spp. and isolates isolated from Ornamentals Advice Centre samples.

Column Number	*Pythium* species	Origin
56	*Pythium sylvaticum*	-
184	*Pythium sp.*	Verbena
198	*Pythium sp.*	Compost
176	*Pythium sp.*	Pot plant
161	*Pythium irregulare*	Antirrhinum
164	*Pythium irregulare*	Antirrhinum
172	*Pythium* sp.	Antirrhinum
201	*Pythium sylvaticum*	Poinsettia
200	*Pythium torulosum*	Poinsettia
180	*Pythium sp.*	Alyssum
177	*Pythium sp.*	Antirrhinum
174	*Pythium sp.*	Plugs
182	*Pythium sp.*	Plugs
175	*Pythium sp.*	Roses
181	*Pythium sp.*	Alyssum
135	*Pythium*	-
183	*Pythium*	Verbena
178	*Pythium sp.*	Pot plant
179	*Pythium sp.*	Celosia
170	*Pythium sp.*	Impatiens
185	*Pythium sp.*	Geranium
165	*Pythium irregulare*	Antirrhinum
163	*Pythium irregulare*	Antirrhinum
173	*Pythium sp.*	Antirrhinum
171	*Pythium sp.*	Antirrhinum
202	Non *Pythium sp.*	Poinsettia

- Not known

© 1998 Horticultural Development Council

Fig. 4



© 1998 Horticultural Development Council

Discussion

Pab 95/10/2 probably represents a considerable improvement on commercial antibodies in tests presently available. Selective recognition of the major pathogens seen in this work was a good feature. Non-recognition of members of the *P. aphanidermatum* complex was a major negative, because although they were not seen in this work, logic says that they will be of importance somewhere in ornamentals. When the work moved to compost-based tests, it was obvious that the antibody bound to *Pythium*-free compost in ELISA, and it was felt likely that mis-diagnosis of pathogen would occur.

To resolve the aspect in a logical way, it was decided in a meeting between HDC, MAFF and HRI to utilise the antibody in the test which is described at Appendix 1. Further, MAFF-funded work considered the need for an antibody which would recognise *P. aphanidermatum* and associated species in tests with new technology where results would not be compounded by reactions with compost. This was done with Mab PA6 raised by Jon Green at Birmingham University, which was found to selectively recognise both *P. aphanidermatum* and *P. torulosum*, and to show little response to members of the common fast-growing pathogen group. A test with the two antibodies will be assessed in the second phase of the project.

Conclusions

1. Pab 95/10/2 represented a considerable improvement over those antibodies in commercial assays in that it selectively recognised pathogenic *Pythium* sp in one half of the genus, to the exclusion of some non-target species.
2. Because of our advancing knowledge of the genus, we know that species with filamentous sporangia and smooth oogonia probably have a different origin to the common fast-growing pathogens. The antibody did not recognise the former, although some are likely to be of importance in this area.
3. Problems with ELISA-based tests and recognition by the antibody of *Pythium*-free compost made it necessary to consider different options for tests in the present context. These will be considered in the second phase of the project.

© 1998 Horticultural Development Council

References

WHITE, J G, LYONS, N F, PETCH, GM (1996). Development of a commercial diagnostic test for the pathogens which cause cavity spot of carrot. In *Proceedings BCPC Symposium on Diagnostics in Crop Production No. 65*, pp. 343 - 348.

© 1998 Horticultural Development Council

B. PART 3 - FUNGICIDE RESISTANCE AND REDUCED SENSITIVITY

Introduction

The last major survey of fungicide resistance in *Pythium* was that of White, Stanghellini and Ayoubi (1988) who looked at the status of metalaxyl resistance in 261 isolates covering 17 different species. This work showed that resistance to metalaxyl was becoming common, and that isolates of *Pythium* resistant to metalaxyl were also resistant to the other acylalanines including furalaxyl. Since that time, resistance developed under furalaxyl usage has been seen regularly in non-edible horticultural crops, and the number of alternative fungicides growers could use has decreased. This has left the situation that ornamentals growers may use as alternatives etridiazole (Aaterra), fosetyl-Al (Alliette) or propamocarb (Filex). Of older technology fungicides the protectant copper ammonium carbonate (Fungex) is still available. As noted above, collaborative growers of this project generally used only etridiazole or propamocarb, with minor use of furalaxyl. It was therefore decided to assess the status of resistance to the three fungicides. There is currently no test for resistance to fosetyl-Al.

Materials and Methods

Isolates of *Pythium* from the culture collection from this project were chosen to give a spread of the major species, across the growers and from different sources in nurseries. For reasons of confidentiality, sources of isolates are given only as the surface or substrate from which they came. A reference isolate of *P. ultimum* var *ultimum* which has been in culture for 60 years was used as the control species.

A standard growth test was used to determine response to fungicides which involved inoculating agar with different concentrations of etridiazole, furalaxyl and propamocarb with the isolates and monitoring growth over a 2-7 day period. Plates were prepared for each fungicide with the range of concentrations 0, 0.1, 1, 5, 10, 50 and 100 μg/ml. There were four replicates for each fungicide concentration/isolate combination. Data were analysed by a Genstat 5 programme which fitted a logistic curve and provided the ED50 values.

© 1998 Horticultural Development Council

Results

The results of the exercise are shown in Table 1. The control *P. ultimum* gave ED50 values of 33.0 μg/ml for etridiazole, 0.7 μg/ml for furalaxyl and 3.1 μg/ml for propamocarb. Data for etridiazole suggests species differences in response to the fungicide, with some species being highly sensitive and others not so. The data for furalaxyl are much as would be expected, with some isolates within species with very low ED50's and others clearly resistant. The fact that all isolates of the HS Group were resistant to furalaxyl was consistent with early observations that the HS Group showed up as the first resistant commercial isolates shortly after introduction of the fungicide in the mid 1980's. Data for propamocarb are less easy to interpret, and at the time of writing the manufacturer regards this effect as a phenomenon of reduced sensitivity, rather than a genetic switch as with furalaxyl. It is noted that these tests are *in vitro* laboratory tests, and it will be necessary to assess performance of the fungicides in compost with *Pythium* and seedlings of an ornamentals subject.

© 1998 Horticultural Development Council

Table 1. Responses of *Pythium* species (ED50*) to etridiazole, furalaxyl and propamocarb

Species of *Pythium*	Source	ED50 Etridiazole	Furalaxyl	Propamocarb
P. irregulare	Plant	9.4	96.5	21.5
P. irregulare	Plant	-	85.5	20.6
P. irregulare	Plant	-	0.3	58.5
P. paroecandrum	Gravel	16.9	2.1	2.3
P. paroecandrum	Mypex	42.1	69.0	12.4
P. paroecandrum	Floor	-	63.6	31.1
P. paroecandrum	Benching	86.7	45.7	3.5
P. rostratum	Mypex	16.6	52.1	19.1
P. rostratum	Water	6.2	48.4	21.8
P. sylvaticum	Matting	7.8	0.6	5.6
P. sylvaticum	Mypex	7.3	>100	6.1
P. sylvaticum	Danish trolley	3.6	0.4	6.4
P. sylvaticum	Soil	6.9	0.5	5.3
P. ultimum	HRI collection	33.0	0.7	3.1
P. ultimum	Matting	39.5	2.4	34.9
P. ultimum	Benching	-	2.6	24.5
P. ultimum	Used pot	66.2	2.0	15.8
HS Group	Used compost	13.4	64.8	4.0
HS Group	Mypex	47.2	84.6	6.9
HS Group	Shredder	29.4	84.2	29.5
HS Group	Floor	7.6	78.6	29.5
HS Group	Used compost	-	96.6	3.0
HS Group	Used compost	32.8	>100	4.3
HS Group	Used compost	6.1	95.3	2.7
HS Group	Benching	67.3	76.2	5.4
Species unknown	Plants	54.5	2.6	36.9
Species unknown	Plants	-	>100	2.4
Species unknown	Plants	68.7	1.3	39.3
Species unknown	Plants	-	39.6	4.3

* ED50 is the concentration of fungicide in µg/ml which reduces growth of a fungus to half of that of the untreated control.
\- Not tested

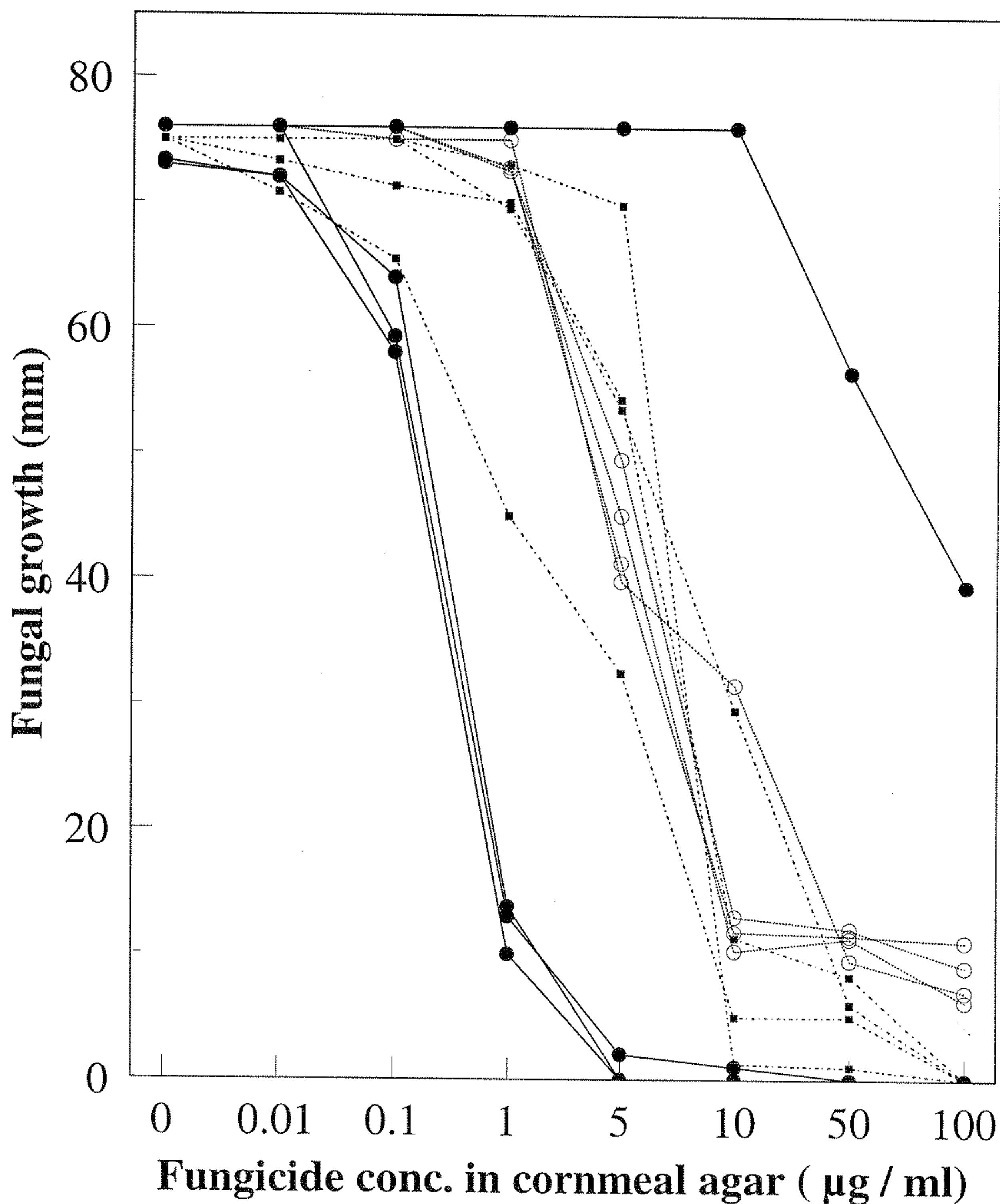
© 1998 Horticultural Development Council

Figure 5. The responses of *Pythium sylvaticum* to furalaxyl, propamocarb and etridiazole

Data for furalaxyl show three sensitive isolates and one with high level resistance. Data for propamocarb and etridiazole show the generally higher concentrations of those fungicides required to reduce growth of the fungus.

© 1998 Horticultural Development Council

Figure 5. *Pythium sylvaticum*



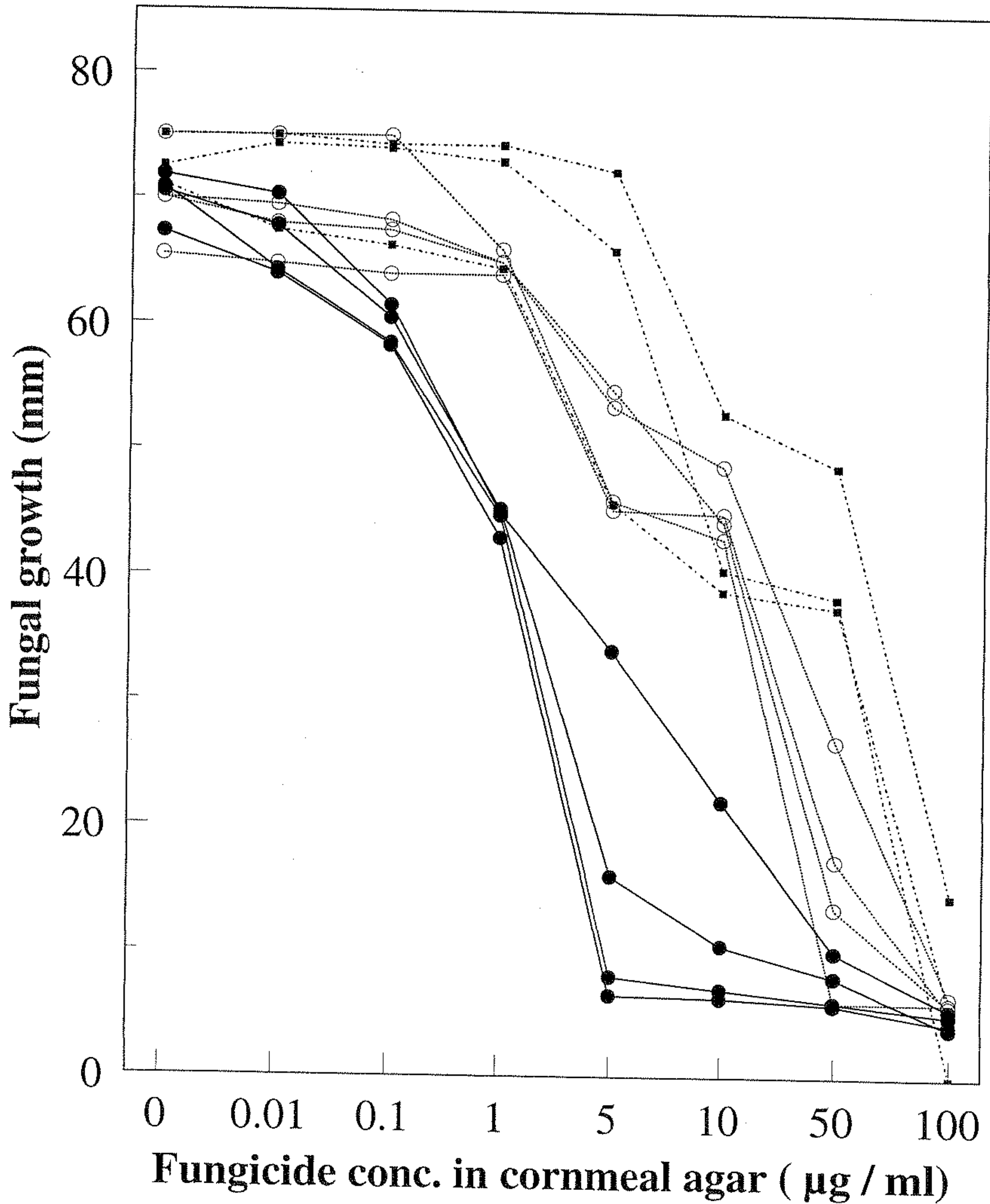
© 1998 Horticultural Development Council

Figure 6. The responses of *Pythium ultimum* to furalaxyl, propamocarb and etridiazole

Data for furalaxyl shows slightly reduced responses while that for propamocarb and etridiazole shows reduced response compared with *P. sylvaticum*.

© 1998 Horticultural Development Council

Figure 6. *Pythium ultimum*



FURALAXYL PROPAMOCARB ETRIDIAZOLE

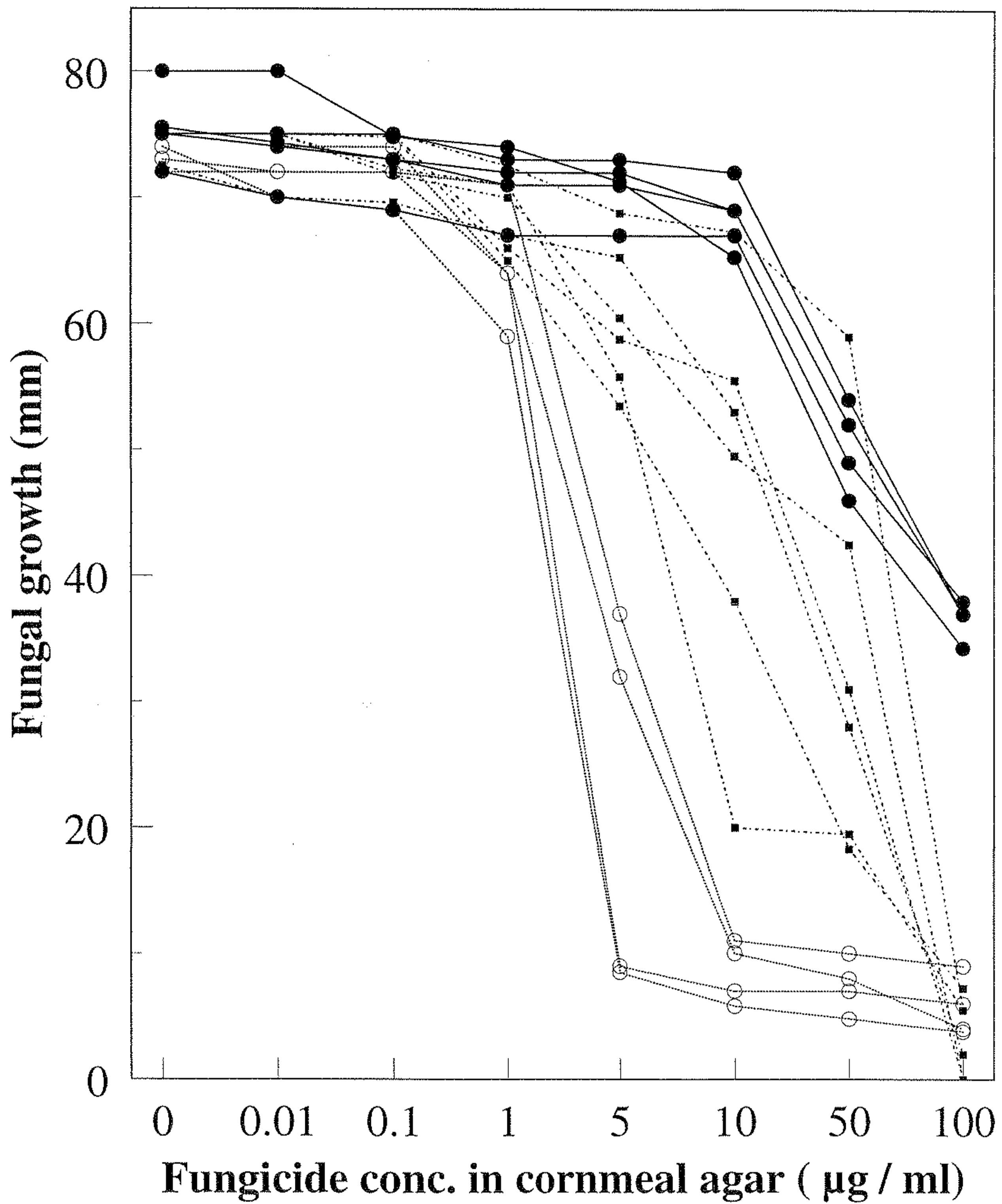
© 1998 Horticultural Development Council

Figure 7. The responses of *Pythium* HS Group to furalaxyl, propamocarb and etridiazole

Data for furalaxyl shows four isolates with high level resistance, while that for propamocarb shows relative sensitivity and for etridiazole a range of responses from relatively sensitive to tolerant.

© 1998 Horticultural Development Council

Figure 7. *Pythium* H.S. group



© 1998 Horticultural Development Council

Discussion

Resistance in *Pythium* to furalaxyl has been with us for more than 10 years. To some extent this has focussed growers down onto a pair of fungicides which are less effective than furalaxyl originally was, and which because of increased use may themselves be under pressure or developing resistance in the pathogen. The results for furalaxyl were as expected, and as it is known that the resistance is not reversible, abstaining from use will not create a situation where the fungicide will again be effective. The grower has the option to kill inoculum with resistance by the use of surface sterilants, or fumigants where soil-based culture is involved, and by combining other hygienic practices should be able to restore use of the fungicide.

There is not sufficient good information on etridiazole or propamocarb to aid interpretation of the present results. The range of ED50's for etridiazole from 3.6 - 86.7 µg/ml suggests a range from susceptible to resistant, but the value of 33.0 derived for the reference *P. ultimum*, which could never have encountered the fungicide, suggests tolerance at the very least. Similarly, the range of ED50's for propamocarb which was 2.3 - 58.5 µg/ml would generally be accepted as a range from susceptible to resistant. Further work is clearly required in this area.

Both fungicides are applied at relatively high rates, and it is likely that both would control the isolates with high ED50's in this study. Whether the values here represent the start of resistance becoming apparent, or whether it is 'reduced sensitivity' is a debating point. What is obvious is that the situation must continue to be monitored as the industry does not really have another good fungicide on which it can rely.

As noted above, collaborating growers did not use fosetyl-Al for control of *Pythium* because of doubts about its efficacy. The new phase of this project will consider whether resistance exists in *Pythium* from ornamentals nurseries to that fungicide.

© 1998 Horticultural Development Council

Conclusions

1. Results for furalaxyl showed the expected high-level resistance known to be present in *Pythium* to the fungicide. Theoretically it should be possible to re-instate effective use of furalaxyl by killing inoculum of the pathogen with resistance, and then maintaining good hygiene.

2. Results with both etridiazole and propamocarb showed a wide range of responses in *Pythium in vitro*. Not enough is known about these fungicides to say whether the results shown developing resistance of the type seen with furalaxyl, or a drift in the response of *Pythium* to the fungicides. Further work is necessary.

3. A test is necessary to allow us to characterise fosetyl-Al, the only other fungicide growers might wish to use, both for inherent ability to control *Pythium* and to determine whether resistance to its active principle exists.

References

WHITE, J G, STANGELLINI, M E & AYOUBI, L M (1988). Variation in the sensitivity to metalaxyl of *Pythium* spp. isolated from carrot and other sources. *Annals of Applied Biology*, **113**, 269-277.

© 1998 Horticultural Development Council

B. PART 4 - SURFACE STERILANT CHEMICALS

Introduction

A primary consideration in nursery hygiene must be to reduce to the lowest level possible inoculum of *Pythium* which is on glasshouse structures, dormant in bed soil, or surviving in dead organic matter. The latter is seen as the most difficult inoculum to kill as the fungus is protected by the material in which it is growing, and may be producing thousands of resistant oospores which will eventually represent widespread sources from which new infestations may result. Therefore, effective chemicals must be identified to give the growers the best chance to reduce future problems. Growers have, in the course of this work, indicated their lack of confidence in some of the products available. We have therefore chosen to examine the efficacy of five true surface sterilant products, and commercial bleach in killing inoculum of five pathogenic species of *Pythium* growing in dead organic material.

Materials and Methods

Five surface sterilants and commercial bleach were assessed for their efficacy in killing the *Pythium* species in dead organic material:-

Panacide M (Coalite Chemicals) - a formulation containing dichlorophen (28 %) as its sodium salt, and sodium hydroxide (8.6 %). A concentration of 0.03 % is recommended to inhibit surface growth on inert surfaces, although a 1 % solution is recommended for the disposal of diseased crops or washing of equipment.

Panaclean 736 (Coalite Chemicals) - a formulation based on dichlorophen 3.7 % w/w. The recommended rate of application is 0.2 % to disinfect floors and structures by spraying.

Jet 5 (Hortichem Ltd) - containing peroxyacetic acid (5 %), hydrogen peroxide (20 %), acetic acid (10 %) and water. The product is recommended as a tray dip as a 1.1 % solution.

© 1998 Horticultural Development Council

Menno-Ter-Spezial (Jiffy Products Ltd) - with active ingredients dihydroxyalkylamine and quaternary ammonium compounds with surface active substances. Recommended for the disinfection of cultivation containers using a 1 % solution.

Reciclean (Kemira-Agro) - consisting of separate solutions of hydrogen peroxide and formic acid which must be mixed 8 hours before use. The new product is marketed as a disinfectant for irrigation pipework, and a concentration of 50 ppm (0.025 %) is recommended to control *Pythium*.

Contact Bleach (Premiere Products) - containing sodium hypochlorite and giving 6 % available chlorine.

All products were tested at four different concentrations based on the recommended rates (where available) for disinfecting trays, equipment or glasshouse structures.

Five *Pythium* species were selected which have previously been identified as pathogenic on ornamental crops. They were *P. ultimum*, *P. paroecandrum*, *P. intermedium* (isolate HDCCR2), *P. sylvaticum* (isolate OI from HRI collection) and a Hyphal Swelling Group isolate (HS128).

A cork borer (14 cm diameter) was used to cut disks from the blades of Reed Canary grass (*Phalaris arundinacea*). The grass disks were heat sterilised in distilled water at 5 psi for 5 minutes. They were then placed into Petri dishes (10 disks/dish) containing Petri's Medium (0.4 g $Ca(CO_3)_2$, 0.15 g $MgSO_4.7H_2O$, 0.15 g KH_2PO_4, 0.06 g KCl, 4.0 g d-glucose, 0.02 g Thiamine per litre). Two plates were prepared for each *Pythium* isolate per surface sterilant, allowing 4 replicates at each of 4 concentrations and 4 untreated controls. Each plate was centrally inoculated with one *Pythium* species, grown on corn meal agar, using 1 cm^2 of agar. The plates were incubated for 7 days at 24°C, until the mycelial mat had covered all the disks of grass blade.

The surface sterilant concentrations were prepared in Repli dishes (11 mm square, 25 compartments; Bibby Sterilin Ltd). Fresh surface sterilant and a sterile compartment was used for each replicate. Each grass disk with *Pythium* was taken from under the mycelial mat with sterile forceps and then placed into

© 1998 Horticultural Development Council

the surface sterilant. After 60 minutes, the disks were removed and washed in 4 ml of sterile distilled water for 1 minute, after which they were plated onto corn meal agar with rifamycin and pimaricin. After 2 weeks incubation at room temperature, plates were checked for evidence of growth of *Pythium*.

Results

Considerable differences were seen between the surface sterilants in their ability to kill *Pythium* in dead organic material **(Table 2)**. Panacide M and Panaclean 736 were completely ineffective against the range of species tested, even at twice the recommended concentrations. Menno-Ter-Spezial partially controlled *Pythium* at the higher concentrations, although both *P. intermedium* and *P. sylvaticum* survived at twice the recommended concentration. Both Reciclean and Jet 5 are peroxide-based sterilants and gave almost total kill at concentrations of 1.6 % and 1.25 % respectively, with just one replicate of *P. sylvaticum* surviving in each case. A 50 % solution of Contact Bleach was required for complete kill of the range of species tested.

Discussion

These tests were designed to simulate the situation where *Pythium* has colonised dead organic material, and where there is some protection from the sterilant chemical as a result. Two surface sterilants had no effect even at twice their recommended rates. Although this does not mean that they would be generally ineffective against *Pythium* without protection, it would not be realistic to expect them to be more effective than the other products tested here.

Because of the scale of the work, it is difficult to clearly separate Ter Spezial and Reciclean, although more treatments with the latter gave zero survival. Reciclean is a relatively new product designed for work with pipework, but it clearly has some general potential. This will be investigated further in the second phase of the project.

The results with peroxide-based Jet 5 as the most effective surface sterilant are consistent with the work of Dr G M McPherson in earlier HDC funded work (PC 125 Cucumber: evaluation of disinfection treatments for the control of *Pythium* spp. in rockwool slabs). Although not directly intended for use in this context, contact bleach is clearly extremely effective. They clearly represent the materials growers should consider for use in the short-term.

© 1998 Horticultural Development Council

Table 2. The effect of various surface sterilants on the survival of *Pythium* species in dead organic matter (60 minute treatment time)

		Number of replicates surviving (/4)				
Sterilant	Concentration %	*P. ultimum*	*P. paroecandrum*	*P. intermedium*	*P. sylvaticum*	HS Group
Panacide M	2	4	4	4	4	4
	1	3	4	4	4	4
	0.5	4	4	4	4	4
	0.25	4	4	4	4	4
Panaclean 736	0.4	4	4	4	4	4
	0.2	4	4	4	4	4
	0.1	4	4	4	4	4
	0.05	4	4	4	4	4
Jet 5	5	0	0	0	0	0
	2.5	0	0	0	0	0
	1.25	0	0	0	1	0
	0.625	0	2	2	2	0
Ter-Spezial	2	0	0	3	1	0
	1	1	2	2	0	4
	0.5	4	3	2	3	4
	0.25	4	4	4	4	4
Reciclean	1.6	0	0	0	1	0
	0.8	0	2	0	2	2
	0.4	0	2	3	4	3
	0.2	2	4	4	4	4
Bleach (6% available chlorine)	100	0	0	0	0	0
	50	0	0	0	0	0
	25	2	1	3	2	3
	12.5	3	4	4	4	4

© 1998 Horticultural Development Council

Conclusions

1. In the most stringent conditions for obtaining control of *Pythium*, the peroxide-based surface sterilants were found to be most effective with five different species. Three other sterilants were either ineffective at all rates used, or allowed survival of some species of *Pythium* at twice the recommended application rate.

2. Commercial bleach was extremely effective when used at full strength, or diluted by 50 % with water, but it was ineffective when diluted 1 : 4 with water.

3. The exposure period was only one hour, and it was necessary to stop treatment with water to achieve that time. In practice, there will be no limit on exposure period, and it may be possible that lower concentrations of sterilants effective here would be effective in practice.

4. Sterilants not seen to be effective here may still work when the *Pythium* is exposed on hard surfaces such as concrete flooring. This should be tested.

5. For sites of likely high activity of *Pythium*, such as benches for gapping-up, regular treatment with one of the effective sterilants should be a part of normal good practice.

6. Reciclean is a new product designed for use in pipework. On the basis of these results, it can clearly be very effective when *Pythium* is well protected, and should certainly be assessed in the context of its original usage.

© 1998 Horticultural Development Council

C. OVERALL DISCUSSION

The project started from the premise that *Pythium* is a major problem for UK growers of ornamentals and that the boundaries of the problem needed defining. The aspect of unknowing sale of infected plants was a potential cause of damage to the image of the industry. However, within the industry there was internal concern because the present structure works towards the distribution of both *Pythium* and fungicide resistance over the UK. Also, probably into the UK from other countries. In this context, a problem shared is clearly not a problem halved.

On-nursery monitoring showed the level of contamination of eight nurseries, and gave some idea of the species of *Pythium* present. At this stage it is important to stress that the businesses clearly function very well commercially, and that this is in spite of any findings herein. One nursery identified as being well endowed with *Pythium* was able to run some sections of production without recourse to any fungicide. Where particularly sensitive crops such as *Antirrhinum* were lost with some regularity, simple measures such as holding back watering in periods of dull weather enabled good crop production.

There were clearly areas where attention to detail in sensitive parts of production could reduce the need for fungicide use downstream. Hygiene measures which reduced both general inoculum, and those species of *Pythium* with resistance, particularly to furalaxyl, could help by reducing the need to use other fungicides. Potentially, they could restore effective use of furalaxyl on nurseries with species with high level resistance.

The project produced a perspective on the needs of a serological diagnostic test which would benefit the industry by allowing detection of the common fast-growing pathogens and those of the *P. aphanidermatum* group. This will be proven in the new lateral-flow dipstick format in the second phase of the project. Spin-off was the inclusion of the five species antibody into a zoospore trapping immunoassay which uniquely measures the number of viable zoospores in irrigation water. This assay will be assessed with collaborating growers.

© 1998 Horticultural Development Council

The situation on fungicide resistance is one where we have a partial understanding of the subject. Resistance to furalaxyl in the *Pythium* HS Group has been found in samples from the ornamentals industry for some years. Practically, it does not really matter whether the observations reflect one isolate which has been moved around, or a number of repeats of the same single gene switch. It is likely that the switch has also occurred elsewhere with a wider range of species than has been seen in this work. We know that the change is not reversible spontaneously, so to deal with it, effective surface sterilisation is needed.

The situation with etridiazole and propamocarb is currently one where more information is needed. The range of ED50 values obtained with current species suggests a change phenomenon with a drift rather than a switch pattern. Because of the low number of fungicides available, this is very much an area for further study.

Surface sterilants represent a way for growers to reduce inoculum levels on nurseries. The present tests were designed to severely test the chemicals, and in doing so have identified two peroxide-based products as being better than the other materials in efficacy. A function for commercial bleach in cleaning up small expanses of highly contaminated benching was proposed.

A general principle to take onboard from this work is that businesses are running very well with the present situation. With greater attention to detail, and a conscious attempt to reduce inoculum levels, they could probably make efficiency improvements.

D. OVERALL CONCLUSIONS

1. The common fast-growing *Pythium* species are endemic in the nurseries surveyed.

2. The industry is stretched to an unacceptable degree by currently available fungicides, but insufficient use is made of surface sterilants as a means to lower *Pythium* inoculum levels.

3. The genus *Pythium* is too extensive and complicated to be amenable to simple serological

© 1998 Horticultural Development Council

diagnostic tools. ELISA-based systems are unlikely to be of value to the industry. A combination assay with a polyclonal antibody raised to the common fast-growing *Pythium* spp. and a monoclonal antibody which selectively recognises the *P. aphanidermatum* molecular grouping would be of value. A format is to be examined which should provide a rapid, low-cost assay for *Pythium* in the ornamentals industry.

4. The most effective surface sterilants for reducing *Pythium* inoculum were the peroxide-based Jet 5 and Reciclean. Reduction of inoculum by the use of such products should lead to reduced problems downstream.

E. ACKNOWLEDGMENTS

We thank the eight growers who took part in this work. They effectively opened up their nurseries to our probing, and answered what might at the time have seemed trivial questions. They have helped us to set a very firm base for future work with *Pythium* in the ornamentals industry. We look forward to further collaboration with all eight of them in the new phase of the project (PC 97a Ornamentals: sources of *Pythium* inoculum, fungicide resistance, and efficacy of surface sterilants). Thanks also go the company who gave us access to their peat bogs and enabled our isolation work from peat at source. We thank Dr N F Lyons of HRI for producing a most useful antibody to five species of *Pythium*. Also, the chemical companies who provided samples of fungicides for use in the resistance studies.

© 1998 Horticultural Development Council

APPENDIX 1

© 1998 Horticultural Development Council

Ann. appl. Biol. (1997), **131**:427–435
Printed in Great Britain

A novel method for detection of viable zoospores of *Pythium* in irrigation water

By A J WAKEHAM, T R PETTITT* and J G WHITE
Horticulture Research International, Wellesbourne, Warwick CV35 9EF, UK

(Accepted 18 November 1997)

Summary

A membrane filtration test has been developed for the detection of viable zoospores of *Pythium* species. Zoospore suspensions were filtered through 5 (m nitrocellulose membranes and the membranes incubated overnight in 0.07 M glucose, rifamycin (30 mg litre^{-1}) and pimaricin (100 mg litre^{-1}). Zoospore germlings were detected using a polyclonal antiserum, raised to mycelial surface washings of five *Pythium* spp., and visualised with Sigma fast red. The assay gave positive results for all *Pythium* spp. tested and also to zoospores of *Phytophthora cryptogea*. Of 10 fungal species isolated from commercial irrigation water, two were detected by the polyclonal antiserum in ELISA tests but only one produced detectable zoospore germlings. The latter isolate was later identified as a *Pythium* sp. Irrigation water samples collected from commercial UK nurseries yielded zoospores of both *Pythium* and *Phytophthora* spp. which, using the assay, were positively identified. Results indicated greater sensitivity than was seen with conventional plating methods. This is a test which could be adapted for on-site use in commercial nurseries.

Key words: *Pythium*, polyclonal antibody, serological assay, zoospore, irrigation water

Introduction

Over 80 species of *Pythium* are recognised (Plaats-Niterink, 1981), many of which are important plant pathogens. Many pathogenic *Pythium* species are worldwide in distribution and are capable of infecting a wide range of plants. Under wet conditions, dispersal of the pathogen is primarily by asexual, flagellate zoospores which are attracted to plant roots. When conditions are suitable, colonisation of host tissues and disease spread can be rapid. This is particularly true in hydroponic growing systems, where irrigation water or nutrient solution is recirculated (Stanghellini & Rasmussen, 1994). *Pythium* spp. are often associated with pre- and post-emergence damping-off. However, seedlings can often survive early infection only for disease to become apparent and cause severe losses at a later stage. Propagated crops, grown under intensive conditions are particularly at risk from the disease. Such outbreaks of disease are often triggered by specific environmental events and they can occur long after initial infection. Without rapid methods of detection, timely prediction of high infection risks is virtually impossible. Detection methods employed are those of baiting, culture plating, or a

*Horticulture Research International, Efford, Lymington, Hampshire SO41 0LZ, UK

© 1997 Association of Applied Biologists

GLOSSARY OF TERMS ON *PYTHIUM*

Mycelium The white strands which may be seen on compost and plants in severe infection, and by which *Pythium* normally grows.

Oogonia are the structures formed as a result of sexual reproduction in *Pythium* which are generally liberated into substrate as plants decay. Because oogonia have protective walls, they are able to survive until stimulated to germinate by the presence of a suitable host.

Sporangia are the structures *Pythium* forms when it is about to reproduce by asexual means. The sporangium is the site of the multiplication, and gives rise to a vesicle in which zoospores differentiate, and from which they are liberated via a discharge tube.

Zoospores are formed as above, and comprise cytoplasm enclosed in a cell membrane and two flagellae by which the zoospore is able to swim. Zoospores may be liberated in large numbers in wet conditions and can swim towards host material. They are able to swim for a number of hours, and are obviously at home in irrigation water and nutrient solution. When zoospores encounter a host they encyst, germinate and then infect that tissue.

© 1998 Horticultural Development Council

combination of both (Pittis & Colhoun, 1984). These techniques take many days to generate information and are normally too slow to assist growers in disease management decisions. This has led to a situation of routine treatment where fungicides have not been targeted effectively and consequently widespread fungicide resistance has built up (White & Wakeham, 1987).

The recent development of serological detection assays has reduced the time taken to achieve reasonably accurate diagnosis of pythiaceous fungi and has allowed more informed and timely judgements to be made. Kits developed by Agri Diagnostics Associates (2611 Branch Pike, Cinnaminson, New Jersey, USA.) and marketed through Neogen (Lansing, Michigan, USA) and Adgen Diagnostic systems (Watson Peat Building, Auchincruive, Ayr, Scotland) detect soluble antigens of pythiaceous fungi in soil, plant tissue and water. However, the tests fail to distinguish between live and dead propagules of the pathogen. Cahill & Hardham (1994) exploited zoospore taxis of *Phytophthora cinnamomi* Rands with the development of a dipstick immunoassay allowing rapid detection of viable propagules which could be carried out on-site by unskilled operators. The procedure relies upon chemotaxis to capture viable zoospores and only a limited proportion of the total number of zoospores present in a water sample are detected. This limitation could be very important in irrigation water supply where the number of zoospores per unit volume may be very low.

This paper describes the development of a rapid serological method for detection and quantification of the total numbers of viable zoospores of *Pythium* in water samples with a view to identification of contaminated nursery irrigation water supplies. Polyclonal antiserum (PAb), raised to soluble mycelial washings of five commonly isolated pathogenic species of *Pythium*, was used in the development of the test.

Materials and Methods

Antigen preparation

Five 3% V8 juice (Campbells Soups) agar plates, each covered with a PN 6026 Supor 450 membrane filter (Gellman Sciences, Brackmills Business Park, Northampton. Cat No. 60206), were inoculated with a single 0.5 cm disc of actively growing mycelium of *P. irregulare*. Similar plates were prepared with *P. intermedium*, *P. sylvaticum*, *P. ultimum* and the *Pythium* hyphal swelling group (HS group). After incubating for 7 days at 25°C the membranes were removed and 5 ml of PBS was added to each. Mycelial surface washings were prepared by gently stroking the surface of the membrane with a sterile glass rod. Surface washings of each isolate were combined and centrifuged at 13 250 × *g* for 30 min, the pellet was discarded and the supernatant was freeze-dried. The sample was rehydrated in phosphate buffered saline (PBS) (pH 7.2) and divided into 10 aliquots of 0.5 ml each.

Immunisation

The immunisation protocol was as described by Wakeham & White (1996). The resulting polyclonal antiserum (PAb) was coded 95/10/2 and the titre was determined by indirect protein-A ELISA. The PAb was stored at −20°C in 250 μl aliquots.

Indirect protein-A ELISA

The immunogen preparation was adjusted to 2 μg protein ml^{-1} PBS tincture of merthiolate (0.02%) (PBST) and pipetted into paired wells (100 μl per well) of a 96-well Immunolon flat-bottomed microtitre plate (Cat No. 0110103355 Dynatech Lab. Chantilly, Virginia 22021,

Table 1. *Soil fungi used in indirect protein-A ELISA cross-reactivity studies; the media on which they were cultured and A_{450} reading as a percentage of that of* Pythium sylvaticum

Species	Growth medium	% A_{450}
Penicillium waksmanni Zaleski	PDA	3.0
Phytophthora cryptogea Pethybr. & Lafferty	PDA	30.1
Phytophthora cactorum (Leb. & Cohn) Schroet.	PDA	27.3
Pythium ultimum Trow.	V8	88.6
Pythium intermedium de Bary	V8	70.0
Pythium sylvaticum Campbell & Hendrix	V8	100.0
Pythium hyphal swelling group	V8	77.0
Pythium irregulare Buisman	V8	66.0
Verticillium dahliae Kleb. & Reinke & Berthold	PDA	9.1
Fusarium solani (Linford) Snyder & Hansen	PDA	30.8
Sclerotium cepivorum Berk	MEA	2.9
Botrytis cinerea Pers: Fr.	PDA	3.7
Sclerotinia sclerotiorum (Lib.) de Bary	MEA	3.2
Rhizoctonia solani Kühn	PDA	2.6
Sclerotium rolfsii Sacc.	MEA	3.8
Trichoderma viride Pers.	PDA	3.3
Thielaviopsis basicola (Berk. & Br.) Ferraris	PDA	2.3
Saprolegnia sp.	V8	24.4

* PDA, Potato dextrose agar; V8, V8 juice agar; MEA malt extract agar.

USA) and incubated for 1 h at 37°C. The plate was washed four times with 200 µl PBST and blocked with 200 µl per well of 3% Tris casein buffer (TCB) (Wakeham & White, 1996) for 20 min in a Wellwarm shaking incubator (Denley Instruments Ltd, Sussex, UK) at 30°C. Following four 1 min washes with 0.3% TCB, wells were loaded (100 µl per well) with PAb 95/10/2 diluted in PBST 0.3% TCB (1:10) and incubated for 30 min as above. Wells were washed as before and loaded with 100 µl per well protein-A horseradish peroxidase (Sigma P-8651) diluted in PBST 0.3% TCB (1:10) to a protein concentration of 0.31 $\mu g\ ml^{-1}$ and incubated for 90 min as above. Following four 1 min washes with PBST (200 µl per well), wells were loaded with 100 µl 3,3′,5,5′-tetramethylbenzidene substrate (TMB Sigma T-3405). The reaction was stopped with 25 µl per well of 2 M H_2SO_4 and absorbance values were read at 450 nm.

Characterisation of the polyclonal antiserum

The effects of antigen modification with protease and periodate on binding of PAb 95/10/2 were determined by the method of Bossi & Dewey (1992) using mycelial surface washings of *P. sylvaticum* and the *Pythium* HS group. A DIG glycan differentiation kit (Boehringer Mannheim) was used to determine the presence of glycoprotein epitopes. Heat stability was assessed by heating the two *Pythium* preparations to 100°C for 3 min prior to processing through indirect protein-A ELISA.

Cross-reactivity of the polyclonal antiserum

The PAb was evaluated in an indirect protein-A ELISA for cross-reactivity against 10 fungal species isolated from nursery irrigation supplies (two *Alternaria* spp; two *Mortierella* spp; two *Phoma* spp; one *Penicillium* sp; one *Trichoderma* sp.; one *Pythium* sp. and one unidentified separate isolate) and a range of soil fungi (Table 1). Surface washings of each

fungal isolate were prepared as described in 'antigen preparation', and each adjusted to 4 μg protein ml^{-1} prior to indirect protein-A ELISA.

Detection of zoospores in sterile distilled water

Zoospore trapping immunoassay (ZTI)

Each of ten 1-litre doubling dilutions of a *Pythium* zoospore suspension was filtered using a Nalgene reusable bottle top filter unit (Fisons Scientific Equipment, Nechells Business Centre, 31 Dollmant Street, Birmingham, UK; Cat No. FDM-260–040F) through a 5 μm cellulose nitrate membrane filter. The filters were removed, incubated overnight in a solution containing 0.07 M glucose, rifamycin (30 mg $litre^{-1}$) (Sigma R-8626) and pimaricin (100 mg $litre^{-1}$) (Sigma P-0440) solution. The filters were air-dried and blocked in Tris-buffered saline Tween stock solution (TBST) (6 g Trizma base (Sigma T1503), 8 g NaCl, 1 ml Tween 20 made up to 1 litre in distilled H_2O, pH 7.4) containing 5% dried skimmed milk for 1 h on an orbital shaker. Following three 5 min washes in TBST, the filters were incubated at room temperature with PAb 95/10/2 diluted in TBST for 1 h on an orbital shaker. The filters were washed as above and incubated, with shaking, in anti-rabbit IgG whole molecule (Sigma A-8025) alkaline phosphatase conjugate diluted in TBST for 1 h at room temperature. Washing was repeated and the filters were developed in Sigma Fast Red TR/Napthol AS-MX phosphate buffer (Sigma F-4523). Colour development was stopped by washing the filters in distilled water. Examination was by using a binocular microscope (×400 magnification) and the number of viable zoospores per litre of water was determined by counting the number of stained germlings. Zoospore suspensions of *Pythium ultimum var. sporangiiferum* Drechsler, *Pythium torulosum* Coker and Patterson and a *Pythium* isolate which produced only mycelium in culture were filtered and developed as described above. Zoospore suspensions of *Phytophthora cryptogea*, a *Saprolegnia* sp., and 10 fungal isolates collected from irrigation water were also tested as described above.

Detection of viable zoospores of Pythium in commercial water samples

Eight 1-litre irrigation water samples collected from commercial UK ornamentals nurseries were filtered as previously described. Each membrane was divided, one half being developed using the ZTI assay the other half was assessed using a conventional agar plate method.

Conventional plating method

Filter halves were cut into several pieces and placed in universal bottles containing 5 ml of 0.1% water agar, rifamycin (30 mg $litre^{-1}$) and pimaricin (100 mg $litre^{-1}$). The filter residue was re-suspended by agitation on a vortex mixer for 2 min. For each filter suspension, a 1-ml aliquot was pipetted on to each of five plates containing potato dextrose agar, rifamycin (30 mg $litre^{-1}$) and pimaricin (100 mg $litre^{-1}$) and the suspension was spread evenly over the agar surface with a glass spreader. Following 36 to 48 h incubation at 20°C in darkness, the number of colony forming units (cfu) was determined per litre of water sample filtered.

Results

Titre and characterisation of polyclonal antiserum

The PAb was of high sensitivity to the antigen preparation with a dilution end point of 1:102 400. Ability of the PAb to bind to both *Pythium* spp. was reduced after they were

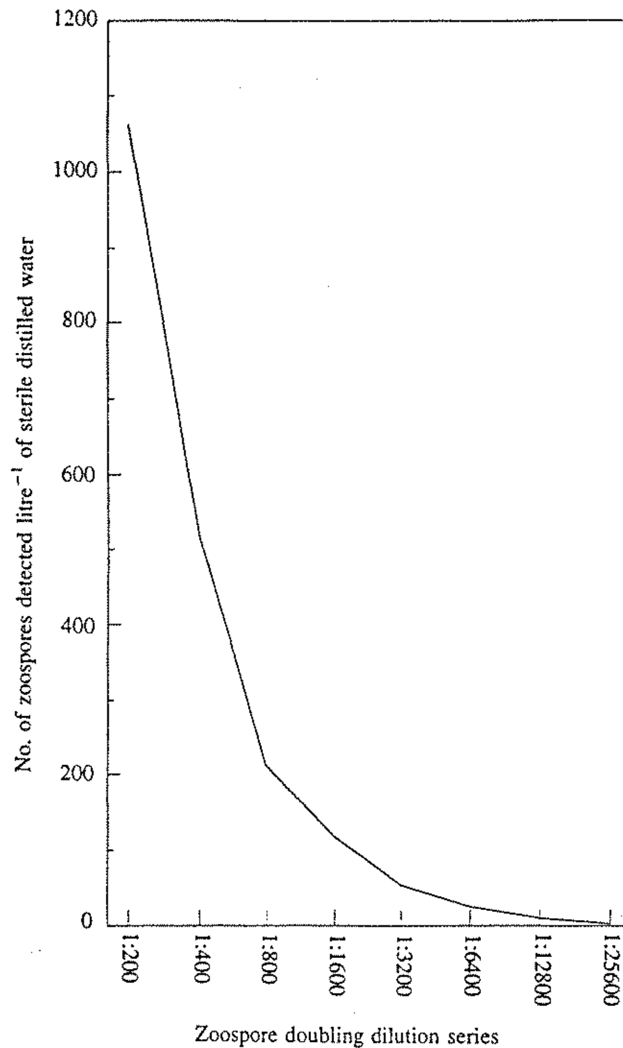


Fig. 1. ZTI assessment of number of viable *Pythium* zoospores detected l^{-1} sterile distilled water.

treated with protease, but a greater reduction was observed after treatment with periodate (Table 2). Compared to that of the controls, binding ability was not affected following heat treatment of *Pythium sylvaticum* but some reduction was observed with antigenic components of the *Pythium* hyphal swelling group. Recognition of glycoprotein epitopes was seen with the DIG glycan kit.

Cross-reactivity of polyclonal antiserum in indirect protein-A ELISA

Of the fungal species isolated from nursery irrigation water, cross-reactivity in indirect protein-A ELISA was limited to isolates 4 (a *Pythium* sp.) and 10 (a *Trichoderma* sp.). Of the soil-borne fungi tested, sensitivity was high to each of the *Pythium* spp. with some cross-reactivity observed to *Phytophthora cryptogea, Phytophthora cactorum, Fusarium solani* f.sp. *pisi* and a *Saprolegnia* sp. (Table 1).

Table 2. *Percentage reduction in binding of polyclonal antiserum following antigen modification with periodate and protease*

Pythium sp.	Periodate oxidation	Protease	
		4°C	30°C
Pythium sylvaticum	77.9	4.5	24.5
Pythium HS group	89.6	41.1	51.81

ZTI sensitivity

Using ZTI, three germinated *Pythium* zoospores were detected in a litre of sterile distilled water representing a dilution of 1:25 600 of the original suspension (Fig. 1). For each of the other filtered *Pythium* spp. both encysted and germinated zoospores (Fig. 2) were readily identified by the red immunological stain. Germinated zoospores of *Phytophthora cryptogea* were also detected, but the intensity of the stain was reduced considerably. Weak labelling of parts of the *Saprolegnia* zoospore walls was observed, but neither their cytoplasmic contents, nor germ tubes (if present) were stained. Of the fungal species isolated from commercial irrigation water only isolate 4 (*Pythium* sp.) tested positive where zoospore germlings were identified.

Detection of viable Pythium *and* Phytophthora *zoospores in commercial water samples*

Viable zoospores were detected in a number of the commercial irrigation water samples tested (Table 3). The ZTI assay was generally more sensitive than the conventional agar-plating technique used.

Discussion

Using a PAb (95/10/2) raised to pooled surface mycelial washings of five pathogenic *Pythium* spp., a zoospore trapping immunoassay (ZTI) has been developed which should prove useful in the detection of viable zoospores of *Pythium* spp. in commercial irrigation water supplies. Isolation media for *Phytophthora* normally contain one tenth of the pimaricin used in this test (Tsao, 1970). Although the two *Phytophthora* spp. specifically investigated germinated in the medium, our experience indicates that *Pythium* species will be the most

Table 3. *Detection of viable* Pythium *and* Phytophthora *zoospores in commercial irrigation water samples employing zoospore trapping immunoassay (ZTI) and conventional plating methods*

Source	No. of zoospores ZTI	No. of cful Conventional	Species
Bore hole water	—	—	
Bore hole water reservoir	3	1	*Pythium*
Pump 1 (quick sand filter)	—	—	
Pump 2 (no filter—direct from reservoir)	5	—	*Pythium*
Pump 3 (no filter—direct from reservoir)	1	1	*Pythium*
Pump 4 (no filter—direct from reservoir)	1	—	*Pythium*
Recycled irrigation water	40	13	*Phytophthora*
Nursery drainage pond	274	36	*Phytophthora*

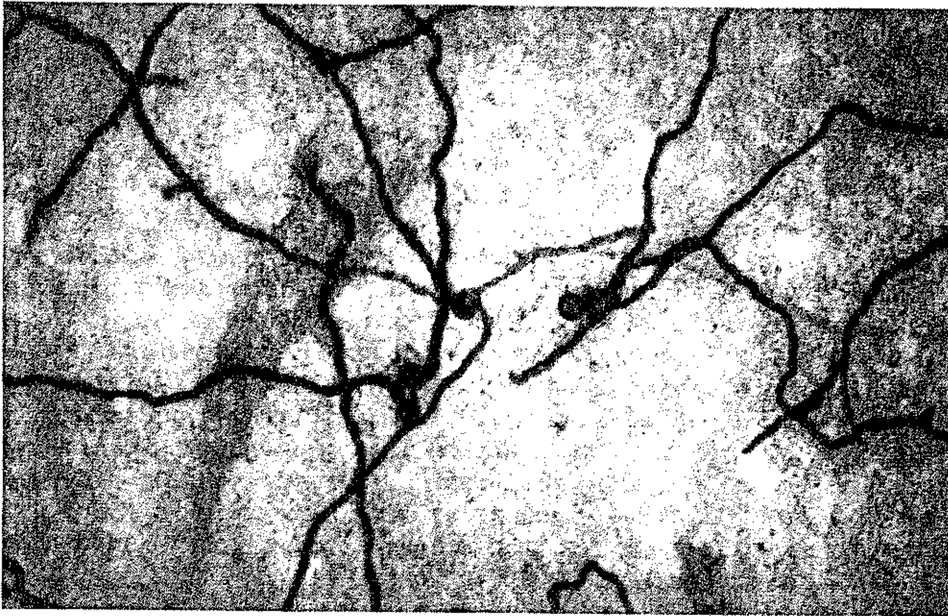


Fig. 2. Detection of viable *Pythium* zoospores employing the ZTI assay.

frequently detected organisms from commercial samples. By manipulation of the rate of pimaricin used, the test might be made appropriate for the detection of some *Phytophthora* spp.

Serological probes for the detection of zoopores of pythiaceous spp. have previously employed monoclonal antibodies (MAbs) (Estrada-Garcia *et al.,* 1989; Cope *et al.*, 1996; Cahill & Hardham, 1994). MAbs are frequently employed in immuno-detection of plant pathogens both in laboratory studies and in commercial assays because of their specificity and reproducibility (Werres & Steffens, 1994). However Hardham *et al.* (1994) reported that comparisons of MAb binding to many isolates and species of *Phytophthora* and *Pythium* revealed that surface components on zoospores and cysts exhibit a range of taxonomic specificities. Surface epitopes may occur on only a few isolates of a species; they may be species-specific; genus-specific, or occur on the spores of both genera. Thus in a commercial test, the specificity obtained with a MAb could be a disadvantage. In this work, PAb 95/10/2 gave a high immune response to the five *Pythium* spp. used as immunogens, and has been shown to recognise species across the genus *Pythium* (Wakeham, unpublished information). The increased potential for cross-reactivity, as seen in the indirect protein-A ELISA, and the reproducibility required on a commercial scale often excludes PAbs from being employed in detection assay systems. Nevertheless, PAbs have been used successfully in immunodetection of plant pathogens in soil (White, Lyons & Petch, 1996; Wakeham & White, 1995, 1996) and in plant material (Lyons & White, 1992; Linfield, Kenny & Lyons, 1995). Cross-reactivity observed in the protein-A ELISA, where soluble mycelial components were probed, did not effect the ZTI assay. The PAb did not bind to conidia, conidophores and structural mycelial cell wall components of the *Trichoderma* sp. tested. Use of PAb 95/10/2, with the inclusion of antibiotics for the selective growth of pythiaceous fungi (White, 1986), enables the test to be

sensitive, specific and quantitative for viable propagules of *Pythium* and it could be extended for use with some *Phytophthora* spp.

ELISA kits developed by Agri-Diagnostics Associates for detection of *Pythium* and *Phytophthora* in irrigation systems are already commercially available in the UK through Adgen Diagnostic Systems (Watson Peat Building, Auchincruive, Ayr, Scotland). The tests have a reported effective detection capability of 17–23 zoospores litre^{-1} (Ali-Shtayeh, MacDonald & Kabashima, 1991). However, the study highlights areas of concern; specificity, sensitivity, reaction intensity and the inability to differentiate between live and dead propagules. In our experience with the ZTI assay, zoospores/germlings stained strongly and could be differentiated from both *Phytophthora* (medium staining) and *Saprolegnia* (weak staining of the zoospore wall only), suggesting that it is possible to differentiate cross-reactors of different genera and also separate live zoospores from dead fungal material.

A dip-stick assay has been developed for the specific detection of zoospores of *Phytophthora cinamomi* (Cahill & Hardham, 1994). The test is based on the phenomena of chemotaxis and electrotaxis to attract zoospores in suspension to a membrane where zoospores encyst and are detected by immunoassay. However the sensitivity of the dipstick assay was determined to be around 40 zoospores ml^{-1} with one or more cysts found on the dip-stick membrane. The report suggests that the true sensitivity is somewhat lower because in an unstirred suspension the zoospores will not be distributed randomly.

The present study has incorporated procedures from both assays to produce a sensitive and viable test which could be carried out on-site. Filtration of a water sample concentrates zoospores on to the filter, and therefore provides the grower with a quantitative assessment of viable propagules. In scientific studies this would be beneficial, enabling disease threshold levels for crops to be determined under different environmental conditions and the rational use of appropriate disease control measures. However, Von Broembsen & Deacon (1992) reported that when zoospores were filtered through a nucleopore membrane they were seen to lyse within minutes. Use of a cellulose nitrate membrane avoided this problem and the adhesive released by the encysting zoospore (Sing & Bartnicki-Garcia, 1975) bound them securely to the membrane throughout the immunoblotting process. Germination potential of the zoospore may be increased by this method, because Donaldson & Deacon (1992) reported that germination occurred at a low level if cysts were held in suspension, but enhanced if newly formed cysts were placed on a support medium.

It is envisaged that the assay would be sufficiently inexpensive to facilitate regular monitoring of nursery irrigation supplies. The increased sensitivity, as compared with culture plating, and the ability to carry out rapid tests on site using non-scientific staff would be a considerable advantage both in the economics of the test and for disease control strategy. Although the present immunoassay system has an overnight incubation stage, it may be possible to decrease this time. Cope *et al.* (1996) reported that following attachment the zoospore rapidly develops a cell wall and germinates within 20–30 min. It would therefore be reasonable to consider reducing the incubation time of the present test to 1 h. Early detection would provide growers with the opportunity to target control measures more effectively, also to localise infections and prevent disease spread.

Acknowledgements

The work was funded by the MAFF ROAME's HH1505SPC and HH1708SHN and HH1712SFV. The polyclonal antibody used was generated in Horticultural Development Council Project PC97.

References

Ali-Shtayeh M S, MacDonald J D, Kabashima J. 1991. A method for using commercial ELISA tests to detect zoospores of *Phytophthora* and *Pythium* species in irrigation water. *Plant Disease* **75**:305–311.

Bossi R, Dewey F M. 1992. Development of a monoclonal antibody-based immunodetection assay for *Botrytis cinerea*. *Plant Pathology* **41**:472–482.

Cahill D M, Hardham A R. 1994. Exploitation of zoospore taxis in the development of a novel dipstick immunoassay for the specific detection of *Phytophthora cinnamomi*. *Phytopathology* **84**:193–199.

Cope M, Webb M C, O'Gara E T, Phillip B A, Hardham A R. 1996. Immunocytochemical comparison of peripheral vesicles in zoospores of *Phytophthora* and *Pythium* species. *Mycologia* **88**:523–532.

Donaldson S P, Deacon J W. 1992. Role of calcium in adhesion and germination of zoospore cysts of *Pythium*: a model to explain infection of host plants. *Journal of General Microbiology* **138**:2051–2059.

Estrada-Garcia M T, Green J R, Booth J M, White J G, Callow J A. 1989. Monoclonal antibodies to cell surface components of zoospores and cysts to the fungus *Pythium aphanidermatum* reveal species-specific antigens. *Experimental Mycology* **13**:348–355.

Hardham A R, Cahill D M, Cope M, Gabor B K, Gubler F, Hyde G J. 1994. Cell surface antigens of *Phytophthora* spores: biological and taxonomic characterization. *Protoplasma* **181**:213–232.

Linfield C A, Kenny S R, Lyons N F. 1995. A serological test for detecting *Botrytis allii*, the cause of neck rot of onion bulbs. *Annals of Applied Biology* **126**:259–268.

Lyons N F, White J G. 1992. Detection of *Pythium violae* and *Pythium sulcatum* in carrots with cavity spot using competition ELISA. *Annals of Applied Biology* **120**:235–244.

Pittis J E, Colhoun J. 1984. Isolation and identification of pythiaceous fungi from irrigation water and their pathogenicity to *Antirrhinum*, tomato and *Chamaecyparis lawsoniana*. *Phytopathology Zeitschrift* **110**:301–318.

Plaats-Niterink A J. 1981. Monograph of the genus *Pythium*. *Studies in Mycology* **21**:1–242.

Singh V O, Bartnicki-Garcia S. 1975. Adhesion of *Phytophthora palmivora* zoospores: detection and visualisation of concanavalin A receptor sites appearing during encystment. *Journal of Cell Science* **19**:11–20.

Stanghellini M E, Rasmussen S L. 1994. Hydroponics: a solution for zoosporic pathogens. *Plant Disease* **78**:1129–1138.

Tsao P H. 1970. Selective media for isolation of plant pathogenic fungi. *Annual Review of Phytopathology* **8**:157–186.

Von Broembsen S L, Deacon J W. 1992. Effects of calcium on germination and further zoospore release from zoospore cysts of *Phytophthora parasitica*. *Mycological Research* **100**:1498–1504.

Wakeham A J, White J G. 1995. Development of an immunological assay for the detection in onion bulbs and soil of *Sclerotium cepivorum* Berk. In *Proceedings of the Fifth International Workshop on Allium White Rot, 11–13 May 1994*, pp. 87–96. Eds A R Entwistle and J M Melero-Varap. 310. Instituto de Agricultura Sostenible, Apartado 3048, 14080 Cordoba, Spain. 310 pp.

Wakeham A J, White J G. 1996. Serological detection in soil of *Plasmodiophora brassicae* resting spores. *Physiological and Molecular Plant Pathology* **48**:289–303.

Werres S, Steffens C. 1994. Immunological techniques used with fungal plant pathogens: aspects of antigens, antibodies and assays for diagnosis. *Annals of Applied Biology* **125**:615–643.

White J G. 1986. The association of *Pythium* spp. with cavity spot and root dieback of carrots. *Annals of Applied Biology* **108**:265–273.

White J G, Lyons N F, Petch G M. 1996. Development of a commercial diagnostic test for the pathogens which cause cavity spot of carrot. In *Proceedings BCPC Symposium on Diagnostics in Crop Production No. 65*, pp. 343–348.

White J G, Wakeham A J. 1987. Responses of *Pythium* spp. associated with cavity spot of carrots to metalaxyl and related fungicides. *Tests of Agrochemicals and Cultivars 8 (Annals of Applied Biology* **110**, *Supplement)*, pp. 52–53.

(Received 25 July 1997)