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Ecophysiology and production of the biocontrol agent *Phlebiopsis gigantea*

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To learn is to live

Abstract

Heterobasidion annosum, the causative agent of the disease “root and butt rot” in conifers, is ubiquitous in the environment and causes severe financial losses to the forestry industry throughout the temperate zone. The normal route of infection is as basidiospores that land on the freshly cut stump surface during thinning operations, the colonisation of the stump providing sufficient inoculum for the fungal hyphae to invade healthy roots of adjoining trees. The most successful disease control measures involve depriving the pathogen the opportunity to become established in the food source of the fresh cut stump. Frequently killing the surface of the stump with a chemical treatment does this, but there is also some success with a biocontrol method. When the antagonist *Phlebiopsis gigantea*, a saprophyte, is inoculated onto the stump surface it rapidly invades the tissue thus preventing the pathogen access to the resource.

Environmental studies have been carried out to assess the fitness of different isolates of the antagonist relative to the pathogen. Competitiveness was affected by environmental factors; water availability, osmotic/matric potentials, temperature, nutrition, and substrate. These indicate that the antagonist is not able to suppress the pathogen under all conditions. Generally, in wet warm conditions the antagonist is dominant, I_D 10:2 antagonist:pathogen, but in drier, cooler conditions the pathogen is dominant, I_D 2:10 on MEA. Interactions *in situ* on wood discs showed the pathogen was restricted to 0-2% of the treated portion and 2-10% of the un-treated surface at 25 ºC, but at 4 ºC it colonised 2-24% of the former and 34-57% the latter. Consideration needs to be given to possible field conditions at the time of application to maximise the establishment of the antagonist.

Studies have been carried out to examine potential for liquid or solid substrate fermentation systems for optimising production of *P.gigantea*. Liquid culture studies were variable regardless of available nutrients and ecophysiological stresses imposed, producing a maximum of 6.5 $\log_{10}$ CFU ml^{-1}. However, temporal studies on solid substrate based on Pinus sylvestris sawdust gave 8 Log_{10} viable oidia g^{-1} in the best moisture content treatments. Scale up, to 200g substrate produced similar CFUs, however increasing to 400 g showed a reduction in viable oidia, due to lack of moisture equiibration. Analyses of the endogenous reserves to identify specific quality characteristics were inconclusive. Mycelial pellets and submerged conidia produced in fermentation had increased levels of glycerol and erythritol as a_w was lowered, however this was not replicated by oidia produced in solid substrate fermentation. Preservation studies, in a fluidised bed reactor showed, the survival of PG21 spores at up to 60 ºC for 20 min.

Studies were carried out to analyse volatile fingerprints of antagonist and pathogen isolates using an AlphaM.O.S. electronic nose. Volatile fingerprints showed clear grouping of antagonist and pathogen isolates, as distinct from each other. With some isolates, the fingerprints of interactions were co-incidental with the antagonist. It is suggested that this novel technique could be used as a rapid screening method for potential BCAs.

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Nomenclature and abbreviations

a_w	water activity
ANOVA	analysis of variance
BCA	biocontrol agent
C	carbon
$CaCl_2$	calcium chloride
CFU	colony forming units
DFA	discriminate factorial analysis
ECU	European currency unit
EPA	Environment protection agency
HPLC	high pressure liquid chromatography
I_D	index of dominance
IPA	isopropyl alcohol
KCl	potassium chloride
KNO_3	potassium nitrate
MC	moisture content
MEA	malt extract agar
mM	milli molar
MW	molecular weight
N	nitrogen
PEG	polyethylene glycol
PEG 200	polyethylene glycol (MW 200)
PEG 8000	polyethylene glycol (MW 8000)
PG suspension	*P. gigantea* spore suspension
PGS	*P. gigantea* spore suspension

ppm	parts per million
®	registered trade mark
RAMS	random amplified micro satellite
RH	relative humidity
RO	reverse osmosis water
rpm	revolutions per minute
SAS	sensor array system
SE	standard error of mean
SRO	sterile reverse osmosis water
Tween80	polyoxyethylene sorbitan mono-oleate
w/v	weight for volume
w/w	weight for weight

Chapter 1

Introduction and Literature Review

1.1 General introduction

Heterobasidion annosum (Fr.) Bref., formerly *Fomes annosus* (Fr.) Cke., an important tree pathogen, is the major cause of root and butt rot, formerly known as Fomes. This is ubiquitous in the temperate environment and a major source of financial loss to the forestry industry within temperate climate zones. Basidiospores invade cut stumps of conifers during tree-felling, particularly during thinning operations; mycelia spread to un-cut trees through root contact and when inoculum density is high enough, kill them. Financial loss also occurs through the slower production of wood mass due to less photosynthesis in the thinned canopies, as in Plate 1.1. In mature stands there can be much loss of timber volume due to butt rot.

Plate 1.1 Views of severely thinned forest canopy at Bennachie north-east Scotland. Conifers, Sitka spruce, are heavily infected with *Heterobasidion annosum*. Nov 2004.

H. annosum is unlikely to be found in areas absent of trees, or in young plantations, as it does not grow directly in the soil, but inhabits stumps and roots (Rishbeth, 1952).

At present the risk of infection by *H. annosum* is seen as relatively low in British Sitka spruce, which is mainly grown in areas not previously used for conifers on acidic soils in the uplands where there is protection from transmission of the infection through the peat soil. Disease has tended to be a more serious problem in pine on mineral soils with a higher pH, such as that found at Thetford, where disease is harboured in the root systems and can then spread freely to subsequent rotations (Redfern, 2001). Once established in a young pine plantation *H. annosum* infection can be devastating, killing trees up to 25-30 years old outright by girdling the stem base in the cambium and causing butt or heart rot in mature trees (Ingold & Hudson, 1993). If a site has been badly infected with *H. annosum* the only effective treatment at clear-fell, is to de-stump the site to remove the infected roots from the soil before re-planting, an expensive, labour intensive, operation as shown in Plate 1.2.

Plate 1.2 De-stumping of clear-fell site at Thetford forest, East Anglia. April 2003

Stumps are usually treated with chemical agents, such as urea, at the time of thinning and cutting to prevent infection with *H. annosum* (Pratt *et al*., 1998). Rishbeth (1963) devised a method of biological stump protection by the application, at the time of cutting, of the saprophyte *Phlebiopsis gigantea* (Fr.) Jülich, formerly *Peniophora gigantea* (Fr. Ex Fr.) Massee., which can reduce losses from *H. annosum* by out-

competing and antagonising the parasite by hyphal interference on the wood substrate. Enzymes secreted by the fungal hyphae of both species digest the lignin component of the cell wall as well as both cellulose and hemi-cellulose. The digested wood becomes pale and fibrous as it loses up to eighty percent of its mass (Dinwoodie, 2000). Both of these white-rot fungi are basidiomycetes, *H. annosum* forms perennial brackets or sporocarps, which are able to shed aerial spores year round. The fruit body of *P. gigantea* is an annual squat, translucent creamy white, slippery sponge-like structure mainly producing spores in autumn, so spores are not readily available in dry or very cold weather (Meredith, 1959).

The natural population of spores of *P. gigantea* can be augmented by the application of a spore suspension (e.g. PG suspension) to the stumps at the time of tree felling. Formulated from locally collected isolates PG suspension has proven efficacy in colonising pine and has been in continual use in East Anglian pine plantations since 1962 (Webb, 1973). PG Suspension gained approval, as a forestry pesticide on 2/4/98, to be used to control *H. annosum* on pine, under regulation 5 of the UK Control of Pesticides Regulations 1986 (Pratt, 1999). PG suspension was one of the world's first fungal biocontrol agents registered as a pesticide in the UK (Pratt *et al*., 1999).

1.1.1 The pathogen *Heterobasidion annosum* (Fr. 1821) Bref. 1888

(a) Historical perspective:

In the early 19th Century butt rot was seen as a natural part of the life cycle of the conifer, being part of the senescence and decay that preceded death, but also as a disease that could precipitate early death when trees were grown in unsuitable conditions (Borkhausen, 1800; Hartig, 1833.) Indeed Theodor Hartig (1833) while

noting that fungal hyphae were associated with both brown and white rot suggested that the decay was caused by wood cell structure break down into balls that re-aligned, fused and formed fungal hyphae. In his book, “The microscopic enemies of the forest”, (1866-1867), Willkomm described the path of infection, noting that it started from the roots or wounds. He observed that hyphae grew out from spores, forming mycelium and was able to show, through staining methods, that the lignin component of wood was degraded preferentially. He suggested that spores were translocated to sites of infection by wind or on animals; one of his suggestions to control the disease was to uproot and burn all infected trees.

Robert Hartig, in 1874, described the fruit body of the fungus causing butt rot in his book, “Important diseases of forest trees: contributions to Mycology and phytopathology for botanists and foresters” (Trans. Merrill, W., Lambert, D. and Liese, W.). This he named *Trametes radiciperda* R. Hrtg. although it had been previously described and named *Polyporus annosus* (Fr.) by Fries (1821); the latter had not made the connection between the fruit body and the disease. Hartig found the fruit bodies under the soil attached to the roots and rootstock of the affected trees, not only of Scots pine, but also on juniper and some hardwoods. In his microscopical observations he noted that the colourless hyphae “are sparsely septate and easily bore through the walls of phloem and xylem vessels”, reproduced with kind pemission as Plate 1.3. He postulated that the sudden death of an otherwise seemingly healthy tree was due to the phloem vessels becoming rapidly blocked by the fungal mass and the rapid spread of mycelium through the roots. He noted that in the following years the disease affected neighbouring trees, causing large patches of dead trees within the forest. Not knowing whether the infection was spread by mycelial outgrowth through

the soil or by the dissemination of spores he agreed with the earlier recommendation of Wilkomm that roots of affected trees should be removed. Hartig noted that the mycelium extruding through bark cracks was initially the size and shape of pinheads. The fruit body, which enlarged centrifugally, nestled closely against the bark with no stalk. Hymenium-bearing tubes arose on the under surface, hyphae grew out at right angles to the surface becoming club-shaped basidia, but only some formed small white spores. The hymenial canals lengthened by growth of the lower surface of the walls and the older upper parts of the canals became blocked by the outgrowth of hyphae from the walls. Hartig described the sterile upper surface of the fruit body as being covered in a grey rind with a strongly swollen margin.

There were several name changes due to difficulties in deciding to which taxonomic group this fungus should be allocated. Initially it was *Polyporus annosus* (Fr.) (Fries, 1821), Robert Hartig named it as *Trametes radiciperda* (Hartig, 1874). Karsten named it twice as *Fomes annosum* (Fr.) Karst. and *Fomitopsis annosa* (Fr.) Karst., then in 1885 Cooke renamed it *Fomes annosus* (Fr.) Cke. The name was finally settled, in 1888, when Oscar Brefield, who was the first scientist able to describe the complete life cycle of this fungus and was able to grow it in the laboratory, and was the first to notice the unusual asexual sporulation mechanism, named the fungus *Heterobasidion annosum* (Fr. 1821) Bref. 1888 (Hüttermann and Woodward, 1998).

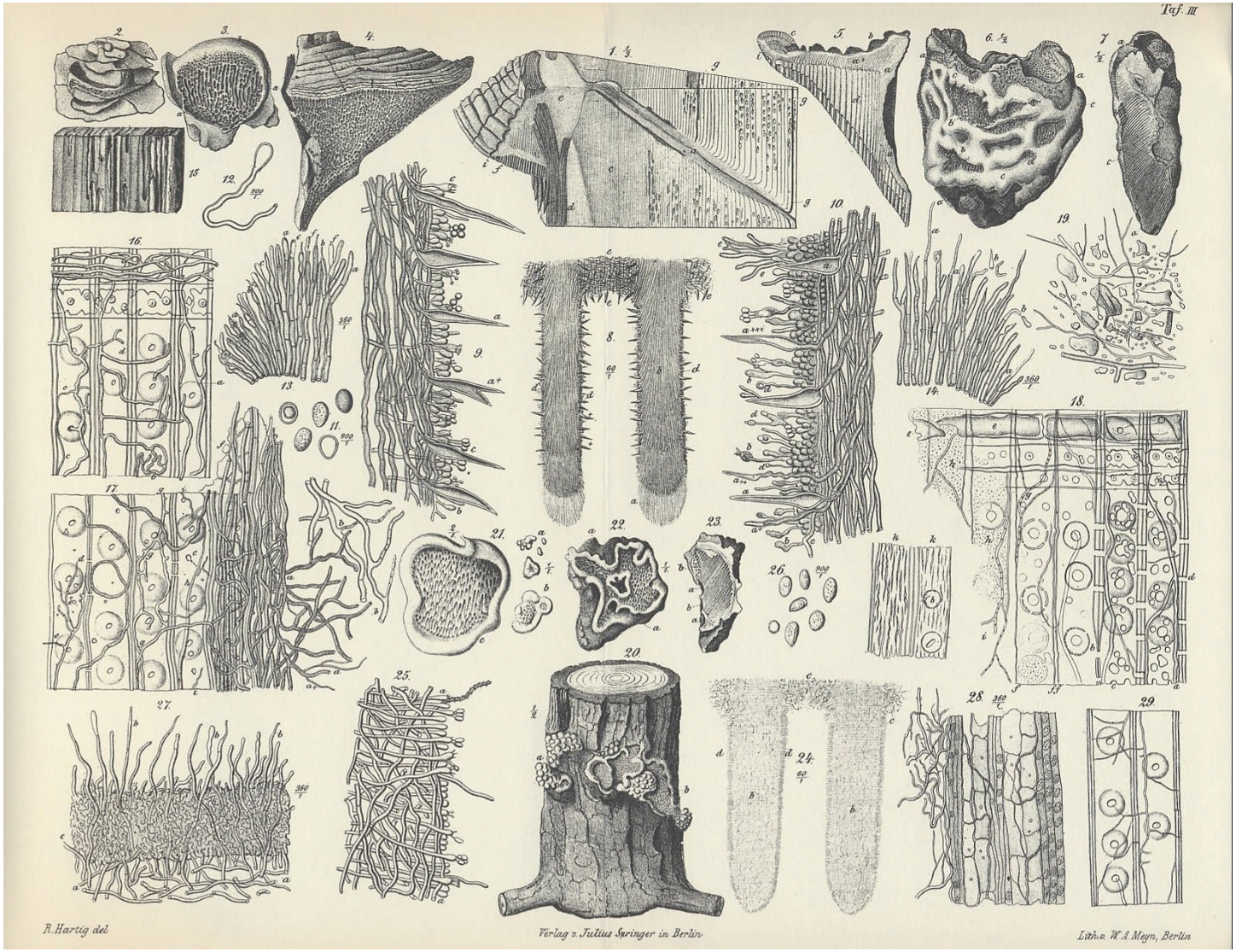

Plate 1.3 Drawings of *Heterobasidion annosum*, then known as *Trametes radiciperda*, R. Hartig (1874) Descriptions of illustrations next page

Explanations of illustrations; (Plate III. Fig. 20-29)
Fig. 20. Root stock of a young Scots pine with a vigorously developed fruit body *Trametes radiciperda*. a. Young fruit bodies clustered together grape-like; b. older fruit bodies.
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Fig. 26. Spores.
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Fig. 29. Root fibres of Scots pine with mycelium of *T. radiciperda*.

Plate 1.3 reproduced from "IMPORTANT DISEASES OF FOREST TREES Contributions to Mycology and Phytopathology for Botanists and Foresters" by Dr. Robert Hartig (Trans. Merrill, W., Lambert, D. and Liese, W., 1975) with kind permission.

(b) Fruit body:

The *H. annosum* basidiophore fruit body, shown in Plate 1.4, found close to ground level on dead trees, stumps and near surface roots, is a perennial bracket, which is woody / rubbery in texture. The upper surface is dark reddish brown to black and the underside cream coloured and covered with tunnels, or tubes, in which the basidiospores are produced. The perimeter between the upper and lower surfaces is a distinctive bright white. Conidiophores, shown in Plate 1.5, are produced, in damp areas and cavities, during an asexual stage of reproduction of *H. annosum.* They are produced from mycelium forming into small pincushion–like structures, the heads of

the pins being the site of conidia formation. Conidia are easily produced in laboratory conditions.

Plate 1.4 Fruit body of *Heterobasidion annosum* growing from the base of a Sitka spruce tree and from a litter covered root. Bennachie, north-east Scotland. Nov 2004.

Plate 1.5 *Heterobasidion annosum* conidia formation as "pin cushions". Grown on a wood disc, as observed through dissection microscope, x10 magnification. 2003.

(c) Spore dispersal

Basidiospores are released throughout the year, except during periods of severe frost (Bjørnkaer, 1938; Kallio, 1970; Meredith, 1959) and high temperature, i.e. above 38°C (Edmonds and Leslie, 1983). Small basidiospores, typically three to five micrometers diameter, are easily dispersed great distances in air and lesser distances by rain droplets (Doverall, 1991) and being airborne, are able to infect new trees through wounds, particularly that of a freshly cut stump surface. Rishbeth (1959) whilst monitoring in the heavily infected area of Thetford Chase, during an eighteen-month period of 1957-1958, found deposition of viable spores up to a rate of two hundred and eighty viable spores per one hundred square centimetres per hour. In about half of the exposures of fresh pine discs that he used for the survey he found from one to twenty viable spores per one hundred square centimetres per hour. Dispersal of the spores depends on air turbulence, but they have been found at great distances from any known basidiocarps or disease centres, spores have been detected in the Shetland Islands, approximately one hundred and eighty miles from the nearest known area of major infection sources (Rishbeth, 1959). Basidiospores are able to survive in the soil for a long time, viable spores have been stored in dry soil for sixteen months (Rishbeth, 1959); on one site trees planted twenty years after clear-felling were infected and those planted after a seven year gap were severely diseased (Rishbeth, 1952). Rishbeth (1959), after inoculating terminal branches with spore suspensions noted their decline in viability was strongly affected by a period of alternate freezing and thawing. Conidia may be less likely to be released into the atmosphere than basidiospores as the conidiophores are found in sheltered places and there is less air turbulence (Redfern and Stenlid 1998); they usually remain viable for

a short time such as a few weeks. Conidia may be spread over short distances by raindrops, but it is also suggested that insects may play a part in dispersal.

1.1.2 The antagonist *Phlebiopsis gigantea*

The fruit bodies of *Phlebiopsis gigantea*, which can be found on dead wood within the forest, have been described as a "grey gelatinous fructification" (Rishbeth, 1952), but in Plate 1.6 can be seen to be a more creamy/pink to beige colour. It is thin and grows close to the substrate, holding firm whilst moist and still growing, but curling up away from the edges once it dries. As a primary coloniser of dead wood *P. gigantea* usually produces fruit bodies the year after colonisation, continuing to produce them for up to three to four years (Rishbeth, 1963).

Plate 1.6 *Phlebiopsis gigantea* covered stump, Thetford, 2006. K. Tubby (neé Thorpe), Forest Research.

Rishbeth (1959) studied the deposition of viable spores in an area heavily infected with *H. annosum*, Thetford Chase, during an eighteen-month period of 1957-1958. In about two thirds of the exposures of fresh pine discs that he used for the survey he found from one to twenty viable *P. gigantea* spores per one hundred square centimetres per hour. In one sample he found a rate of more than one hundred and

fifty viable spores per one hundred square centimetres per hour, but in sixteen out of the eighty-five discs sampled there were no viable spores. Distribution of *P. gigantea* spores is affected by the weather: basidiospores were not released during periods of dry weather (Meredith, 1959); during a short dry period in spring or summer very few viable spores were found on the discs used in this study. Rishbeth also noted that after severely cold weather there was a notable drop in spore deposition, which he suggested was caused by the drying of the fruit bodies. Rishbeth (1959), after inoculating terminal branches with spore suspensions, noted spore viability decline was strongly affected by a period of alternate freezing and thawing; from a rate of five hundred viable spores per gram of foliage at the time of inoculation in only two weeks the viability had dropped to one or two viable spores per gram of foliage.

1.2 Biocontrol

Biocontrol can be defined as the use of living agents to control, or reduce the effects of pests and diseases. This can include the introduction/augmentation of the pest's natural predator, infection of the pest species or introducing/augmenting a competitor to the pest. However, it must be remembered that any interference with nature may have unexpected consequences. An example of biocontrol gone wrong was the introduction of cane toad to Australia to control sugar cane beetles. It was so successful in adapting to its new environment that it has become a pest in its own right. The myxoma virus was deliberately introduced into the wild rabbit population of Australia, but the virus has been able to jump continents; thought to have been accidentally introduced to the UK on rabbit carcases, it is now not unusual to find rabbits suffering from myxomatosis in the English countryside and it is a danger to

pet rabbits. As almost the entire population of rabbits died this had a disastrous effect on their predators. Before any new organism can be introduced to the environment it needs to be tested both in laboratory and field trials for possible risks to human health and the organisms of the natural ecosystem (Holdenrieder and Greig, 1998). Starting in the latter part of the 20th century, driven by an increased desire to reduce the use of pesticides in the environment, much progress has been made in the biocontrol of agricultural pests e.g. the use of wasps (*Encarsia formosa*) to control whitefly (*Bemisia spp*.) in tomato crops, ladybirds to control greenfly and parasitic mites (*Hypoaspis miles*) to control swamp gnats (*Sciarid* flys) in glasshouses. In Canada whitefly control on outdoor vegetable rows has been achieved by the use of *Beauvaria bassiana* sold as Mycontrol® (Jackson, 1997).

1.2.1 Biocontrol of the pathogen *Heterobasidion annosum*

It is known that competition with other fungi at the time of cutting the tree can influence the colonisation of *H. annosum* on the stump surface and biological methods have been used to reduce the success of *H. annosum* (Rishbeth, 1952). As well as sexually produced basidiospores, *P. gigantea* has an asexual stage, producing uninucleate arthroconidia (oidia) from fragmentation of mycelia (Pratt *et al.*, 1998). Oidia are freely produced in laboratory culture (Cram, 11/10/02). During the late 1940s and early 1950s Rishbeth conducted a series of experiments on East Anglian Pine, involving the comparison of passive stump protection by excluding the pathogen basidiospores by painting the stump surfaces with either household paint or creosote or covering with soil or by active protection, initially with the use of wedges of wood from pines naturally infected with *P. gigantea*, later with a *P. gigantea* spore suspension consisting of “truncate spores or oidia”. Applying the soil was counter-

productive in that there was a greater incidence of disease than in the control stumps; the passive stump protectants gave an average of 90% protection from incident disease, but no protection from infection arising through the roots; from an infection rate of 78% in untreated stumps, the active protection afforded by the wedges reduced this to 47% and that by the spore suspension was 13% (Rishbeth, 1952). The spore suspension treatment developed by Rishbeth has been in continuous use on vulnerable pines in East Anglia since 1962 and is credited as being the first biological control agent of a plant pathogen that was commercially available (Baker & Cook, 1974: Pusey, 1996).

Biocontrol products have been used widely on *Pinus* species for over 30 years, suggesting a cost benefit in controlling losses to *H. annosum*. In a survey of nine European countries there was found to be a growing interest in the use of stump treatment at final felling as well as thinning; in some countries it is thought to be a strategic benefit as a means to reduce the overall production of *H. annosum* inoculum, thus benefiting all woodland, not just the treated areas (Thor, 2002). During field trials Nicoletti *et al.,* (1999) compared some biocontrol and chemical treatments against *H. annosum* on Norway spruce; they found that each treatment tested reduced the incidence of *H. annosum*. The most successful treatments in their study were the fungicide propiconazole, and *P. gigantea*, which reduced the rate of stump infection with *H. annosum* by 56 and 50% respectively. When comparing the proportion of surface area colonised by the pathogen they found an 85% reduction of infection when compared with the control when inoculated with *P. gigantea*; the propiconazole produced a 96% reduction. *P. gigantea* has been shown to be not as effective on spruce as on pine where it offers almost complete protection. Treatment of pine

stumps in early thinning reduced losses compared to untreated control areas where 43% of trees were lost (Sierota, 1997).

Greig (1976) reported on field trials in which *P. gigantea* oidia were mixed with the chainsaw lubrication oil. He found that the surface area colonised by this method compared well with the standard method of hand application of spore suspension with a brush. He suggested there would be a cost benefit of this method by the reduction of labour, together with an assurance that all stumps would be treated as he acknowledged the possibility of some stumps missing the treatment when applied by hand after felling had taken place.

To provide effective control on pine stumps 5×10^5 viable *P. gigantea* oidia m^{-2} are usually required (Rishbeth, 1963). However, pine is more easily colonised by *P. gigantea* than spruce. The latter requires an inoculum of at least 5×10^6 CFU l^{-1} (Korhonen, 2001). *P. gigantea* inoculum is currently available as PG Suspension (formerly produced by Omex Ltd, currently being produced in-house by Forest Research) in the UK and as a freeze-dried product, Rotstop, marketed by Verdana Oy, formerly Kemira Oy, in Finland. In the USA it has not been available since 1995 when the EPA notified the Forest Service that *P. gigantea* products would have to be registered as a biological pesticide, which is a very expensive process (Cram, 11/10/02). *P. gigantea* strains indigenous to the UK have been tested on Norway and Sitka spruce in the 1970s; these were not successful as stump colonisation was poor (Holdenreider & Greig, 1998). PG suspension is not regularly used to protect spruce in the UK and is currently used only on pine, although the Finnish product Rotstop is successfully used on spruce in Scandinavian countries. In large areas of contamination

with *H. annosum*, i.e. heavily diseased pine plantations, the following crop is not protected by the *P. gigantea* inoculum used to treat the previous crop stumps on felling.

Roy *et al.* (2003), working in Canada, compared the efficacy of *P. gigantea* and *Phaeotheca dimorphospora* as antagonists for *H. annosum*. Although the *P. dimorphospora* performed well in laboratory studies, in the field trials it promoted colonisation by the pathogen whereas the *P. gigantea* suspension was found to completely inhibit the pathogen.

1.3 Competition between fungi and antagonist mechanisms

Fungi have evolved adaptations to allow them to survive in a wide variety of ecological niches. Those which occupy similar niches may have adapted different strategies to allow their survival e.g. the ability to use specific nutrients or the ability to survive environmental stress (Magan, 1997); some have developed the ability to actively discourage the growth of other fungi or micro organisms in their immediate environment. The three major ecological strategies could be summarised as (i) combative, - persistent, long-lived, capable of defending resources, (ii) stress-tolerant, - adapted for stressful conditions and able to persist as long as the stress conditions remain, (iii) ruderal, - ephemeral species that rapidly utilise resources, sometimes only capable of utilising easily assimilable nutrients and reproduce rapidly (Cooke and Rayner, 1984). Both the pathogen and antagonist used in this study are of the long-lived combative type.

As wood decay fungi inhabit a solid matrix that is both their “living space” and their food it is difficult to separate the type of competition between them as exploitation of food reserves type or that of territory. However there is a distinction between primary resource capture and combative antagonism to defend the food resource or to invade substrate already occupied by another organism (Boddy, 2000). A freshly cut tree stump gives a changing environment to potential colonisers. Initially some cells are still alive and the tree might be able to maintain a degree of resistance to invasion; this could be an advantage for a facultative parasite versus a saphrophyte. This would enable the parasite to form a substantial biomass from which it is able to defend its territory and mount a hostile attack on healthy trees (Cooke and Rayner, 1984).

The competitiveness of individual fungi is affected by the environmental conditions in which they grow. Magan & Lacey (1984c) showed that water availability, substrate, nutrients and temperature influenced the antagonism and dominance of individual fungi within a community grown on stored grain. Interspecific fungal interactions can occur, when hyphae of two species meet. These range from mutual intermingling, through mutual antagonism, to one species dominating the other at a distance. Magan and Lacey (1984a) studied the interactions between field and storage fungi; they found that dominance of one species over another changed when environmental conditions were altered. Each interaction was given a score ranging from 1-5 such that when added together they gave an Index of Dominance (I_D) with the higher scores indicating the higher relative rate of competitiveness under the different environmental modifications.

Production of secondary metabolites that diffuse across the matrix, or when direct contact between hyphae of antagonist and pathogen occurs, compromise the integrity of parasite hyphae, can be an important factor in antagonism and can contribute to the success of the antagonist by excluding other species from the available resource.

As these are secondary metabolites their production is affected by the growth conditions (Magan, 1997).

1.4 The concept of water availability

Life processes occur in aqueous solution, thus the osmotic pressure within the cell needs to be maintained for cellular processes to proceed. If the exterior environment is not correct for these processes the cell must have the ability to control its internal environment rather than to remain at osmotic equilibrium with the exterior. Some fungi have evolved to be able to survive in extreme conditions such as high salt concentrations or arid regions (Magan, 1997). Water is bound by the physical and chemical structure of the substrate and water which is bound in this way is not freely available for use by micro-organisms. The availability of water, necessary for life processes, is an important environmental factor influencing the colonisation of wood by wood-decay fungi. This is affected by osmotic potential, the result of solute and matric potential, the force holding water molecules to interfaces with solid particles within the matrix of the wood (Mswaka & Magan, 1999). Free water is less tightly bound to the substrate and is thus more available for use by growing microorganisms. Lee *et al*., (2000) noted that the total water content of a substrate is often known, but that this does not define the actual amount of water available for growth of microorganisms. In the colonisation of wood by wood-rot fungi there generally needs

to be water content of more than 20% (Ingold & Hudson, 1993). To be able to study the interactions between fungi in their niche it is important to know the optimal water activity (a_w) for growth and the whole range of a_w that allows sub-optimal growth (Magan and Lacey, 1984b).

Scott (1957), while looking at conditions suitable for growth of food spoilage microorganisms defined water activity (a_w) as a fundamental property of aqueous solutions;

$$a_w = P/P_o \text{ (by definition)}$$

Where P = the vapour pressure of water in a substrate and P_o = vapour pressure of pure water at the same temperature and pressure.

a_w is numerically equal to the corresponding relative humidity (RH) expressed as a fraction, i.e. RH/100. The a_w of pure water is 1, a solution has an a_w that is less than 1 due to the solutes within. A one molar solution of an ideal solute has 98.2 % of the vapour pressure of pure water at the same temperature and pressure; this equates with a water activity of 0.982 a_w and corresponds to an osmotic pressure of over twenty-four atmospheres.

In a solution the water molecules are closely associated with solute molecules, all in a state of ionisation. In concentrated solutions microorganisms are surrounded by large numbers of solute molecules, each hydrated by water molecules, so there are few free water molecules within the substrate. Over the entire range of a_w permitting microbial growth, the microorganisms compete with solute molecules for the water required for growth (Scott, 1957). Magan (1997) listed terms used for organisms that are able to

tolerate and grow in conditions of high water stress as xerotolerant, osmotolerant, osmophilic, halophilic and xerophilic; he suggested that the most suitable terms in use for fungi are osmophilic for a specialised group of salt tolerant yeasts and xerophilic for dry-loving. Some microorganisms can grow at a_w levels as low as 0.65 (Scott, 1957), but it has been generally accepted that a xerophile is a fungus that is able to grow during some part of its life cycle at a limit of 0.85 a_w (Pitt, 1975).

As a means to control the intracellular osmotic potential, fungi are able to accumulate compatible solutes, some by manufacture of secondary metabolites and some by absorption from the growth medium. Parasitic fungi have been shown to convert plant host glucose to polyols thus preventing the host from utilising it (Deverall, 1981).

1.5 Principles of environmental manipulation as a means to study ecophysiology of the antagonist and pathogen

Modifying the water activity, nutrients and temperature of the growth media alters environmental conditions for the growth of microorganisms during culture. “Moisture is probably the most important condition which controls the germination of spores. Most spores require moist air for their germination and some must have free liquid, as rain drops or dew films” (Butler (in Tomkins 1929)). Tomkins (1929) set out to devise experiments to quantify the range of vapour pressures that allowed for the germination and growth of spores. He set up growth chambers in which the only available moisture was provided by known solutions of potassium hydroxide, using tables of humidity to gauge the available humidity. He then incubated the chambers at controlled temperatures, withdrawing them at intervals to measure the germ tube

extensions; finding that both temperature of incubation and relative humidity affected growth rates. It became clear that as the relative humidity was decreased from the one hundred percent of pure water, the range of temperatures that allowed for germination decreased. When mycelia were grown in Petri dishes on dried nutrient agar, but in humidity controlled chambers, the growth rates were similarly affected by both temperature and relative humidity.

Solutes commonly used in laboratory experiments for the purpose of restricting available water are NaCl and KCl (ionic), glycerol, glucose and sorbitol (non-ionic) and Polyethylene glycol (matric potential) (Beecher & Magan, 2000).

Little information is available about the water and temperature relations of *P. gigantea* and *H. annosum* and the effect of abiotic factors on competition.

1.6 Mass production of biocontrol agents, solid substrate, semi-solid and liquid fermentation methods

Forest enterprise needs a reliable supply of PG suspension for treatment of tree stumps at the time of felling. At the start of this project the production of PG suspension was carried out by a single company which was experiencing production difficulties. Production methods need to be low cost with a high yield of viable spores, for the commercialisation of fungal biocontrol agents. To this end the development of liquid culture fermentation for rapid spore production on a large scale would be beneficial (Jackson, 1997). Although *P. gigantea* is usually grown on a solid or semi-solid substrate, as are other biocontrol fungi, there are problems due to gas exchange, temperature control, substrate sterilisation, maintenance of a pure culture

and harvesting of spores. The fermentation time for sporulation is 3 weeks; for some agents it is only days in liquid fermentation. According to Jackson (1997) liquid culture is the most economical method for producing most microbial biocontrol agents. Within liquid culture the internal environment can be maintained and controlled, hence homogenous conditions for nutrition, gas content, pH and temperature can be maintained for quality assurance and the scale-up of production. Jackson (1997) found that nutrition during fermentation could significantly affect the quality and quantity of propagules, later, Inch *et al.* (1986) observed that nitrogen exhaustion induced some fungi to sporulate profusely.

1.7 Quality improvement in biocontrol propagules

Fungi that occupy the same niche are often ecologically similar, thus to improve the competitiveness of an antagonist over a pathogen, to give it an advantage in interactions, an increase in the propagules fitness would be an advantage. Temperature and atmospheric moisture vary greatly within the forest, so tolerance to these fluctuations is necessary for effective biocontrol of the pathogen. The inoculum needs to be able to rapidly establish itself to be able to gain control over resources, thus preventing the development of the pathogen. To this end the antagonist inoculum needs to be able to germinate, make good use of food reserves and to grow rapidly, as well as to be able to survive bouts of relative desiccation.

Jackson *et al.,* (1997) developed a liquid culture medium that produced a large number of desiccation tolerant spores of the biocontrol agent (BCA) *Paecilomyces fumosaroseus* used to control whitefly. They tested various media with differing

concentrations of C and N sources and found the highest yield of blastospores, asexual buds from submerged hyphae, to be when the medium was rich in both C and N (glucose > 20 g l^{-1}, amino acids > 13.2 g l^{-1}). However desiccation tolerance was dependent on the amino acid concentration with the greatest proportion of desiccation tolerant spores produced from media in which the concentration of amino acids was between 13.2 and 40 g l^{-1}. This contrasted with other studies which showed that nitrogen limitation improved the viability and effectiveness against the target organisms (Lane *et al*., 1991), and Schisler *et al*. (1990) who in a study of *Colletotrichium truncatum*, a mycoherbicide, showed that the use of a carbon:nitrogen ratio of 10:1 produced effective spores. Frey and Magan (2001) found the addition of $CaCl_2$ to the culture medium, oatmeal extract broth, enhanced the production of *Ulocladium atrum* spores.

Magan (1997) found that xerotolerant fungi were adapted for the conditions of low water availability, e.g. 0.70 a_w by the accumulation of low molecular weight alcohols, polyols, such as glycerol and erythritol. These compatible solutes allow enzymes to function normally within the cell. However, most fungi used as BCAs are unable to germinate and grow at less than 0.90 a_w. When grown on media with lowered water availability polyols and trehalose accumulated in the spores of entomopathogenic fungi, used as BCA, and these were shown to be more tolerant and have enhanced germination in low a_w conditions over un-modified spores (Hallsworth and Magan, 1995). Similarly spores of *Metarhizium anisopliae* from natural substrates, dead insects, have been shown to have increased virulence, against insect hosts, than spores produced in rich artificial media, with significantly different profiles of polyols and sugars (Magan, 2001). When spoilage fungi such as *Aspergillus flavus* and *A.*

ochraceus were exposed to water stress they accumulated significant quantities of polyols in their conidia as well as in the mycelium (Neschi *et al*., 2004).

1.8 Methods of production of PG suspension and other formulations

Laboratory grown *P. gigantea* spores are harvested from MEA, then suspended in a sucrose/fructose mixture which acts as a preservative medium inhibiting growth of other micro-organisms. Each PVC sachet contains approximately 20 x 10^6 viable spores which when diluted are applied at a rate of approximately 1 x 10^6 spores m^{-2} of stump surface (Pratt, 1999)

The earliest formulation of PG in the UK, consisted of inoculated pinewood blocks, incubated for 10 days, sealed in polythene bags, sent fresh out to forests daily (Rishbeth, 1963; Pratt *et al.*, 2000). Prior to use, the blocks were shaken in water to release oidia (ca. 10^6 ml^{-1}) that were applied to stumps. Block shelf life was 8 weeks at room temperature, with no appreciable loss of viability. A further development in making an easily useable product was that of freeze-dried suspensions of oidia in 20% sucrose stored at 10°C for 8 months. The method was not suited to large-scale production. A new formulation was developed; pills of oidia suspension, sucrose, talc and carboxymethyl cellulose. The product, easy to handle, was successfully used for 5 years. Attempts at other formulations were unsuccessful until 1970 when the current formulation of *P. gigantea* Suspension (PGS), oidia in sucrose, in PVC sachets was developed (Pratt *et al*., 2000).

The current manufacturing system for PG suspension is labour intensive. For each production run approximately 150 Petri dishes of MEA plus Phostrogen® are prepared, the first 5-10 of these are used as master dishes, having been inoculated with fresh culture as supplied by the Forestry Commission and incubated at 22ºC for 1-2 weeks. From these dishes oidia are picked off with a sterile wire loop and inoculated onto the remainder of the Petri dishes, which are then incubated at 21-24ºC for 18-21 days. To harvest, the surface growth is manually scraped off each plate into sterile distilled water to which sucrose and honey are then added. The suspension is stirred vigorously for 30 minutes before being added to a bulk suspension syrup, which has been boiled and cooled, and is thoroughly stirred for a further 30 minutes to ensure even distribution. After further additions and coarse straining the mixture is then ready for sealing into PVC tubing (The Forestry Commission, 1998).

Rotstop, the Finnish equivalent, is batch cultured in fermentation vessels, freeze-dried and the sealed packs have a shelf life of 12 months at 8°C (Webber 30.4.01). In Finland, in the 1970s, Kemira supplied Petri dishes of culture direct to forests where the spore suspensions were prepared on the site immediately prior to use. In 1987 a *P. gigantea* strain isolated from a Norway spruce log, in Finland, was found to be effective against *H. annosum* on Norway spruce and pine stumps. Kemira formulated the product Rotstop in 1991 (Korhonen *et al*., 1994). IBL, the Polish product developed in the 1970s, consists of a mixture of oidia and mycelium grown on sterile beech sawdust in polythene bags/bottles. In the USA there were several preparations of *P. gigantea* but these have now been withdrawn as a pesticide and are no longer available in the USA (Pratt *et al.*, 2000).

Quality control is not standardised between products, but Rotstop & PGS are tested for CFUs and viability after storage. Each product is also checked for contamination by *E.coli* and other pathogens, contaminated containers are rejected.

1.9 Strain variation between *P. gigantea* isolates and monitoring for effectiveness against *H. annosum*

P. gigantea isolates vary in effectiveness in controlling the pathogen *H. annosum*; ten different isolates have been used in formulations of PG suspension for over 30 years of use. New isolates are taken periodically from forests throughout the UK and tested. In Poland new isolates are collected each year from untreated areas of forest (Pratt *et al*., 2000). The Finnish preparation of *P. gigantea*, Rotstop, was chosen from approximately 30 isolates used in pilot tests. Isolated from a spruce stump in Loppi, Finland, the isolate KK91002 has proved to be as effective as urea in controlling *H. annosum* on both pine and spruce in Scandinavian countries (Korhonen, 2001). This isolate has still been found to be the most effective of the European isolates that have been tested by Korhonen (2001).

As isolates vary in rate of spore production, and with their ability to suitably antagonise the pathogen *H. annosum*, they are screened before being used in production. There is no standard protocol for screening and methods vary. Each is checked for growth rate on MEA. In Poland and the UK the number of oidia produced in these dishes are checked with a haemocytometer; in the UK there are spore viability assessments, whereas in Finland the amount of decolouration of a lignin-like dye is assessed. In each case this preliminary test is followed by trials on fresh wood;

in the UK assessment of stain on fresh pine discs, in Poland weight loss of pine blocks inoculated either with the *P. gigantea* strain, or the *P. gigantea* and *H. annosum* in pairs. The Finnish strains are tested on spruce as well as pine using log pieces to model growth in stumps, half sprayed with *P. gigantea* and completely sprayed with *H. annosum* then the growth of *H. annosum* is assessed (Korhonen, 2001). Each of these is followed by field trials on stumps and an assessment of the growth of *H. annosum* (Pratt *et al.*, 2000). As yet, there is no recognised method for screening for continued effectiveness (Webber 2001, Pratt *et al*., 2000). According to Rykowski and Sierota (1983), the best isolates for biocontrol are able to thrive on beech wood (Pratt *et al.*, 2000).

1.10 Environmental implications

There is no evidence of pathogenicity, toxicity, or allergic reactions caused to mammals by *P. gigantea* formulations. Indeed, it is classified as edible (Worgan, 1968). The relevant data from animal tests performed for Kemira, on Rotstop, were made available to Forest Research for use in their pesticide application. It had also been used for many thousands of man-hours without any harm to health according to the detailed health records of the employees (Pratt *et al.*, 1999).

Questions have been asked about the natural distribution of the control microorganism, and what impact its ability to spread widely after application in the field might have. Before being introduced as a BCA *P. gigantea* was widely spread throughout the UK, living spores had even been recovered from the atmosphere above the Atlantic ocean (Rishbeth, 1959). Applications of working strength dilutions from

the commercial preparation are applied by hand or by the mechanical harvesting machine. The latter can cause a problem of contamination of butt logs, the lowest portion of the trunk immediately above the cut made at harvest (Pratt *et al.*, 2000), but PG suspension has been used for 30 years in Forestry Commission plantations with no observed environmental problems (Pratt, 1999). In a 60 year rotation period within the forest approximately 3 x 10^7 viable spores ha^{-1} are applied, whereas the natural deposition is far greater, $>10^{12}$ spores ha^{-1} (Pratt *et al.*, 1999).

Isolates used in the BCA preparations were all collected from the wild. Master cultures of *P. gigantea* have been isolated from dead pinewoods in the UK. Isolates collected from sporocarps, decaying stumps and logs and spore captures on exposed discs, are presumed to be heterokaryotic, but have not been tested. In Finland the same heterokaryote has been used in Rotstop since 1991. In Poland, isolates are collected from sporocarps in untreated areas of forest during April-October (Pratt *et al.*, 2000).

Recently UK and Scandinavian isolates of *P. gigantea* were compared using RAPD and RAM molecular analysis (Webber and Thorpe, 2002a,b). They found a high level of variation within the UK population, but the Scandinavian isolates were not significantly different in terms of molecular markers. Lack of distinct groups consisting of similar banding patterns when using DNA Random Amplified Micro satellite (RAMS) markers suggests that *P.gigantea* is a single European breeding population with large genetic variation (Vainio *et al.*, 1998)

Vaino *et al*. (2001) investigated the population of *P. gigantea* isolates in treated Norway spruce and Scots pine stands to see if the use of Rotstop would reduce the level of polymorphism of *P. gigantea*. They found that the survival time of the isolate used as a biocontrol agent differed between spruce and pine stumps; at six years after treatment the BCA strain was still present in about half the spruce stumps, but in pine had been replaced by other fungal species. They concluded that the use of Rotstop as BCA is unlikely to cause loss of diversity of natural *P. gigantea* populations, but that it is important to monitor possible changes of diversity in managed forests.

Vasiliauskas and Stenlid (2001) reported at the Rotstop seminar held in Finland that the Rotstop isolate of *P. gigantea* was well established in 4 year treated stumps inside wood and fruiting bodies were present, whereas the Rotstop isolate was only found in 10 % of stumps treated 6 years previously and no fruit bodies were found. The *P. gigantea* strains isolated from non-treated, control, stumps were not identical with Rotstop. The natural behaviour of *P. gigantea* is as an early coloniser that disappears from the substrate after 5-6 years. Treatment with Rotstop did not appear to affect fungal diversity on stumps, treated stumps and those naturally colonised with wild *P. gigantea* strains, contained the same species of microfungi. In forest stands where Rotstop was used in previous thinning events a wide diversity of fungi have been isolated, but not the *P. gigantea* isolate Rotstop.

1.11 Aims and objectives of this study

The aim of this project was to improve the production and efficacy of the biocontrol agent: thus, to study the fundamental ecophysiology of the antagonist and pathogen under varied environmental conditions; to use knowledge gained to inform investigation and development of production methods for *P. gigantea* spore suspension (PG suspension). As free moisture is required for the spore to germinate and penetrate the host, the effect of culture to reduce time needed and to equip the spore with sufficient reserves to allow for germination and initial growth of germ tube is worth investigating. Figure 1.1 shows the components of research to achieve thesis objectives, the details of which are in this report. In production and preservation trials the *P. gigantea* isolate used was PG21 as this is the isolate currently in use for PG suspension.

To achieve these overall goals the following strategies were carried out:

- Fundamental studies into comparison of different isolates of *P. gigantea* and *H. annosum* in relation to environmental stresses including water, temperature and solute relations.
- Interactions in relation to environment, water availability and temperature, for a range of isolates.
- Evaluation of differing substrates for the production of PG suspension. Solid substrate, semi-solid and liquid fermentation methods.
- Evaluation of preservation methods.
- Investigation of volatile fingerprints as a possible rapid screening method for new isolates and their potential efficacy.

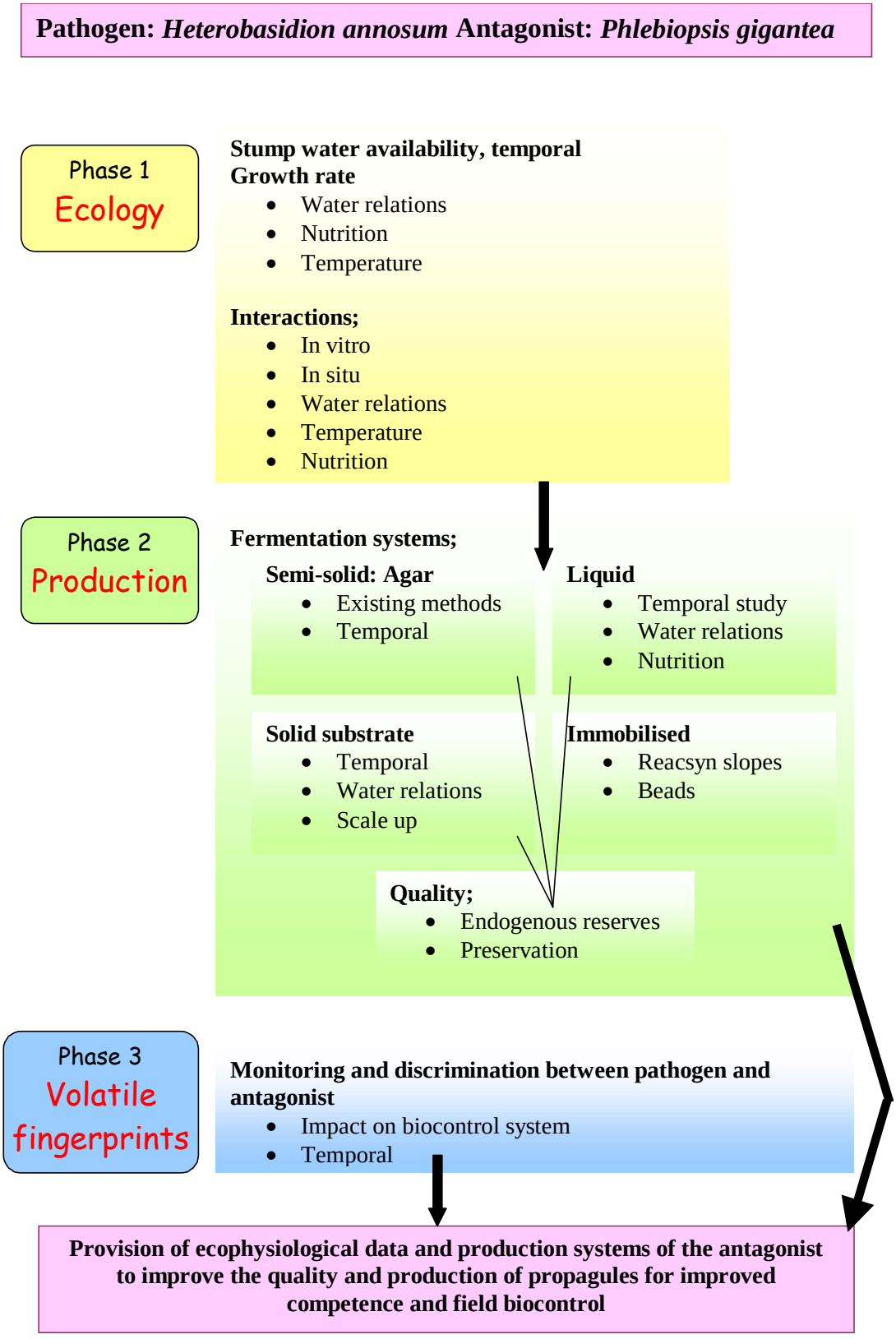

Fig 1.1 Flowchart to show components of research to achieve thesis objectives

Chapter 3

Results: Ecology

3.1 Water availability in the surface of tree stumps

The available water, measured as water activity (a_w), in the surface half a centimetre of freshly felled *Pinus sylvestris*, was monitored over a four-week period. Two of the sampled stumps are displayed in Plate 3.1, (x) is representative of the group of dryer looking stumps, (y) is representative of those with a coating of viscous, syrup like liquid. The results are displayed in Figure 3.1 and show that the available water remained in the region of 0.965 to 0.990 a_w during this critical period of time. ANOVA of the effect of visual appearance group, time since harvest and two-way interaction showed no statistical difference between the groups, but there was a significant difference ($p < 0.001$) at each precipitation event when the moisture content rose. Samples were harvested during February, and early March, during a time of changeable, but wintry weather. The arrows in the figure denote precipitation before or during sampling; day 7 there was light rain both before and during sampling, the day 15 samples followed a day of heavy rain, during the following week there was much snow, the samples taken on day 25 had the surface wiped clear of lying snow prior to sampling, the snow continued, but by day 29 was beginning to thaw and there was a layer of semi-frozen snow on the stumps and the surfaces were visibly wet.

Plate 3.1 *Pinus sylvestris* (30 ± 2 years) tree stumps at the time of the first sampling, group (x) appeared to be slightly dry; group (y) stumps were coated with a viscous liquid. Arrows mark sampling point.

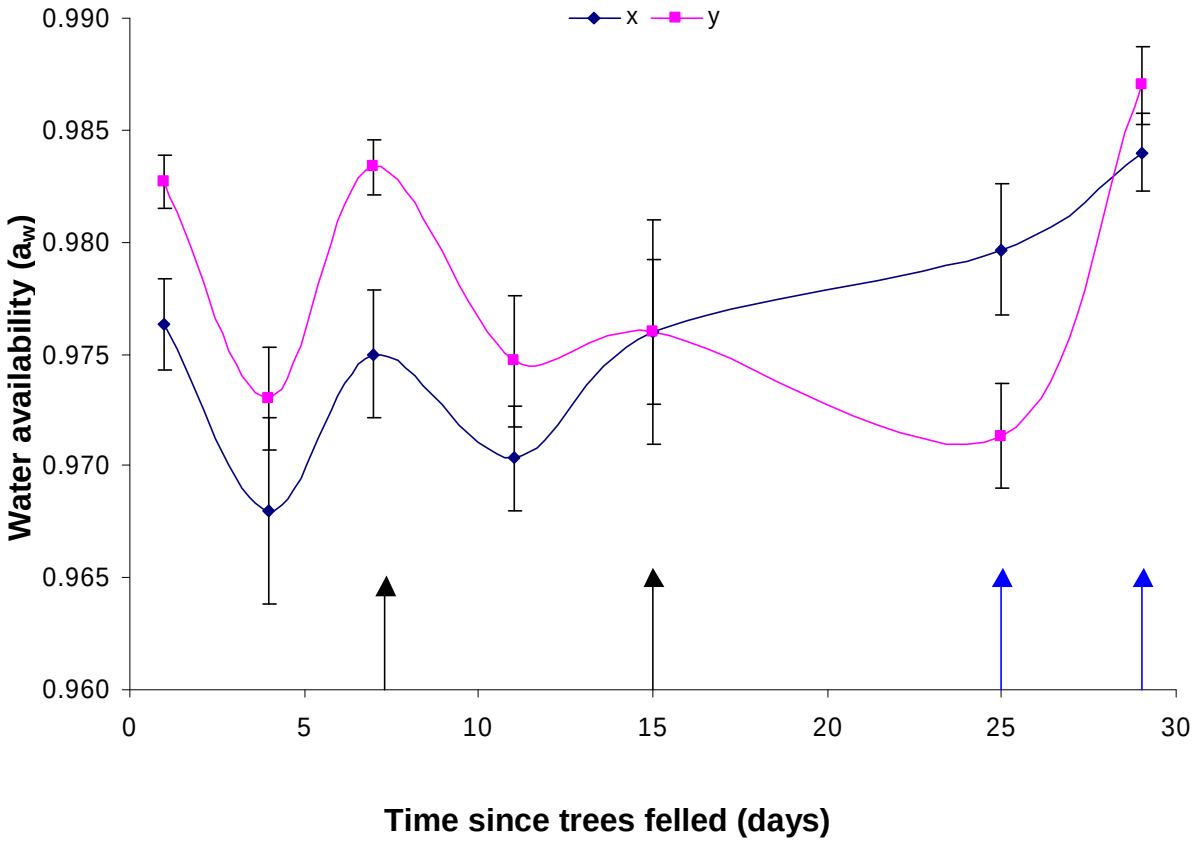

Fig. 3.1. Tree stump water availability (a_w) measured at the cut surface of stumps of *Pinus sylvestris* for a period of 4 weeks after felling. Key: x = dry, y = viscous surface. Arrows denote inclement weather at the time of sampling; black arrows indicate rain before and during sampling, blue arrows indicate snow.
Error bars represent standard errors of the means, ANOVA showed no statistical difference between groups x and y.

3.2 Investigation of the effect of water availability and solute type on the growth of isolates of *P. gigantea* and *H. annosum*

Figures 3.2 and 3.3 show the effect of water activity (a_w) and solutes on the mycelial extension rates of one isolate each of *P. gigantea* and *H. annosum*. In both Figures the data for *P. gigantea* and *H. annosum* have been presented as plots of mean radial extension per water activity. No growth of either species was observed at a_w levels <0.945 a_w, during the experimental period of 10 days. The organisms were grown on malt extract agar (MEA) at 25 ºC with the a_w modified with glucose, glycerol or

potassium chloride (KCl). The linear growth phases were used to calculate growth rates (mm day^{-1}). In Figure 3.4, the relative growth rates of the two fungi grown with the three solute treatments, in relation to a_w is shown. This shows that with freely available water *P. gigantea* grew more rapidly than the *H. annosum* isolate. When water stress was imposed to limit available water the growth rate was reduced for both fungi. ANOVA of the effect of species, solute and a_w group and two and three-way interactions showed that there were no significant difference in the response of the two species but all other factors and interactions were significant ($p<0.001$).

The biocontrol agent, *P. gigantea*, was very sensitive to glucose and KCl, with a reduction to 33 and 34% of its growth rate at 0.984 a_w and a further reduction down to 13 and 10% respectively of potential growth at 0.974 a_w. It was more tolerant of lower a_w levels when using glycerol as the solute, with a reduction to 55% of growth rate at 0.984 a_w and down to 24% of growth rate at 0.974 a_w.

The pathogen, *H. annosum* was most sensitive to glucose, with a reduction to 55% of its growth rate at 0.984 a_w and a further reduction down to 16% at 0.974 a_w. With KCl as solute the reduction in growth rate was reduced to 54% at 0.984 and 24% at 0.974% a_w. It was more tolerant of lower a_w levels when using glycerol as the solute, with a slight reduction to 91% and 37% growth rate of that at 0.984 a_w and 0.974 a_w respectively.

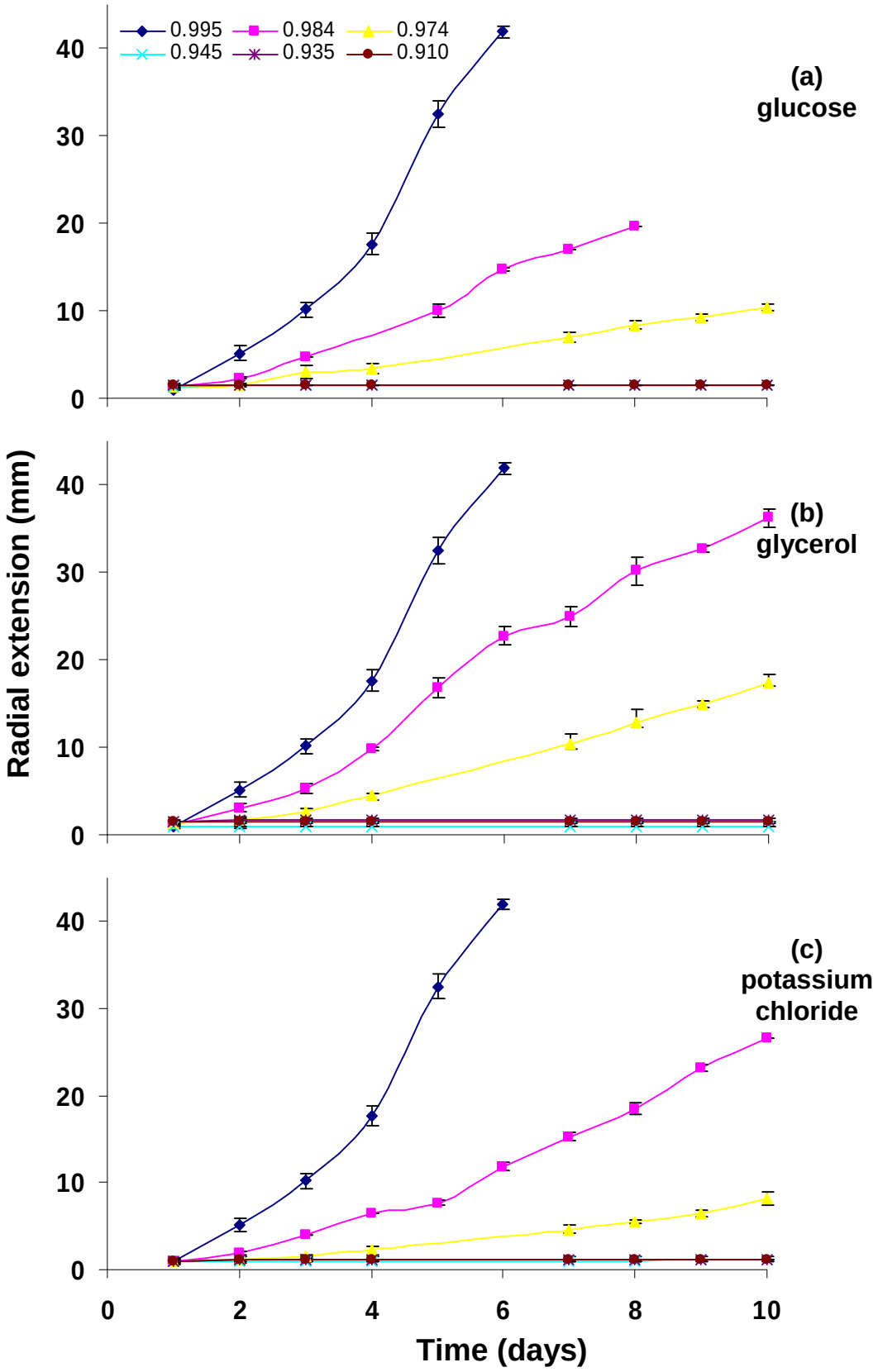

Fig 3.2 Effect of solute on mycelial extension rates of *Phlebiopsis gigantea*. Grown at 25 ºC on malt extract agar, water activity (shown in key) modified with (a) glucose, (b) glycerol and (c) potassium chloride. Bars represent standard errors of means.

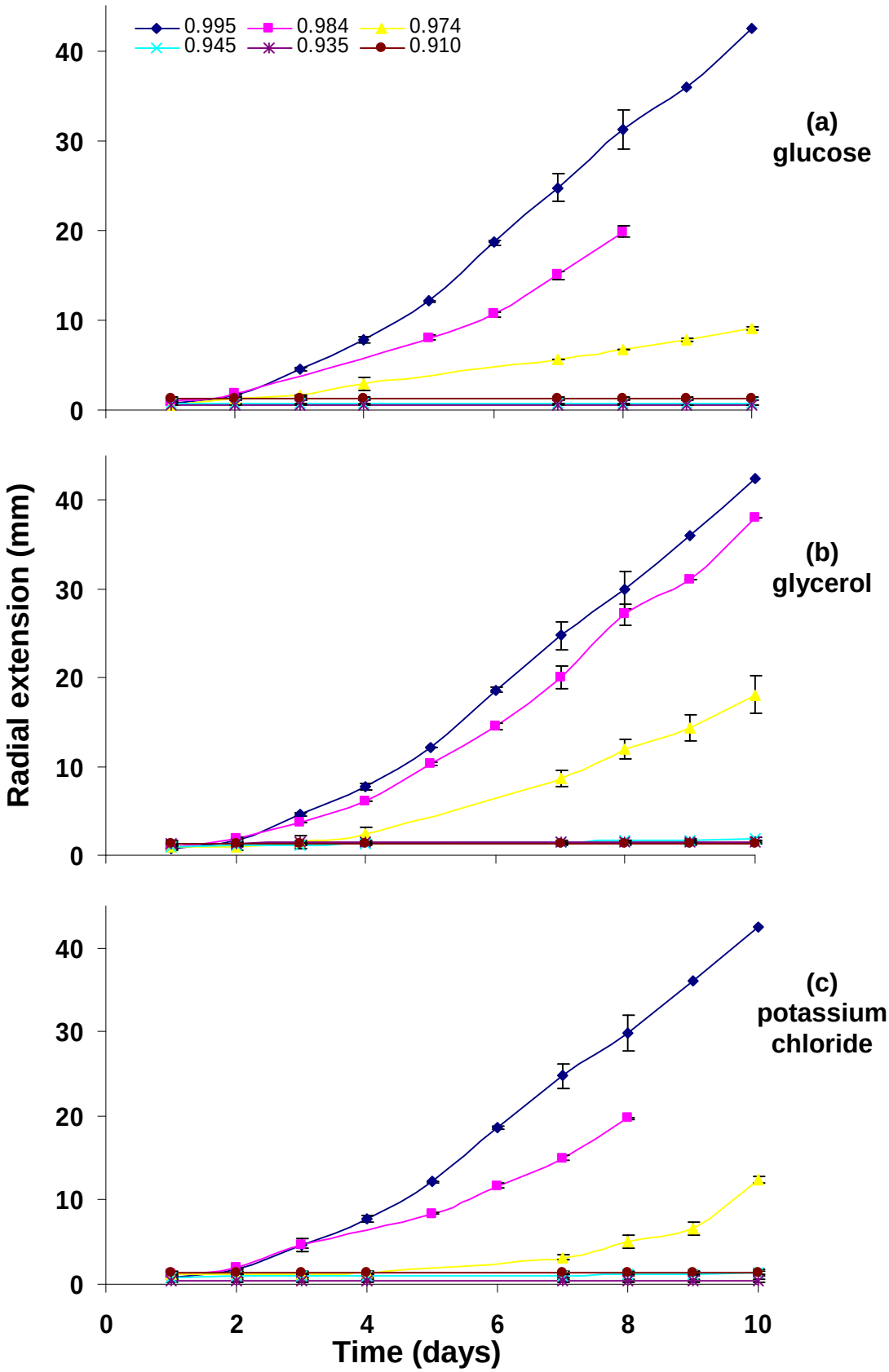

Fig. 3.3 Effects of solute on mycelial growth rates of *Heterobasidion annosum*, when grown at 25 °C on malt extract agar. Water activity (shown in key) modified with (a) glucose, (b) glycerol, (c) potassium chloride. Bars represent standard error of the means.

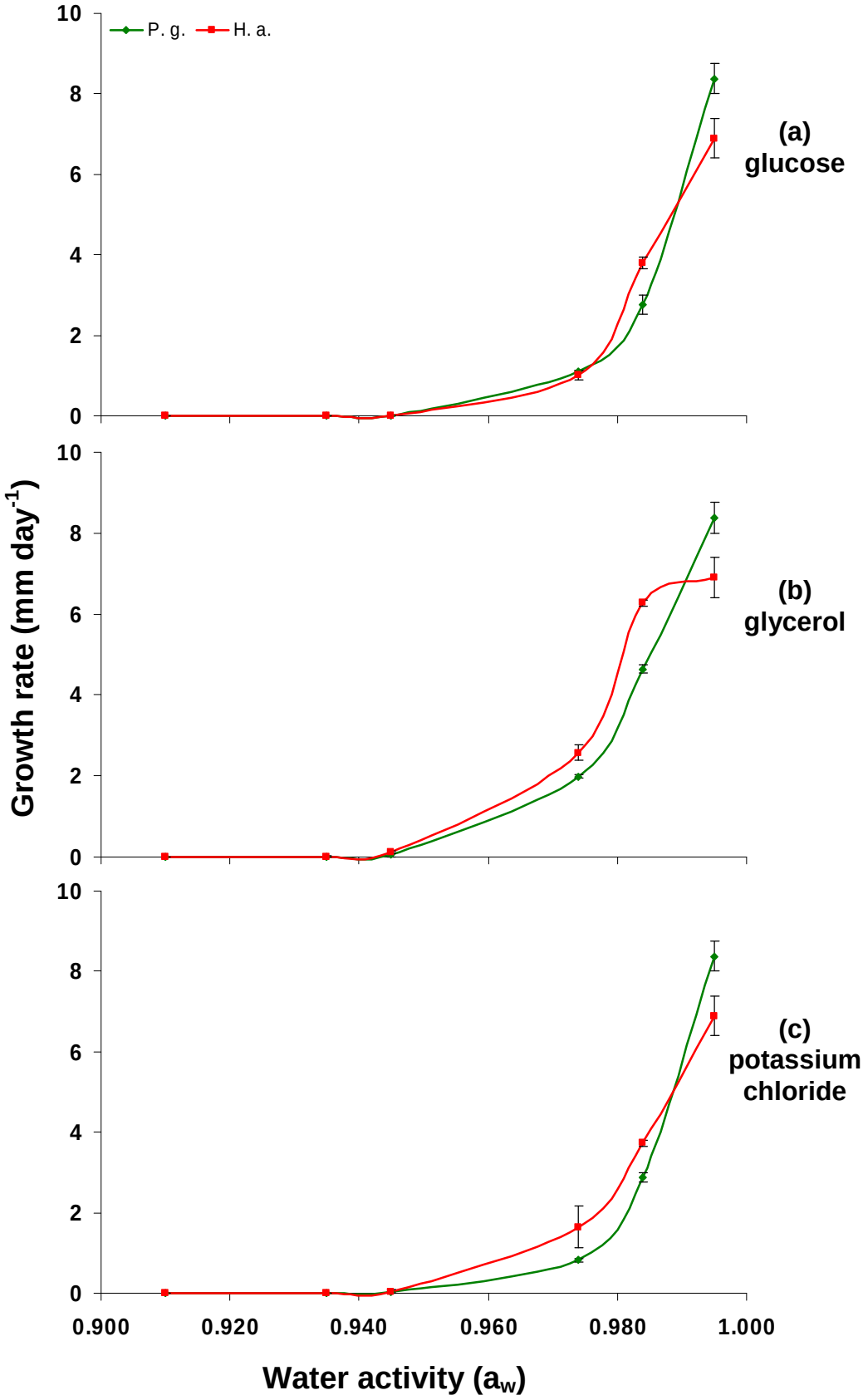

Fig. 3.4 Effect of water activity and modifier on growth rates of *Phlebiopsis gigantea* (P.g.) and *Heterobasidion annosum* (H.a.) when grown at 25 ºC on malt extract agar. Water activity modified with (a) glucose, (b) glycerol and (c) potassium chloride. Bars represent standard error of means.

3.3 Comparison of osmotic and matric stress on a range of *Phlebiopsis gigantea* and *Heterobasidion annosum* isolates

Figure 3.5 shows the effect of a_w on the growth of 8 different isolates of the antagonist, *P. gigantea,* and 3 of the pathogen, *H. annosum*. The isolates were grown at 25°C, on malt extract agar with a_w modified with glycerol (a) and (b), or supported on cellophane discs over malt extract broth with a_w modified with polyethylene glycol molecular weight 8000 (PEG 8000) (c) and (d).

With fully available water, on MEA, all the biocontrol isolates of *P. gigantea* grew faster than those of the pathogen *H. annosum*. For example, *P. gigantea* isolates grew at approximately 7-9 mm day^{-1}, while the *H. annosum* isolates grew at approximately 4-4.5 mm day^{-1}. At 0.982 a_w the *P. gigantea* isolates had a reduced growth rate of 4-6 mm day^{-1}, but the *H. annosum* isolates were relatively unaffected, and still grew at 3.5-4.5 mm day^{-1}. At 0.974 a_w, *H. annosum* isolates grew faster than the *P. gigantea* isolates, approximately 2.5-3.5 and 1-2.5 mm day^{-1} respectively. At even lower a_w the antagonist isolates were unable to grow or grew slowly when compared with the pathogen isolates. For example, LAEL.19 grew at 1.5 mm day^{-1} at 0.957 a_w. With fully available water the fastest growing antagonist was PG 21, but at lower a_w levels this was superseded by other isolates (e.g. B20/5, Rotstop, PG15, 9.12.2). The slowest growing *P. gigantea* isolate at all a_w treatments was 342.

Figure 3.5 also shows the effect of matric stress on growth of the different isolates of *P. gigantea* and *H. annosum*. Generally isolates of both the biocontrol strains and the pathogen were more sensitive to matric than solute stress. The growth rates on

unmodified media were 3-5.5 mm day^{-1} for the antagonist isolates and 2-3.5 mm day^{-1} for pathogen isolates. At 0.995 (-0.7 MPa) a_w PG 342 and Rotstop grew fastest with a growth rate of 5-5.5 mm day^{-1}. At 0.982 (-2.8 MPa) a_w PG21 was the only *P. gigantea* isolate able to grow faster than two of the *H. annosum* isolates. At 0.974 (-4 MPa) a_w none of the antagonist isolates were able to grow faster than 0.5 mm day^{-1} and the pathogen isolates were restricted to less than 1 mm day^{-1}. The pathogen isolate FERN8 was still the most tolerant of the pathogen isolates tested. ANOVA of the effect of isolate, solute and a_w group and two and three-way interactions were significant (P <0.01).

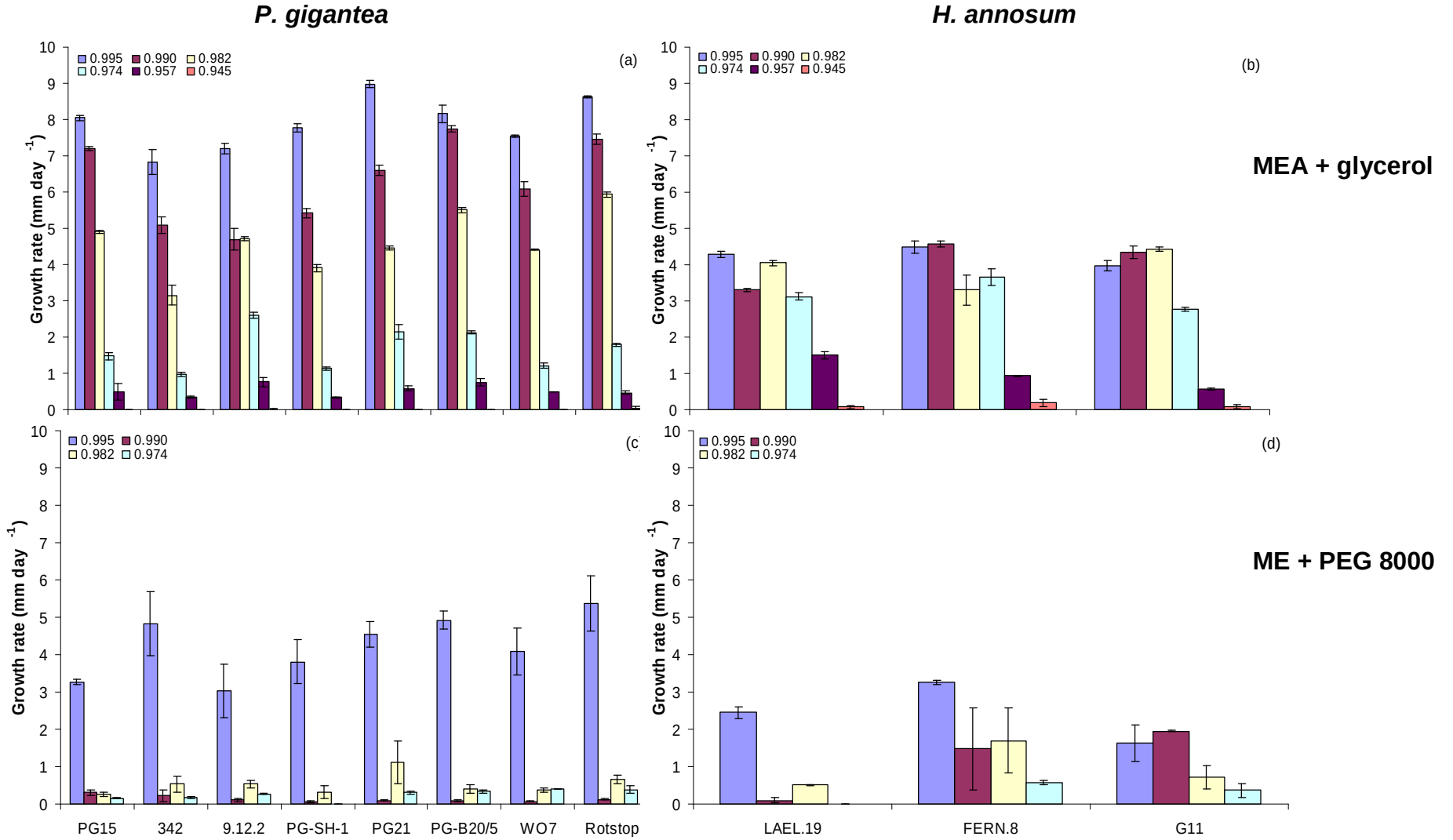

Fig. 3.5 Effect of osmotic versus matric stress on the growth rate of (a) & (c) *Phlebiopsis gigantea* and (b) & (d) *Heterobasidion annosum* isolates, grown on (a) & (b) MEA modified with glycerol and (c) & (d) malt extract broth modified with PEG 8000, at 25 ºC. Key shows water activity of medium. Bars represent standard errors of means.

3.4 Determination of colonisation of wood-based agar by *P. gigantea* and *H. annosum*

Figure 3.6 shows the effect of medium on the growth rates of (a) the antagonist *P. gigantea* (PG21) and (b) the pathogen *H. annosum* isolate (Fern8). PG21 grew fastest (>5 mm day^{-1}) on MEA, plain wood agar, wood + cellulose, wood + soy protein and wood + KNO_3; it grew at approximately 4 mm day^{-1} on plain technical agar and wood + glucose. Fern8 grew fastest on technical agar, with MEA, wood + cellulose and wood + potassium nitrate ($KNO_{3)}$ all at > 3 mm day^{-1}; on wood + soy protein and wood + glucose it grew at < 3 mm day^{-1} and was slowest growing on plain wood agar >2 mm day^{-1}. ANOVA of the effect of species and media and two-way interactions between them were significant (P <0.01)

Plates 3.2 and 3.3 compare the colonisation of wood-based agar by *P. gigantea* (PG21). This shows that the colonies grew and sporulated more rapidly when there was a readily available carbohydrate food source. On the wood agar there was good growth on areas where the wood particles were at the surface of the technical agar support medium. Growth was very sparse on both wood agars with the addition of either soy protein or KNO_3 and on plain technical agar.

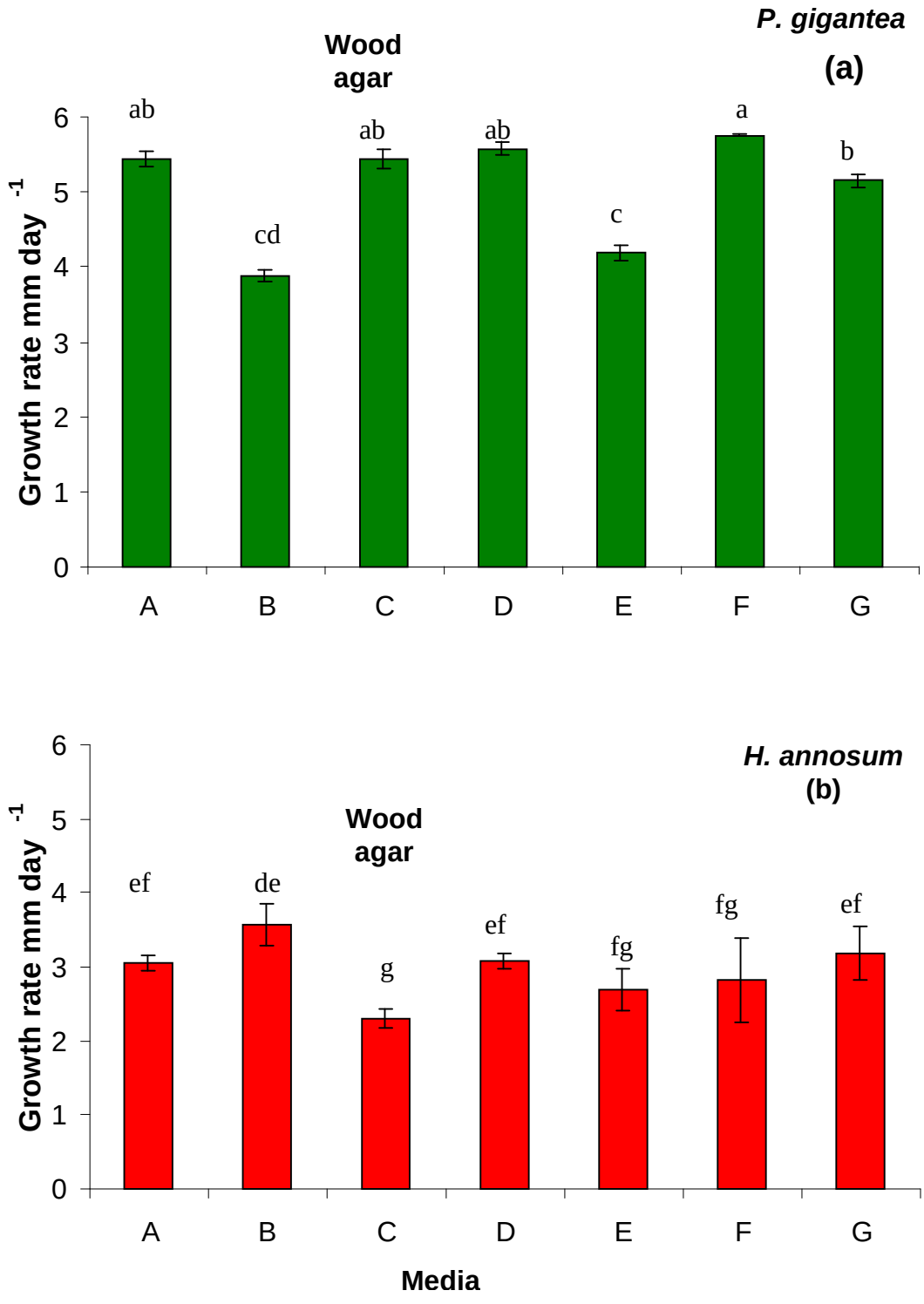

Fig. 3.6 Effect of media on growth rates of (a) *Phlebiopsis gigantea*, PG21, and (b) *Heterobasidion annosum*, FERN8.
Agars: A, malt extract plus chloramphenicol; B, technical agar no 3; C, wood; D, wood plus cellulose; E, wood plus glucose; F, wood plus soy protein; G, wood plus potassium nitrate.
Bars represent standard errors of means, different letters between two means indicate significant differences (*P* <0.05)

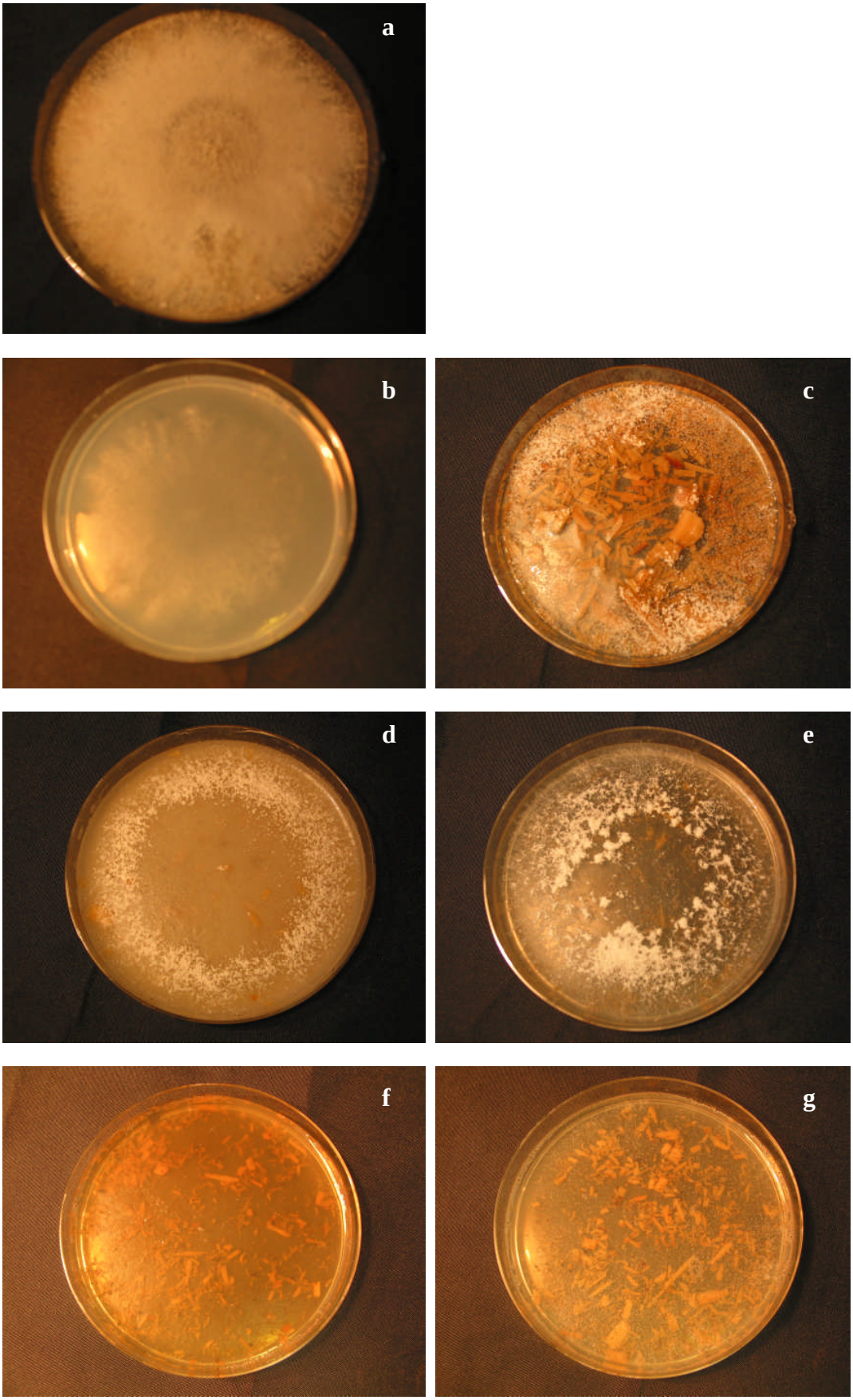

Plate 3.2 *Phlebiopsis gigantea* (PG21) colonisation of modified wood agar. a. MEA, b. Technical agar no 3, c. wood agar, d. wood + cellulose, e. wood + glucose, f. wood + soy protein, g. wood + KNO_3.

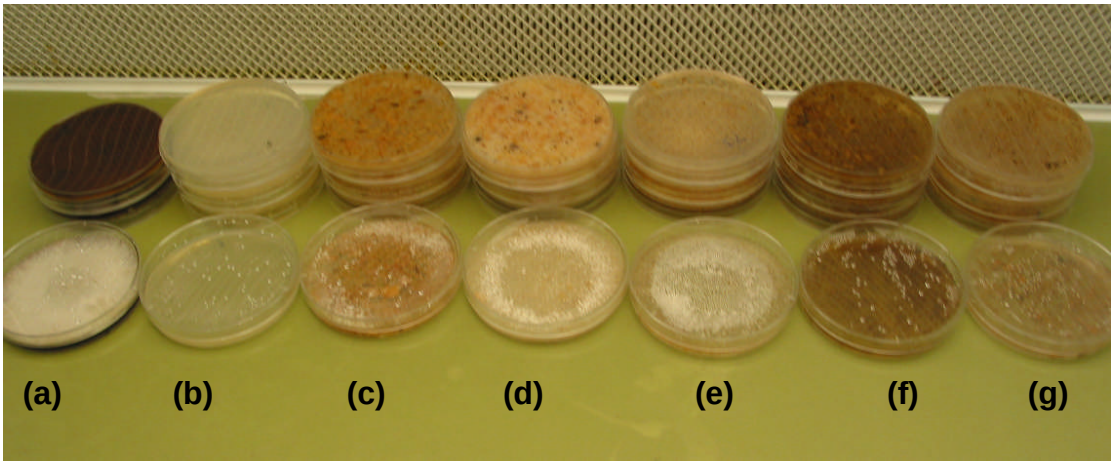

Plate 3.3 *P. gigantea* (PG21) growing from point inoculation on a range of wood agar, front row. Non-inoculated samples of agars, back row.
Agars: (a) MEA, (b) technical, (c) wood, (d) wood + cellulose, (e) wood +glucose, (f) wood + soy protein, (g) wood + KNO_3

3.5 Effect of temperature and medium on the growth rate of *Phlebiopsis gigantea* and *Heterobasidion annosum* isolates.

Fig 3.7 shows the effects of temperature and medium on the growth rates of isolates of *P. gigantea* and *H. annosum*. Both species were grown at 15, 20 and 25 ºC, on both malt extract agar and wood agar. ANOVA of the effect of isolate, medium, temperature and two and three-way interactions were significant (P <0.05).

It has been shown that isolates of both species grew fastest at 25 ºC when compared with growth at both 20 and 15 ºC. At 25 ºC, *P. gigantea* isolates grew at a rate of 6.5-8 mm day^{-1} on MEA and from 4.5-7.5 mm day^{-1} on wood agar; the *H. annosum* grew at 2-8 mm day^{-1} on MEA and 3.5-4.5 mm day^{-1} on wood agar, the range being due to variation between isolates. On MEA the *P. gigantea* isolates were not different between 15 and 20 ºC with radial extension being in the range of 4-5.5 mm day^{-1}, whereas the *H. annosum* showed a reduction in growth rate between the two temperatures, the range being 1.5-5.5 mm day^{-1} at 20 ºC and 2-3.5 mm day^{-1} at 15 ºC.

On wood agar there was a small, but significant reduction in growth rates of *P. gigantea* isolates at the lower temperature. The *P. gigantea* isolates at 20 ºC had a range of 3.5-4.5 and at 15 ºC 2.5-4.5. On wood agar there was less difference between the pathogen isolates and an insignificant difference between growth rates at 15 and 20 ºC, the range of isolates grew at a rate of 3-3.5 at 20 ºC and 2-3.5 mm day^{-1} at 15 ºC.

Antagonist isolates showed differing responses to a reduction of incubation temperature and a change of growth medium. The fastest growing antagonist isolates were PG15, PG21 and Rotstop on MEA at 25 ºC, 342, PG-SH-1 and PG21 at 20 ºC and PG15 at 15 ºC. On the wood agar WO7 was the fastest growing isolate at 25 ºC with 342 being the fastest at both 15 and 20 ºC. On MEA at all temperatures used in this study the fastest growing pathogen isolate was FERN8.

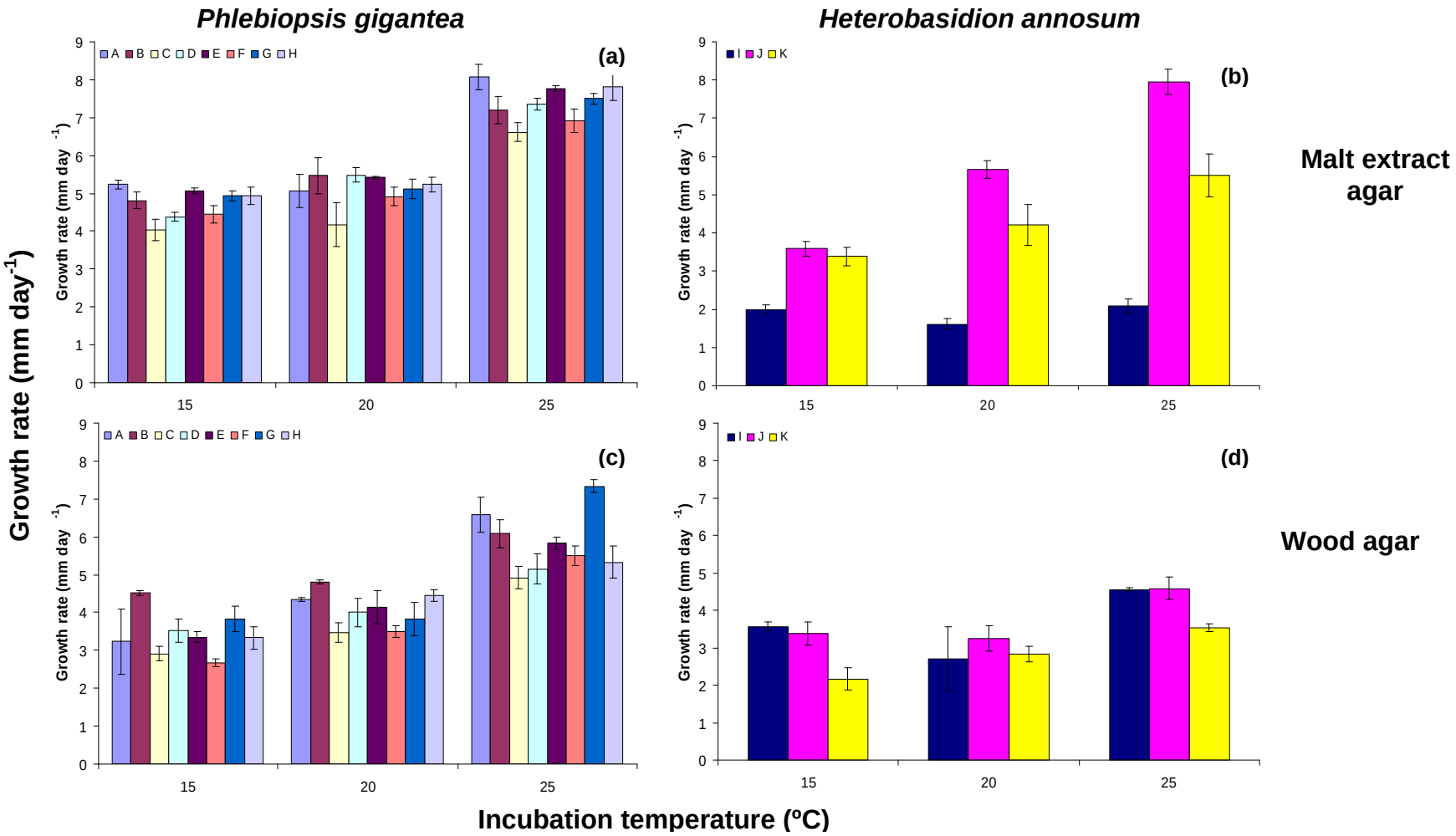

Fig 3.7 Effect of temperature and medium on the growth rate of (a) and (c) *Phlebiopsis gigantea* and (b) and (d) *Heterobasidion annosum* isolates. Cultures were grown on (a) and (b) malt extract agar and (c) and (d) wood agar at 15, 20 and 25ºC.
Key; A, PG15; B, 342; C, 9.12.2; D, PG-SH-1; E, PG21; F, PG-B20/5; G, WO7; H, Rotstop; I, LAEL.19; J, FERN8; K, G11.
Bars represent standard errors of the means.

3.6 Effect of pairing in competition on growth rates

Figure 3.8 shows the relative growth rates of three pathogen and antagonist isolates when grown in competition with each other on MEA at 15 and 25 ºC at 0.995 and 0.973 a_w. ANOVA of the effect of isolates, temperature, aw and two and three-way interactions were significant (P <0.01) except for the two-way interaction between isolate and temperature, which was not significant. At 25 ºC and 0.995 a_w all antagonist isolates grew significantly (P <0.05) more quickly than the pathogen isolates. However, when the available water was reduced to 0.973 a_w the pathogen growth rates were not significantly altered while those of the antagonist isolates were dramatically reduced to 1-1.5 mm day^{-1}. At the lower temperature all growth rates were suppressed, but this was most marked in the antagonist isolates with freely available water; their growth rates decreased to 2.5-3.5 mm day^{-1}, and under water stress to 0.5 mm day^{-1}. For the pathogen isolates at 0.995 a_w the growth rates were 1.5-3.5 mm day^{-1} and with restricted available water were 0.5-1 mm day^{-1}.

Figure 3.9 shows the effect on growth rates of one pair of isolates when grown in isolation and when paired in competition. These were grown at 25 ºC with freely available water and that restricted to 0.973 a_w. The data for individual colonies, grown in isolation, were taken from that used in figure 3.5. With freely available water there was a decrease in growth rate of *P. gigantea* from 9 mm day^{-1} when grown in isolation to 5.6 mm day^{-1} when grown in competition with the pathogen. When the water was restricted this was reduced to 2 mm day^{-1} in isolation and only 1 mm day^{-1} when grown in competition. However, the growth rate of the pathogen was not so markedly affected; with freely available water there was an initial growth rate of 4.5

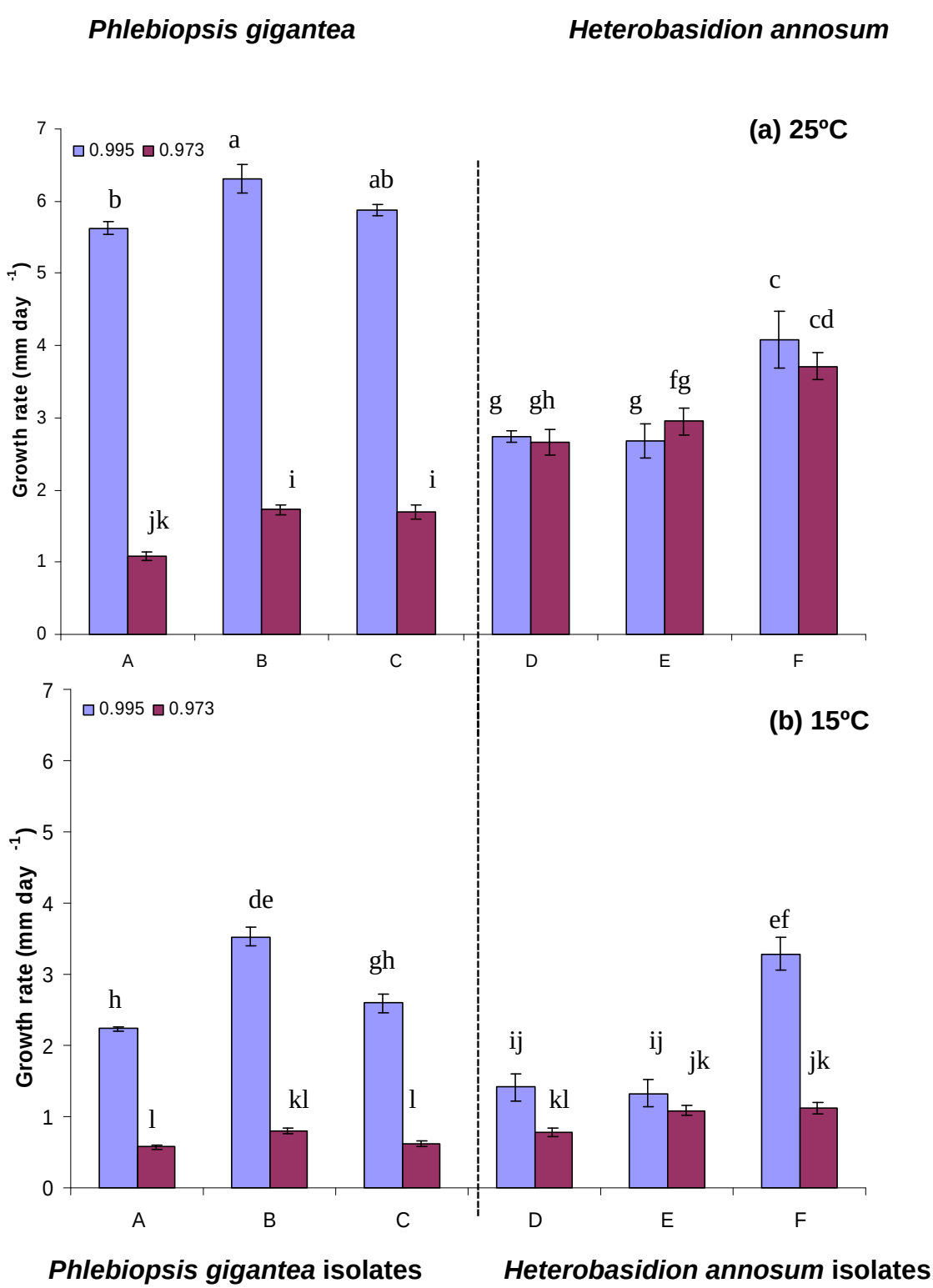

Fig. 3.8 Effect on growth rate of *Phlebiopsis gigantea* (A B &C) and *Heterobasidion annosum* (D E & F) isolates in interaction studies, at (a) 25 and (b) at 15ºC on malt extract agar at 0.995 and 0.973 a_w. Water activity modified with glycerol.
Isolates: A, PG21, B, Rotstop, C, PG-B20/5, D, G11, E, LAEL.19, F, FERN8. Bars represent standard errors of the mean, different letters indicate significant differences (*P* <0.05) between the means.

mm day^{-1} when grown in isolation, it was increased to over 5mm day^{-1} when grown in competition. When available water was lowered to 0.973 a_w the growth rate was unaffected between being grown alone or in competition.

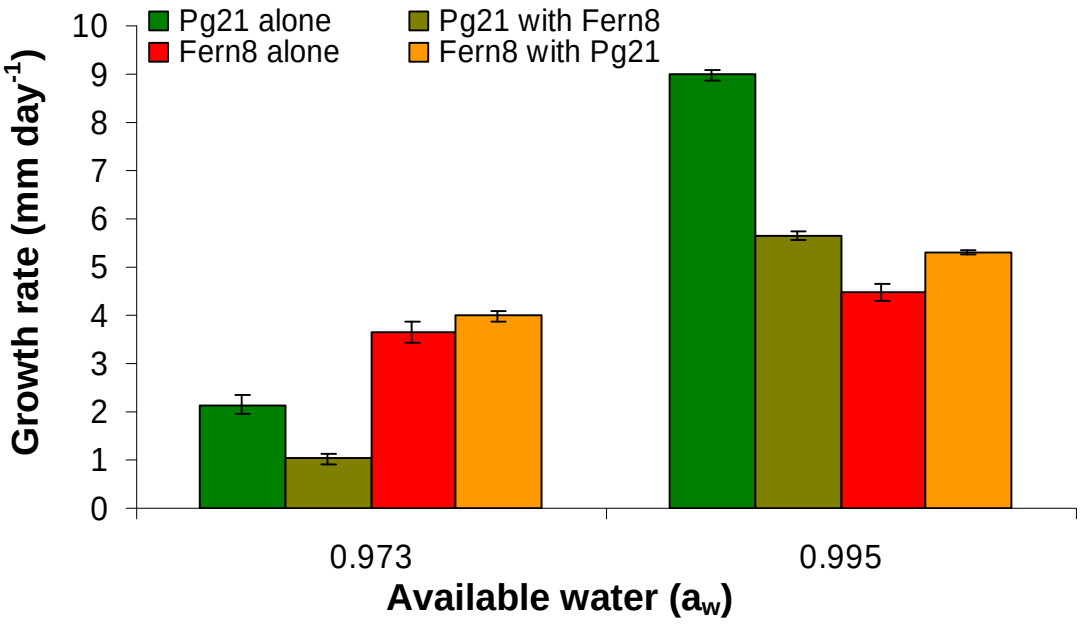

Fig. 3.9 Effect on growth rate of interactions between *Phlebiopsis gigantea* (PG21) and *Heterobasidion annosum* (FERN8), in comparison with growth rates from single colonies. Grown on malt extract agar at 25 ºC, at 0.995 and 0.973 a_w. Water activity modified with glycerol.
Bars represent standard errors of the mean.

3.7 Interactions between *P. gigantea* and *H. annosum*

3.7.1 Initial screening for interactions: *in vitro*

Plate 3.4 shows the effect of screening the antagonist *P. gigantea* isolates in vitro for their effectiveness against 3 isolates of the pathogen *H. annosum* when grown together on MEA at 25 ºC for 7 days. From the central inoculation point the antagonist has grown out towards the pathogen, the 3 points of inoculation being visible in the photographs. In plate 3.4(a) antagonist 342 can be observed to have vigorously interacted in the zones where the species met. The 3 associated photomicrographs, taken through the ocular of a dissection microscope, after 8 days incubation, are of the interaction zones with each pathogen isolate. (b) shows the antagonist isolate PG-SH-1, a less vigorous isolate, with its associated interaction zones. In all cases the *P. gigantea* growth margin was curved or concave, prior to contact with *H. annosum* colonies. Growth was denser once *P. gigantea* was growing on pre-colonised agar. Many droplets of moisture were visible on *P. gigantea* mycelia. By day 9: Four *P. gigantea* isolates had grown over the inoculation point of FERN.8. The most competitive *P. gigantea* isolates at this stage were W07, Rotstop, PG21 and PG - B20/5. The latter had made the furthest advance over the *H. annosum* isolates. The densest growth in an interaction zone was that of PG21. Table 3.1 shows a semi-quantitative measure of these interactions, after 17 days of incubation, under these conditions the PG-B20/5 isolate was the most competitive biocontrol agent and 9.12.2 and PG-SH-1 were the least competitive.

Table 3.1 Comparison of effectiveness of isolates of the biocontrol agent *Phlebiopsis gigantea* to control the pathogen *Heterobasidion annosum* (isolates LAEL.19, FERN8, G11) The scoring is semi-quantitative.

***Phlebiopsis gigantea* isolate**	**Dominance over *Heterobasidion annosum* isolates**
PG15	++
342	++
9.12.2	+
PG-SH-1	+
PG21	++
PG-B20/5	+++
WO7	++
Rotstop	++

3.7.2 Photomicrography of hyphae

Plate 3.5 shows the development of the pathogen *H. annosum*, and the antagonist *P. gigantea*, incubated at 25°C on MEA for up to a month. The photomicrographs show progressive development of *H. annosum*, the thin shiny hyphae at 13 days, immature conidiophores at 3 weeks and mature conidiophores surrounded with attached conidia at 4 weeks of incubation. *P. gigantea* is already showing signs of maturation at 10 days of incubation with aerial hyphae above the mycelium separating into oidia. By 3 weeks incubation time there were many more oidia developing and at 4 weeks many mature oidia had separated from the parent hyphae. Magnification was 400x on an Olympus BH-2 microscope using an Olympus camera body.

Plate 3.6 shows the effect of interactions between the antagonist *P. gigantea* 342 and the mycelia of the pathogen *H. annosum* (a) FERN8, (b) LAEL.19. Orange/red stained hyphae indicated that membrane integrity was compromised, allowing the entry of methyl red stain, while intact hyphae remained unstained. In 3.6 (a) a stained conidiophore of the pathogen, marked C, can be clearly seen.

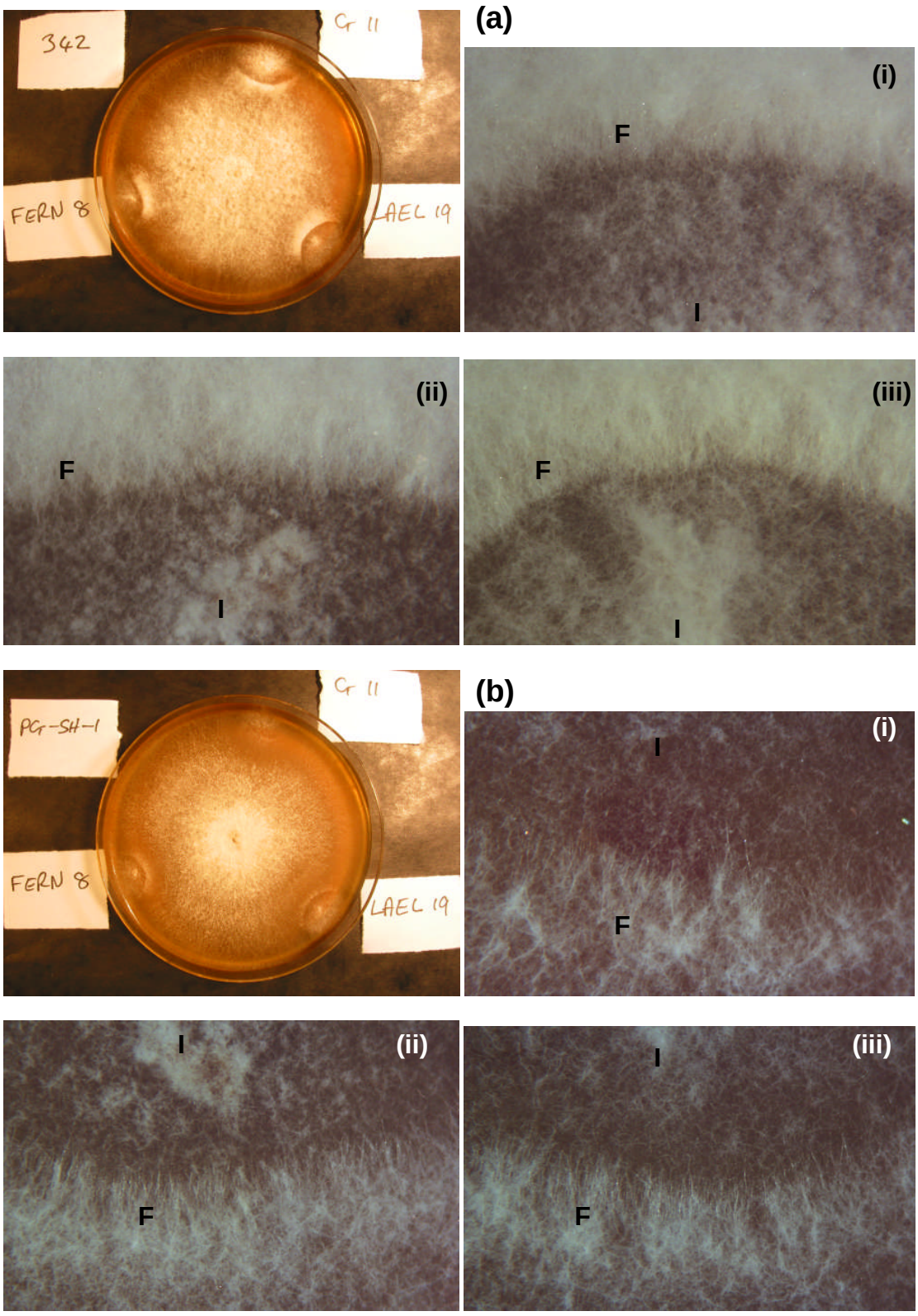

Plate 3.4 Screening of the antagonist *Phlebiopsis gigantea* isolates against a panel of the pathogen *Heterobasidion annosum*. (a) 342 and (b) PG-SH-1 with associated interaction zones, (i) G11, (ii) FERN8, (iii) Lael.19. Points I are the inoculation points of the pathogen, F are the advancing fronts of the antagonist meeting and growing over the area colonised by the pathogen. MEA at 25ºC. Photomicrograph; camera Olympus, using dissection microscope

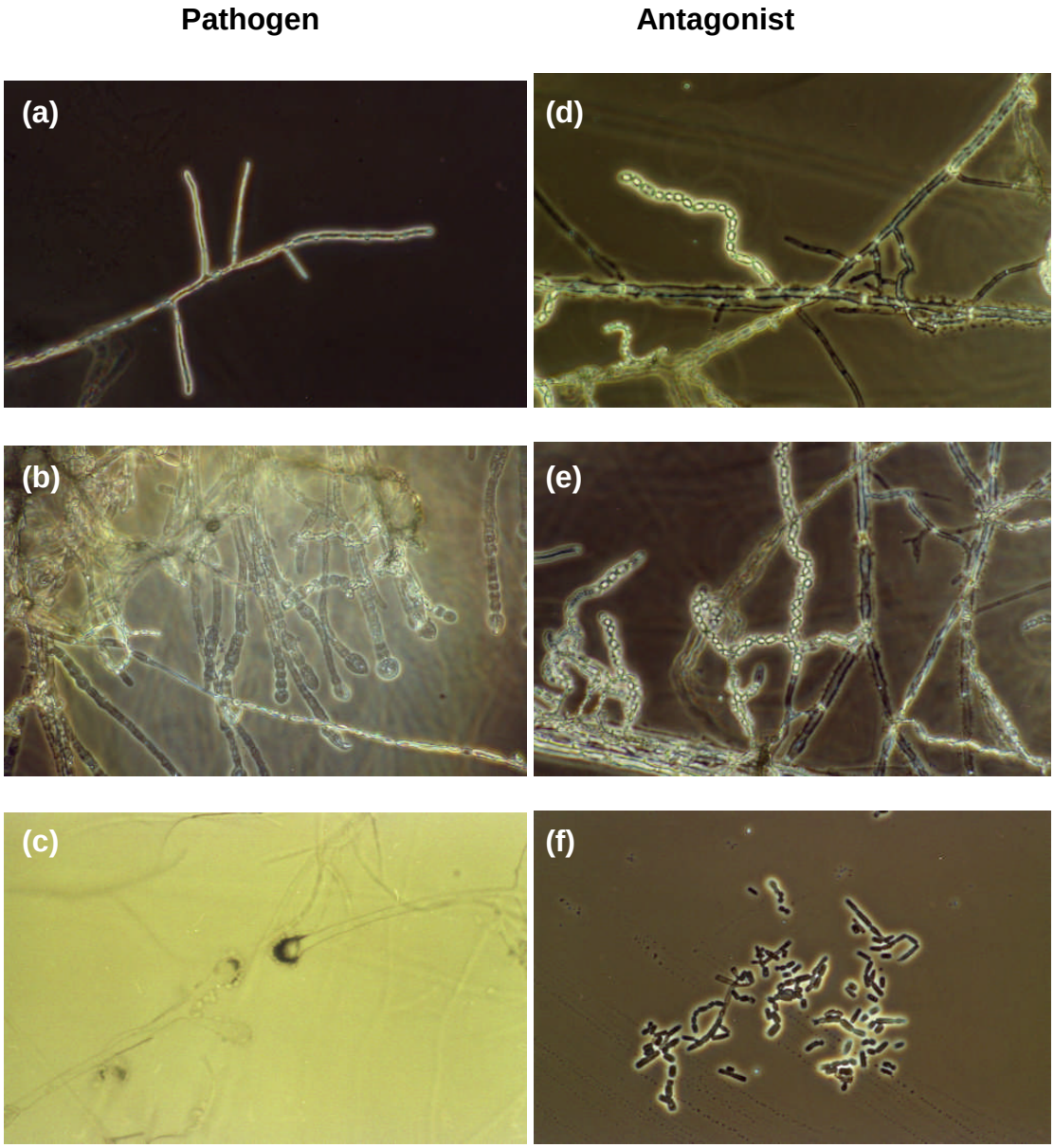

Plate 3.5 Photomicrographs of *H. annosum* (a-c) and *P. gigantea* (d-f). Incubated at 25°C on MEA, incubation time: <2 weeks (a) and (d), 3 weeks (b) and (e), 4 weeks (c) and (f). Magnification 400x on an Olympus BH-2 microscope using an Olympus camera body.
The photomicrographs show the thin shiny hyphae of *H. annosum* at 13 days (a), in (b) the development towards conidiophores at 3 weeks and (c) mature conidiophores surrounded with attached conidia at 4 weeks of incubation. *P. gigantea* is already showing signs of maturation at 10 days of incubation (d), with aerial hyphae above the mycelium separating into oidia. By 3 weeks incubation time (e), there were many more oidia developing and at 4 weeks (f) many mature oidia had separated from the parent hyphae.

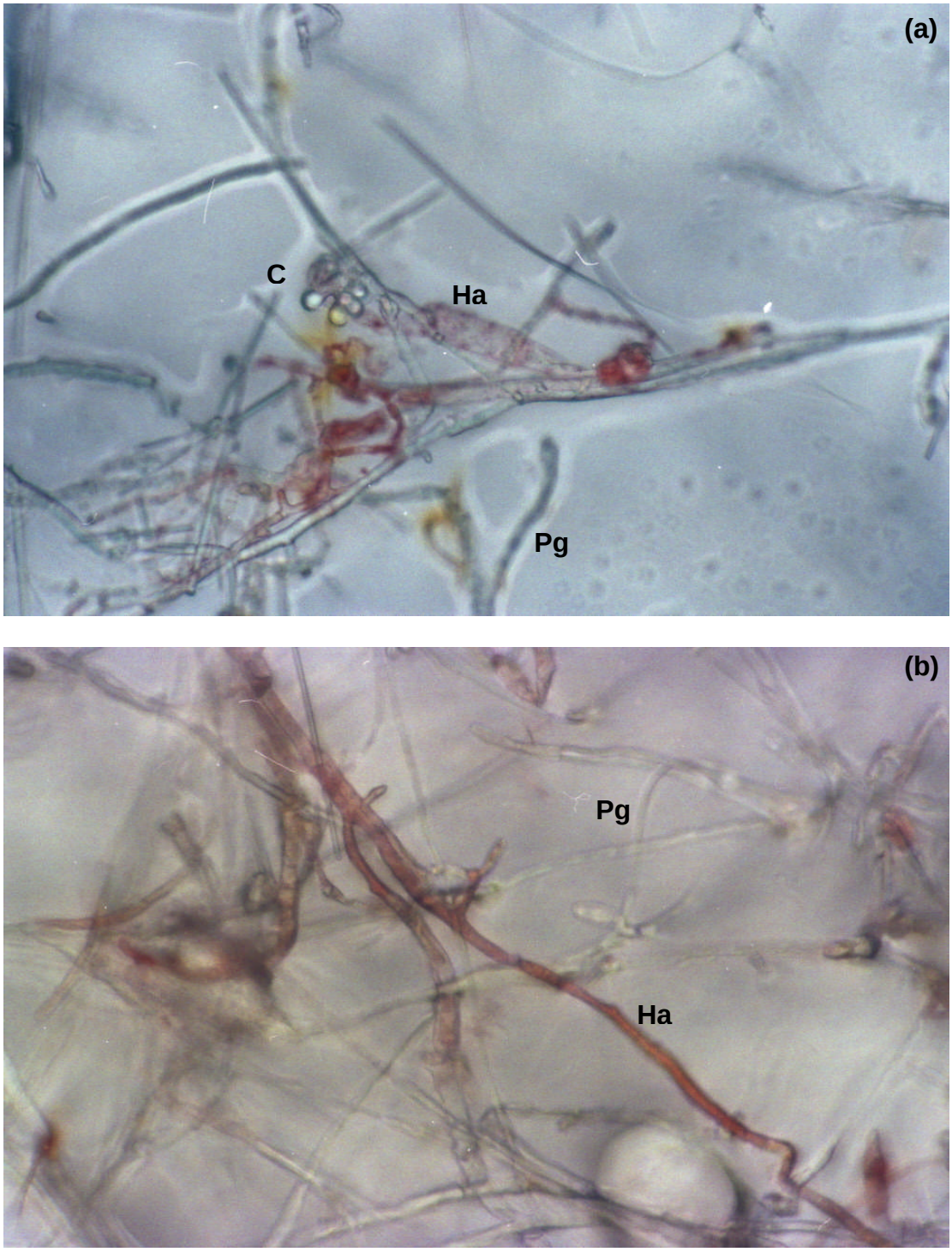

Plate 3.6 Interactions between the antagonist *Phlebiopsis gigantea* PG 342 and the pathogen *Heterobasidion annosum*. (a) FERN.8, (b) LAEL.19. Damaged hyphae are stained orange/red with methyl orange. Antagonist hyphae labelled Pg, pathogen hyphae Ha, pathogen conidiophore C. Photomicrograph: camera Olympus, microscope Olympus BH-2 at magnification 400x.

3.7.3 Quantification of interactions with an Index of Dominance (I_D)

Table 3.2 shows the effect of environmental conditions (15, 25°C and 0.995, 0.973 a_w) on interactions between *P. gigantea* (PG21, Rotstop, PG-B20/5) and *H. annosum* (G11, LAEL.19, FERN8) examined. Numerical values, based on the system developed by Magan and Lacey (1984) were given to each interaction. Mutual intermingling 1:1, mutual antagonism on contact 2:2, mutual inhibition at a distance 3:3, inhibition of one organism on contact, the inhibitor species continues to grow unchanged or at a reduced rate through the inhibited colony 4:0, inhibition of one organism at a distance, the inhibitor species then continuing to grow through the resulting clear zone and the inhibited colony, perhaps at a reduced rate 5:0, (the 4 and 5 being given to the dominant species, 0 to the dominated). The numerical scores were totalled as an Index of Dominance (I_D) that was used as an overall measure of competitiveness. The I_D scores were totalled for each a_w and temperature to give a total I_D. Out of the biocontrol strains tested, PG21 and Rotstop each had a total I_D of 28, while PG-B20/5 was slightly less competitive with a score of 22. For the pathogen strains the I_D scores were 12, 20 and 34 for G11, LAEL.19 and FERN8 respectively. Under the environmental conditions tested, the pathogen strain FERN8 was the most competitive of the fungi observed.

Table 3.2 Interactions between bio-control agent *Phlebiopsis gigantea* (PG21, Rotstop, PG-B20/5) and pathogen *Heterobasidion annosum* (G11, LAEL.19, FERN8) on MEA at 0.973 and 0.995 a_w at 15°C and 25°C. I_D scores are applied to biocontrol agent (left) and pathogen (right).

a_w		Species	Temperature		I_D
			15°C	25°C	
0.995		**PG21:G11**	4:0	4:0	**8:0**
		PG21:LAEL.19	4:0	4:0	**8:0**
		PG21:FERN8	4:0	2:2	**6:2**
	Total I_D		**12:0**	**10:2**	**22:2**
		Rotstop:G11	4:0	4:0	**8:0**
		Rotstop:LAEL.19	4:0	4:0	**8:0**
		Rotstop:FERN8	2:2	4:0	**6:2**
	Total I_D		**10:2**	**12:0**	**22:2**
		PG-B20/5:G11	4:0	4:0	**8:0**
		PG-B20/5:LAEL.19	4:0	2:2	**6:2**
		PG-B20/5:FERN8	0:4	2:2	**2:6**
	Total I_D		**8:4**	**8:4**	**16:8**
0.973		**PG21:G11**	2:2	2:2	**4:4**
		PG21:LAEL.19	0:4	2:2	**2:6**
		PG21:FERN8	0:4	0:4	**0:8**
	Total I_D		**2:10**	**4:8**	**6:18**
		Rotstop:G11	2:2	2:2	**4:4**
		Rotstop:LAEL.19	0:4	2:2	**2:6**
		Rotstop:FERN8	0:4	0:4	**0:8**
	Total I_D		**2:10**	**4:8**	**6:18**
		PG-B20/5:G11	2:2	2:2	**4:4**
		PG-B20/5:LAEL.19	0:4	2:2	**2:6**
		PG-B20/5:FERN8	0:4	0:4	**0:8**
	Total I_D		**2:10**	**4:8**	**6:18**

Species isolate	Total I_D
PG21	**28**
Rotstop	**28**
PG-B20/5	**12**
G11	**12**
LAEL.19	**18**
FERN8	**34**

Figure 3.10, compiled from the data in Table 3.2, shows the effects of a_w and temperature on overall I_D of *P. gigantea* and *H. annosum* isolates. Water availability was shown to have a greater influence than temperature on dominance during competition. When water was freely available the antagonist *P. gigantea* was able to control growth of the *H. annosum* isolates. Interestingly, when the available water was restricted to 0.973 a_w the dominance was reversed and pathogen isolates *(H. annosum)* were able colonise the *P. gigantea* colonies.

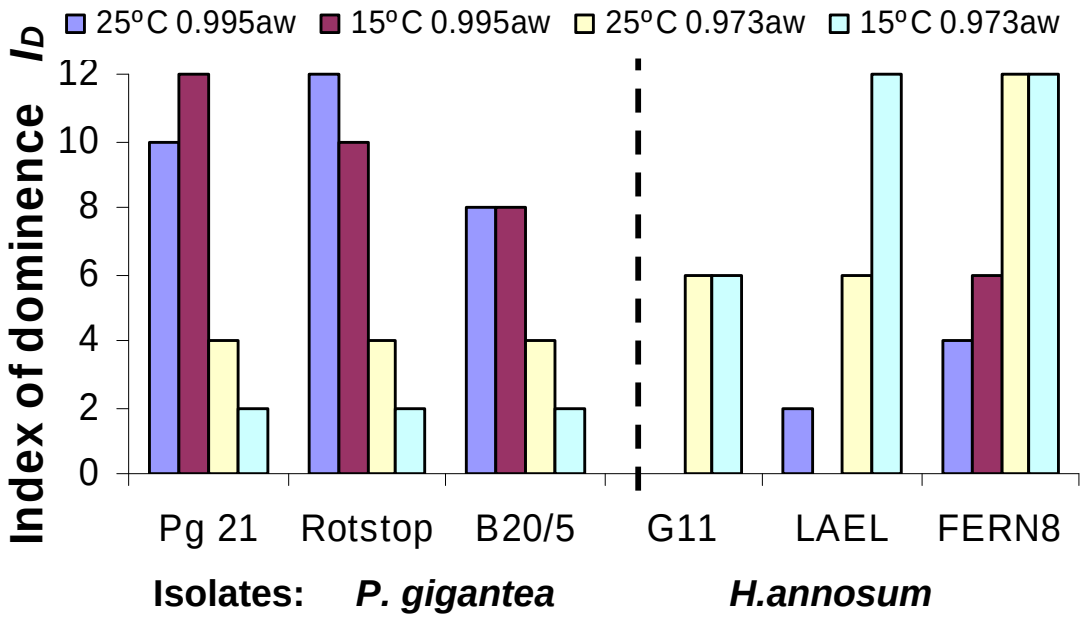

Figure 3.10 Interactions between the biocontrol agent *Phlebiopsis gigantea* (PG21, Rotstop, PG-B20/5) and the pathogen *Heterobasidion annosum* (G11, LAEL.19, FERN8) when grown on MEA at 15 and 25°C at 0.973 and 0.995 a_w. Index of dominance (I_D) is total for each isolate under specified experimental condition.

Plate 3.7 shows examples of interactions of *P. gigantea* paired with *H. annosum* under different environmental conditions. Plate 3.7 (a) is of incubation at 25°C and (b) at 15°C. It can be seen that all isolates grew more quickly at 25 ºC than at 15 ºC. In i – iv both the antagonist isolates PG21 and ROTSTOP can be seen to have grown more vigorously than each of the pathogen isolates, FERN8 and G11, and to have been able to overgrow the pathogen. With available water restricted to 0.973 a_w the antagonist isolates in v and vi can be seen to have been severely restricted in their growth and close inspection of the photographs shows that the pathogen isolates have grown over the margins of the antagonist mycelia. Nine days later, 19 days after incubation, the pathogen isolate, FERN8, had completely surrounded the antagonist isolates and G11 was growing over the colony margins. At the lower temperature, at 0.995 a_w, ix, x and xi, the pathogen is seen to be dominated by PG21, co-mingle with ROTSTOP and to be challenging PG-B20/5 respectively. At 15°C, 0.973 a_w, xii –xiv, the antagonist isolates were severely limited in growth and can be seen to have only just extended beyond the inoculation points, whereas the pathogen has made clearly visible growth. Thus in wet (0.995 a_w) and warm (25 ºC) conditions, the *P. gigantea* isolates controlled *H. annosum* isolates, but as can be seen in Plate 3.7, under drier conditions (0.973 a_w) the pathogen *H. annosum* grew more strongly than the antagonist.

Plate 3.7 Interactions between *P. gigantea* and *H. annosum*, under different environmental conditions.

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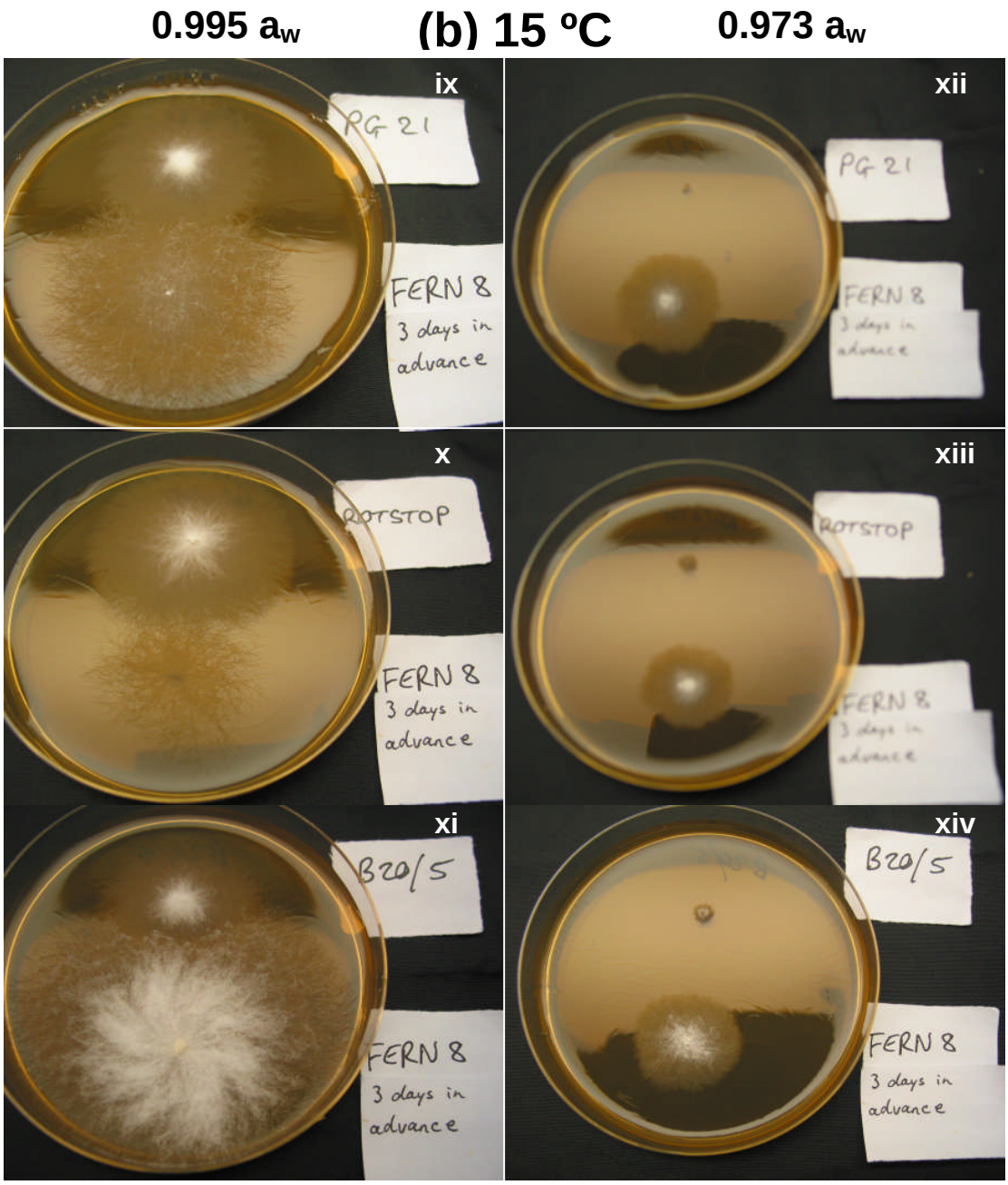

Plate 3.7 Continued from previous page.

Interactions between *P. gigantea* and *H. annosum* under different environmental conditions, incubation at (a) 25 ºC and (b) 15 ºC. i – iv and ix – xi at 0.995 a_w, v – viii and xii – xiv at 0.973 a_w. All photographs were taken at 10 days of incubation except vii and viii taken at 19 days. Each Petri dish shows the inoculation point of the antagonist centre top and that of the pathogen centre bottom. Labels within photographs show each isolate name and if the pathogen was inoculated simultaneously with the antagonist or 3 days in advance.

3.7.4 Interactions *in situ* on wood discs

Figure 3.11 shows the relationship between the growth areas of *P. gigantea* and *H. annosum* when grown in competition on 1 cm thick wood discs, in humid conditions, at three temperatures. The discs were marked into halves, half given a treatment of *P. gigantea* spores, and then the whole disc over-sprayed with spores of the pathogen.

When grown at 25 ºC the antagonist covered from 90 – 100% of the disc halves, both treated and untreated, whichever of the isolates were used and on both pine and spruce. It thus outgrew the pathogen. *H. annosum* was not visible, when viewed through a dissection microscope, on spruce when grown against Rotstop and PG-B20/5 and was restricted to approximately 5% of available space when grown in competition with PG21. On pine the pathogen was not visible on treated areas, but on the untreated it covered from 2 – 10%.

At 15 ºC the antagonist was still dominant with all combinations of isolates and wood, but there were variations between the isolates in the area they visibly covered and this was shown later by the appearance of brown stain. PG-B20/5 completely dominated the area of treatment on both woods, preventing the growth of the pathogen; on the untreated areas it covered 96% allowing the pathogen to grow on only 3% of the pine. However, on spruce it visibly grew on 60% and stained 95% of the surface, allowing the pathogen to occupy up to 15%. Rotstop was able to prevent the growth of the pathogen on treated areas, but on the untreated regions the pathogen was visible on 28% of the pine and 14% of the spruce. PG21 grew to a greater extent on pine, 95% treated and 68% untreated areas; on spruce, 75% treated, 60% on untreated areas.

However, on untreated areas the pathogen was able to colonise 17% of the former and 7% of the latter.

When 4 ºC was the incubation temperature the pathogen was able to dominate some isolates of the antagonist. Again, PG-B20/5 was able to colonise the greatest area of the three *P. gigantea* isolates tested; it dominated the area of treatment on both woods, almost preventing the growth of the pathogen, although the latter was able to colonise 2% of the treated half of spruce. However, on the untreated areas both PG-B20/5 and the pathogen grew on 47% of the pine, but on the spruce the pathogen visibly grew on 37% and the antagonist only 31% of the surface. On pine Rotstop covered 30% on treated but was visible on only 2% of the untreated areas, allowing growth of the pathogen to 18% and 57% respectively. On spruce the situation was similar in effect, Rotstop colonised 59% and 7%, the pathogen 24% and 54%. The growth of PG21 is quite slow at 4 ºC, on pine it was visible on 12% of treated areas, but absent from untreated areas; it grew better on spruce, 36% on treated and 6% on untreated areas, allowing the pathogen to occupy up to 11% of the former and 34% of the latter.

Plate 3.8 shows *P. gigantea* isolate PG-B20/5 grown in competition with three *H. annosum* isolates on discs of *Pinus sylvestris* and *Picea sitchensis.* Incubated in chambers with freely available water at 25 ºC for 2 weeks and 4 ºC for 5 weeks. Growth of *P. gigantea* was heaviest when incubated at 25 ºC on pine. When grown at 4 ºC the growth was mainly restricted to the treated half of each disc, and some weed species have become apparent.

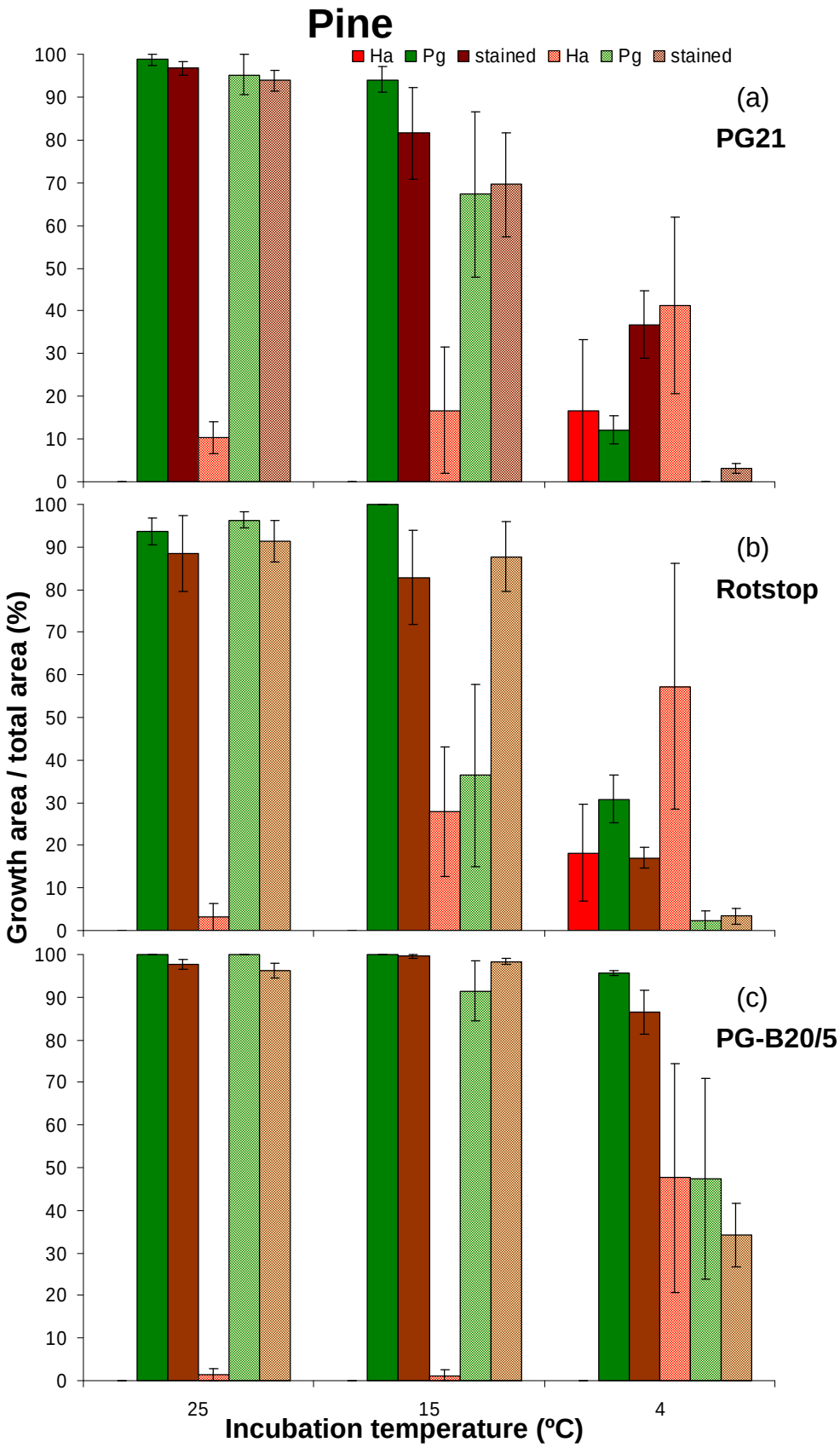

Fig. 3.11 Area of colonisation during interactions between *P. gigantea* isolates, PG21 (a) and (d), Rotstop (b) and (e), PG-B20/5 (c) and (f) and *H. annosum* on Pine (a) to (c) and Spruce (d) to (f) discs, incubated at 25, 15 and 4 ºC with water
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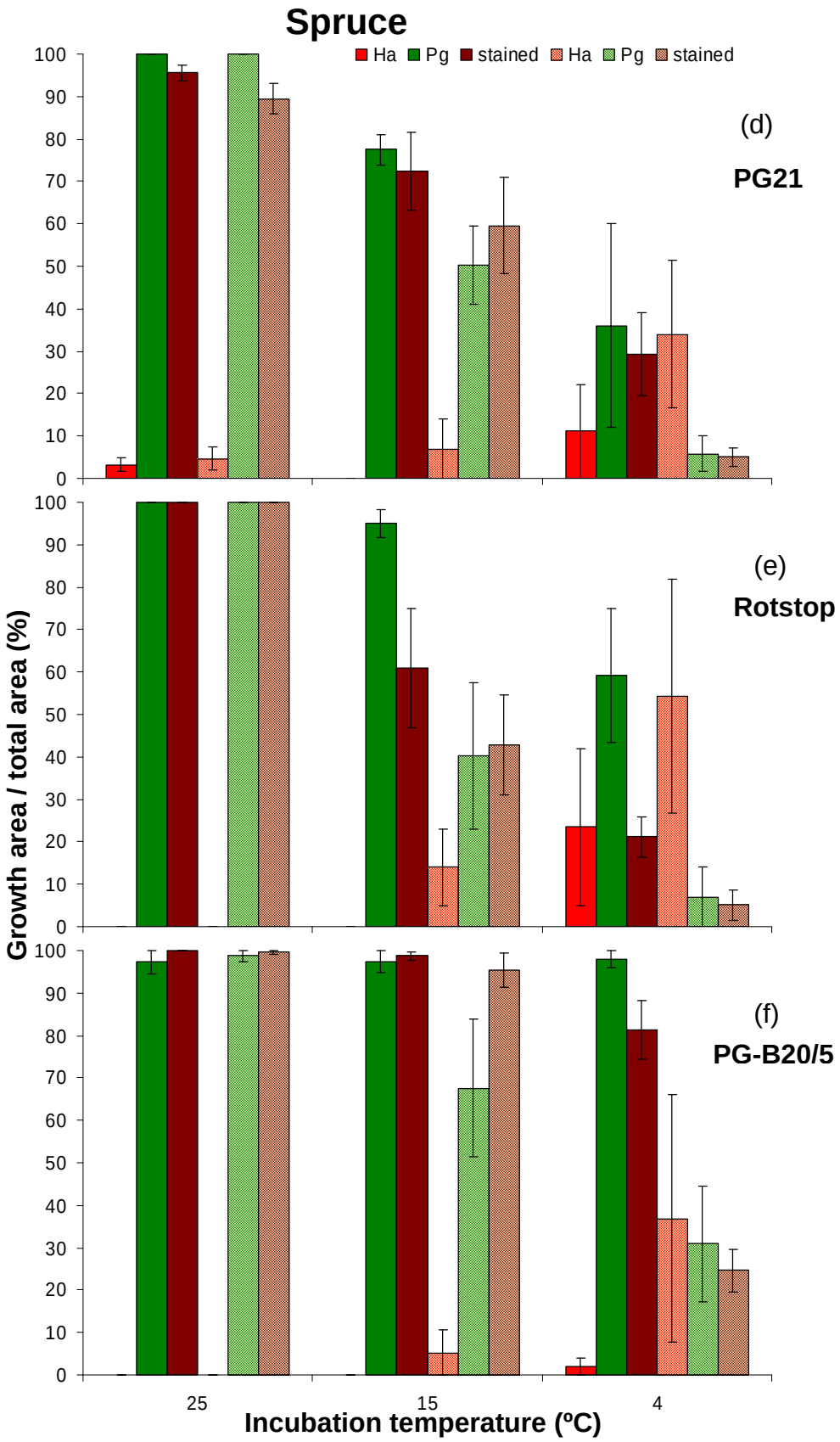


Figure 3.11 Continued from previous page.

freely available. Solid bars show area of colonisation on treated zones and patterned bars that on untreated zones. Error bars represent standard errors.

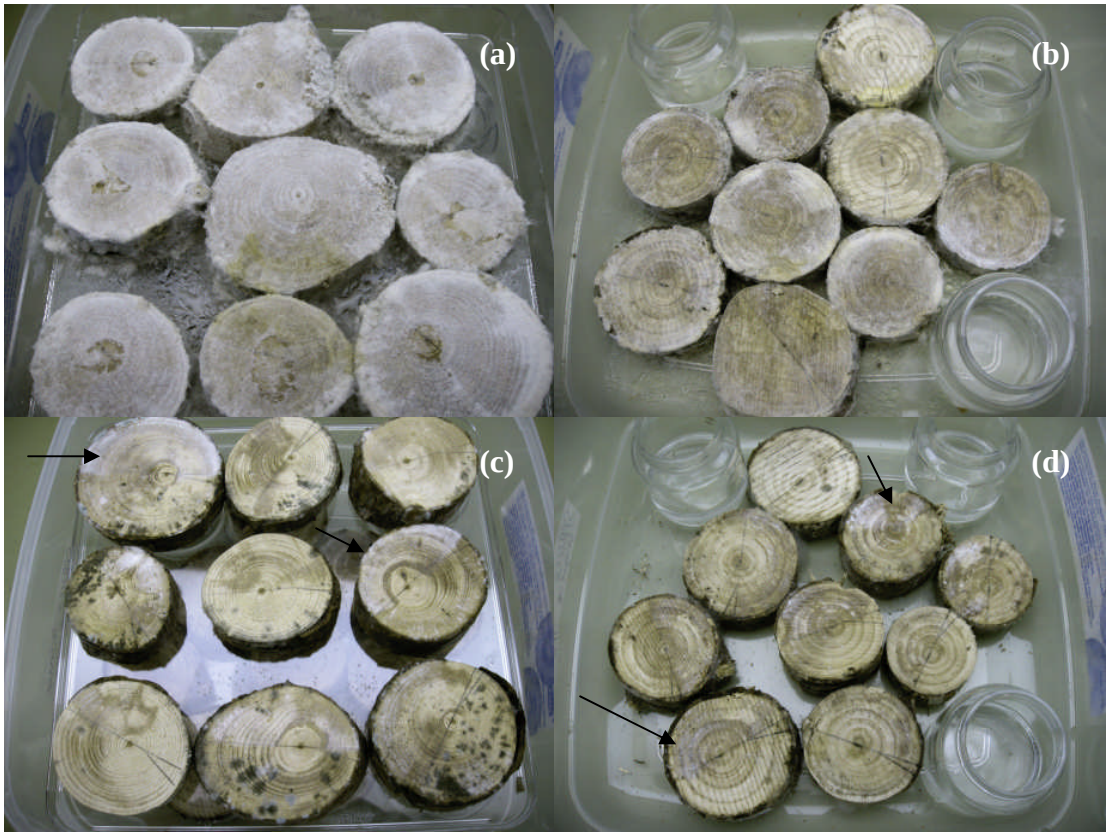


Plate 3.8 *Phlebiopsis gigantea* isolate PG-B20/5 grown in competition with three *Heterobasidion annosum* isolates on discs of *Pinus sylvestris* (a) and (c) and *Picea sitchensis* (b) and (d) with freely available water at 25 ºC (a) and (b) for 2 weeks and 4 ºC (c) and (d) for 5 weeks. Arrows indicate areas of PG-B20/5 growth on treated halves of discs grown at 4 ºC.

Chapter 4

Results: production of *Phlebiopsis gigantea* inoculum

4.1 Production of *Phlebiopsis gigantea* spores: semi-solid media

4.1.1 Simulated trial of existing production methods of PG suspension

Table 4.1 shows the concentration of *P. gigantea* (PG21) spores (oidia) produced for each simulated production trial, when grown on malt extract agar (MEA) at 25ºC for 2 weeks, with the results of viability testing on the spores produced in trial 3. As can be seen from the result of the viability test the number of colony forming units is a factor of 10 lower than the count of spores in the bulk suspension syrup. The result shows that this particular spore suspension would not have been acceptable as a commercial product. Production of spores from growth medium (MEA) was 5.65 $\log_{10}$ CFU g^{-1} media.

Table 4.1 *Phlebiopsis gigantea* spores (oidia) produced from simulation of commercial production

	Spores in primary sugar solution ($\log_{10}$ ml^{-1})	**Spores in bulk suspension syrup ($\log_{10}$ ml^{-1})**	**Colony forming units at 0.995 a_w ($\log_{10}$ ml^{-1})**
Trial 1	8.23	Not tested	Not tested
Trial 2	8.11	7.18	Not tested
Trial 3	8.19	6.86	5

4.1.2 Temporal study: effects of culture age on *P. gigantea* (PG21) spore production on semi-solid culture media

Figure 4.1 shows the yield of *P. gigantea* (PG21) spores from colonies harvested from MEA plates grown at 25°C for 5-25 days. The number of spores harvestable per

colony increased rapidly from 6.2 $\log_{10}$ at five days, peaking at 8.2 $\log_{10}$ on day 13, before slowly declining to 7.7 $\log_{10}$ on day twenty and 6.9 $\log_{10}$ on day 25. The peak production of spores on day 13 equated to 6.9 $\log_{10}$ spores g^{-1} MEA.

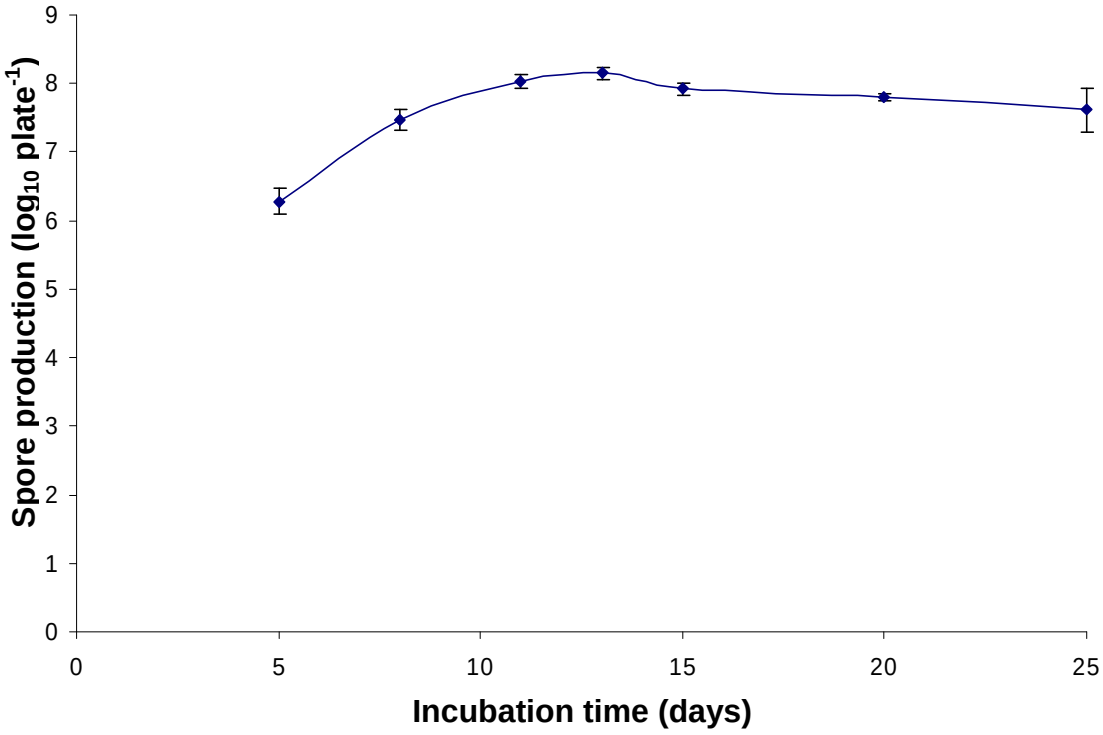


Fig. 4.1 yield of *P. gigantea* (PG21) spores from malt extract agar (MEA) incubated at 25 ºC for 5 - 25 days. Bars represent standard errors of the mean.

4.2 Production of *Phlebiopsis gigantea* spores: fermentation

4.2.1 Investigation of effects of inoculum size

Table 4.2 shows the effect of inoculum size on the production of spores (submerged conidia) and the mycelial pellet size in a malt extract (ME) liquid medium. Within this range, the inoculum size did not have much effect on the level of spore

production, but there was a correlation between inoculum and pellet size; the largest inoculum giving smaller pellets and the lowest inoculum producing the largest pellets.

Table 4.2 Effect of inoculum size on production of *Phlebiopsis gigantea* spores in a liquid malt extract medium

Inoculum (log_{10} ml^{-1})	Maximum spore production (log_{10} ml^{-1})	Mycelial pellet size (mm)
5.10	6.03	3
5.67	6.68	1.2
5.97	6.29	1
6.53	6.33	0.8

Figure 4.2 shows initial inoculum concentration with (a) spore production and (b) dried biomass in submerged fermentation in malt extract broth at 25 ºC. Over the range tested, 5 - 7.5 log_{10} ml^{-1}, there was no correlation between inoculum size and the production of *P. gigantea* (PG21) spores. Spore production lay within the range of 5.5 – 7 log_{10} ml^{-1}. Biomass production was not correlated with the size of inoculum and lay within the range of 7 – 11 mg ml^{-1}.

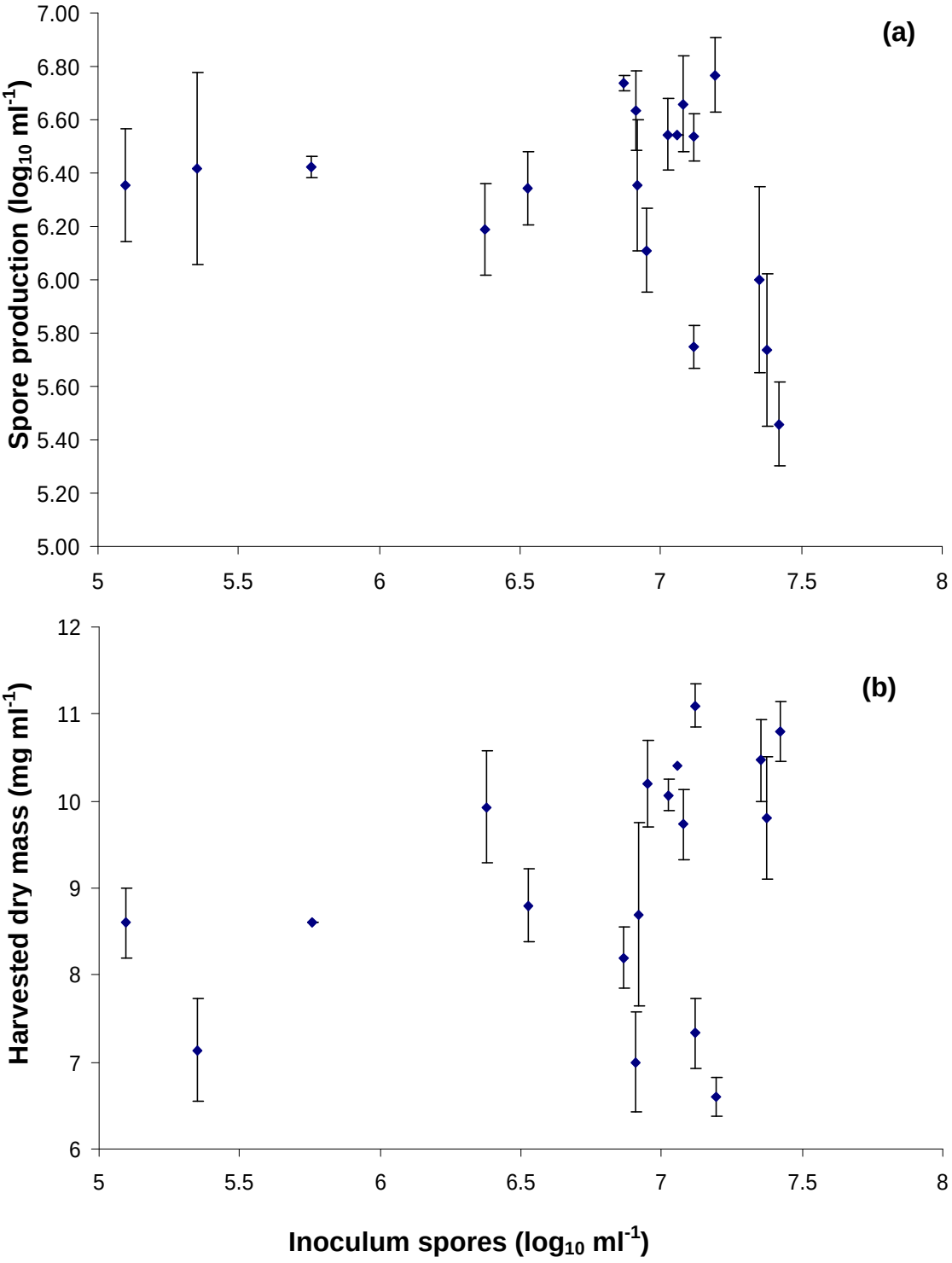


Fig. 4.2 Effect of inoculum size on *P. gigantea* (PG21) (a) spore production and (b) dried biomass. Submerged fermentation, in malt extract broth at 25 ºC. Bars represent standard error of the means.

4.2.2 Temporal study-effects of *P. gigantea* (PG21) culture age on spore and biomass production in submerged fermentation

Figure 4.3 shows the effect of inoculum culture age on production of (a) spores and (b) dry biomass by *P. gigantea* (PG21) in submerged fermentation. The number of spores harvested did not vary greatly with age of inoculum except for those flasks inoculated with spores from cultures harvested at day thirteen, apparently producing fewer spores, by a factor of ten. The production of biomass in submerged fermentation showed a steady increase with age of inoculum spores, reaching a peak of production when the inoculum was 12-13 days old. The data for day 15 showed a lower production of biomass, this could be explained by the use of pre-prepared media on that day, which had therefore been autoclaved twice.

Figure 4.4 shows (a) number of *P. gigantea* (PG21) spores harvested and (b) dried biomass produced from submerged fermentation of malt extract broth, incubated at 25 ºC for 4-14 days. The number of spores produced increased from 5.3 - 6 $\log_{10}$ ml^{-1} between days four and five of incubation. This was followed by a steady rise to 6.6 $\log_{10}$ ml^{-1} by thirteen days growth. In contrast to spore production, the amount of biomass decreased with time of incubation. The harvested dry biomass was 10.6 mg on day four, increased slightly to 11 mg on day five from where it declined down to 6.6 mg when harvested at thirteen days incubation. ANOVA of the effect of inoculum age and incubation time were each significant (P <0.01).

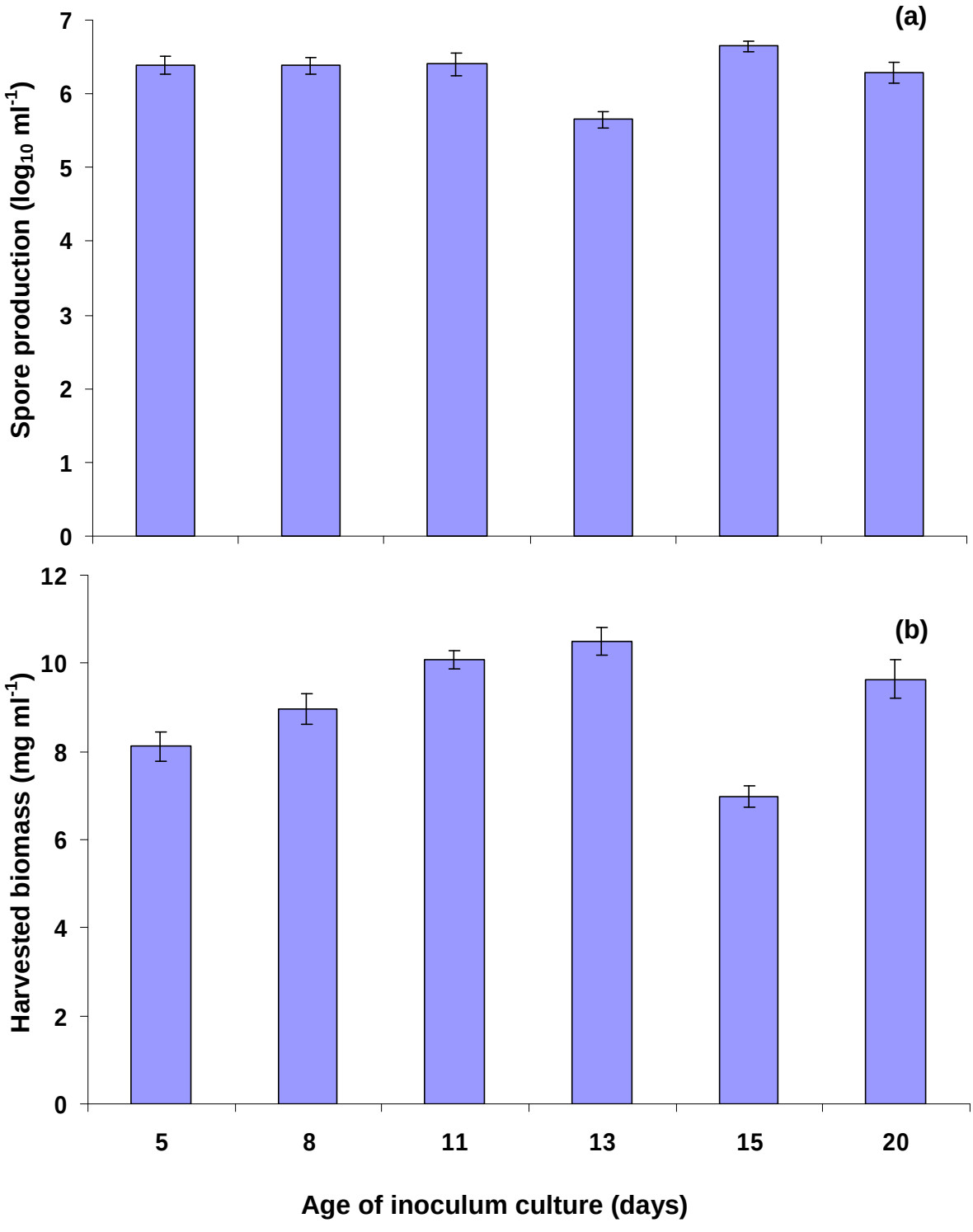


Figure 4.3 Effect of *P. gigantea* (PG21) inoculum culture ages on spore production (a) and harvested dried biomass (b) from submerged fermentation; in malt extract broth at 25 ºC. Bars represent standard errors.

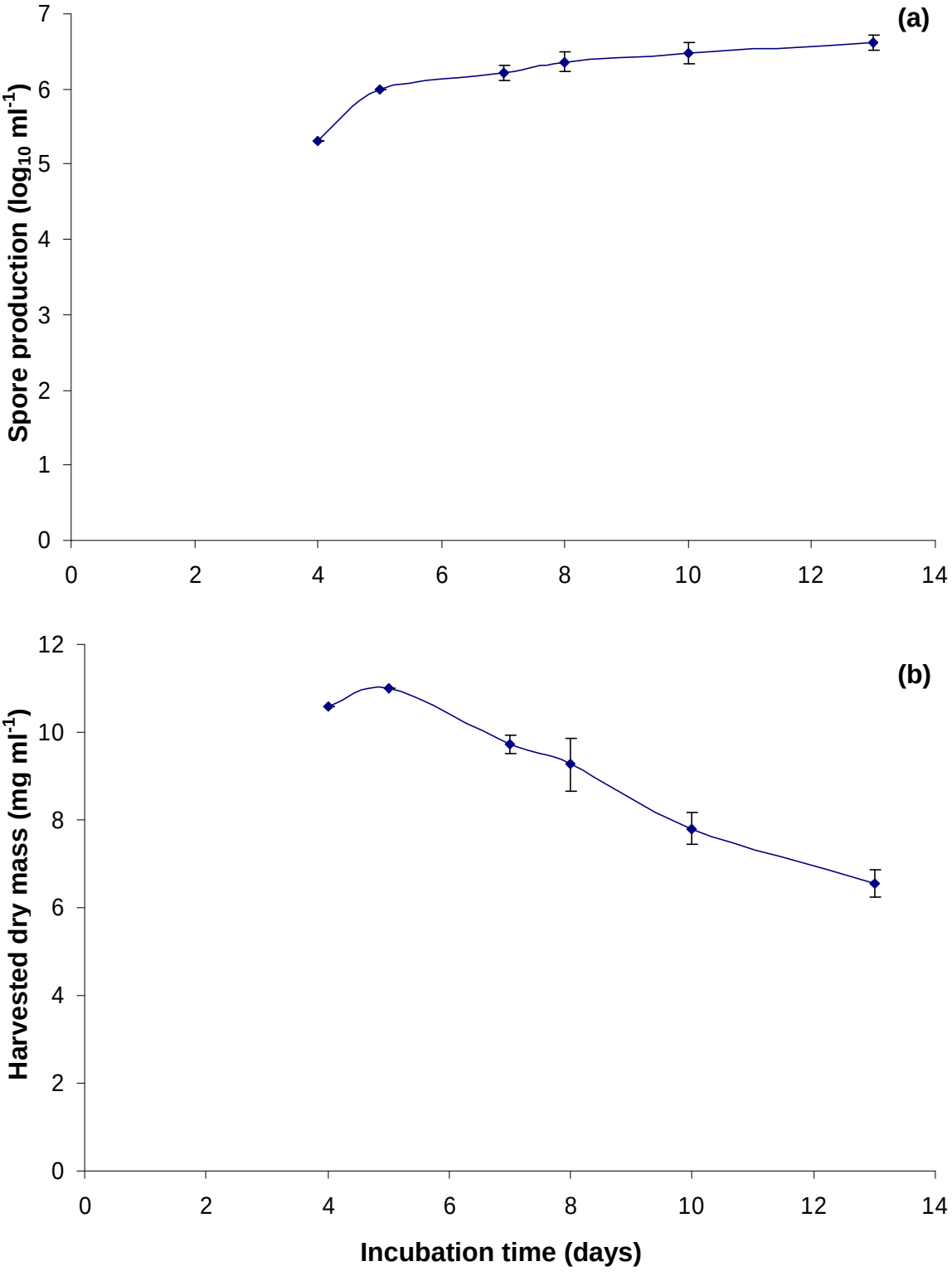


Figure 4.4 Effect of incubation time on the production of *P. gigantea* (PG21) spores (a) and biomass (b). Submerged fermentation of malt extract broth, incubated at 25ºC. Bars represent standard error of the means.

4.2.3 Effect of nutrient modified media on spore production

Table 4.3 shows the effect of modification of nitrogen sources on spore production. Figures are the mean, number of spores harvested, of six replicates per treatment. ANOVA of the effect of medium, incubation time and two-way interaction showed no significant differences between treatments (P <0.05).

Table 4.3 Effect of modifications to nitrogen source on the production of *Phlebiopsis gigantea* isolate PG21 spores (mean ± standard error).

Media	Spore production ($log_{10}ml^{-1}$)
a. ME + Phostrogen® + peptone	6.82 ± 0.43
b. ME + Phostrogen®	7.44 ± 0.21
c. ME + peptone	7.19 ± 0.27
d. ME	7.66 ± 0.16

The effect of more detailed manipulation of nitrogen (N) and carbon (C) sources, over seven combinations of media, are shown in the following figures. Figure 4.5 shows the effect of the media on spore production; production was greatest in the richest, complex medium at 6.6 x log_{10} spores ml^{-1}; with the C remaining high and the use of organic N spore production was 6.3 x log_{10} spores ml^{-1}. Using inorganic N production was 5.9 x log_{10} spores ml^{-1}. There was no significant difference between remaining treatments; the spore production being in the region of 5.5 – 5.8 x log_{10} spores ml^{-1} in each case, when the N sources were omitted and when the amount of C was halved whatever the source of N.

The quality of the spores was assessed in three different ways: growth on a range of agar media, the results of a semi-quantitative measure of "health" by visual appearance (shown in Figure 4.6 (a) and (b)) and growth rates (shown in Figure 4.7(a) and (b)); endogenous reserves are shown in section 4.4. Figure 4.6 (a) shows that

when the harvested spores were grown on wood agar the most highly developed growth, a dense feeding form of penetrative hyphae, was produced from spores cultured in the rich complex medium A and in C, containing organic N. All others showed less luxuriant growth with few aerial hyphae. Figure 4.6 (b) shows the same set of harvested spores grown on MEA modified with glycerol to water activities of 0.940 – 0.994 a_w, spores from the rich complex medium and those with the inorganic N produced the most dense growth at high water activity, but at 0.954 a_w the most dense growth was from spores grown in organic N with the full strength C, with very sparse growth from spores grown in unbalanced, nutritionally depleted, media that is with high C and no N and that with high N and low C. At 0.940 a_w growth was negligible. Figure 4.7 (a) shows that spores grown in medium E, that is reduced C with both organic and inorganic N, were able to most rapidly colonise the woody resources in wood agar, those from the rich complex medium and that with the inorganic N were also quite rapid at colonisation, but those from the full C and either organic N or N absent were the slowest growing. Figure 4.7 (b) shows that below 0.960 a_w the growth rate of the spores was negligible whatever their growth medium had been. Spores from media containing a lower ratio of C with organic N and that with both organic and inorganic N were the fastest growing on MEA with freely available water, but as soon as the water was slightly restricted they were amongst the poorest performers, along with the spores grown in a medium deprived of N. Spores from the rich complex medium and that with the high C plus inorganic N were the fastest growing on water restricted MEA.

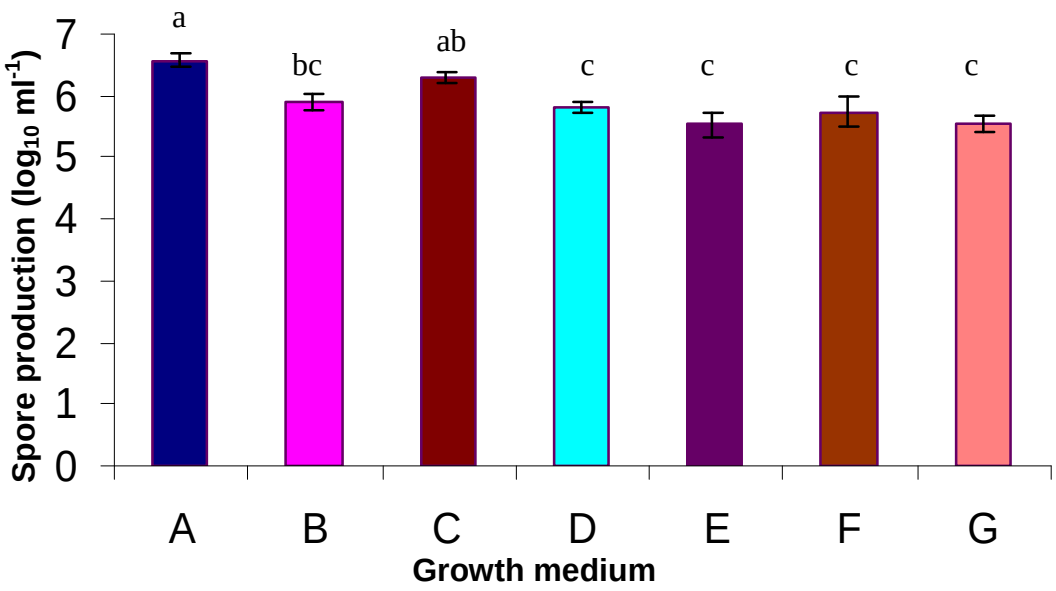


Fig. 4.5 Effect of nutrient modified fermentation media on spore production. Media based on malt extract broth:
A, rich medium, ME + peptone +Phostrogen®; B, ME + Phostrogen®; C, ME + peptone; D, simple medium, ME; E, ½ ME + peptone + Phostrogen®; F, ½ ME + peptone; G, ½ ME + Phostrogen®.
Bars represent standard errors of the means; different letters indicate statistical differences (*P* <0.01) between means.

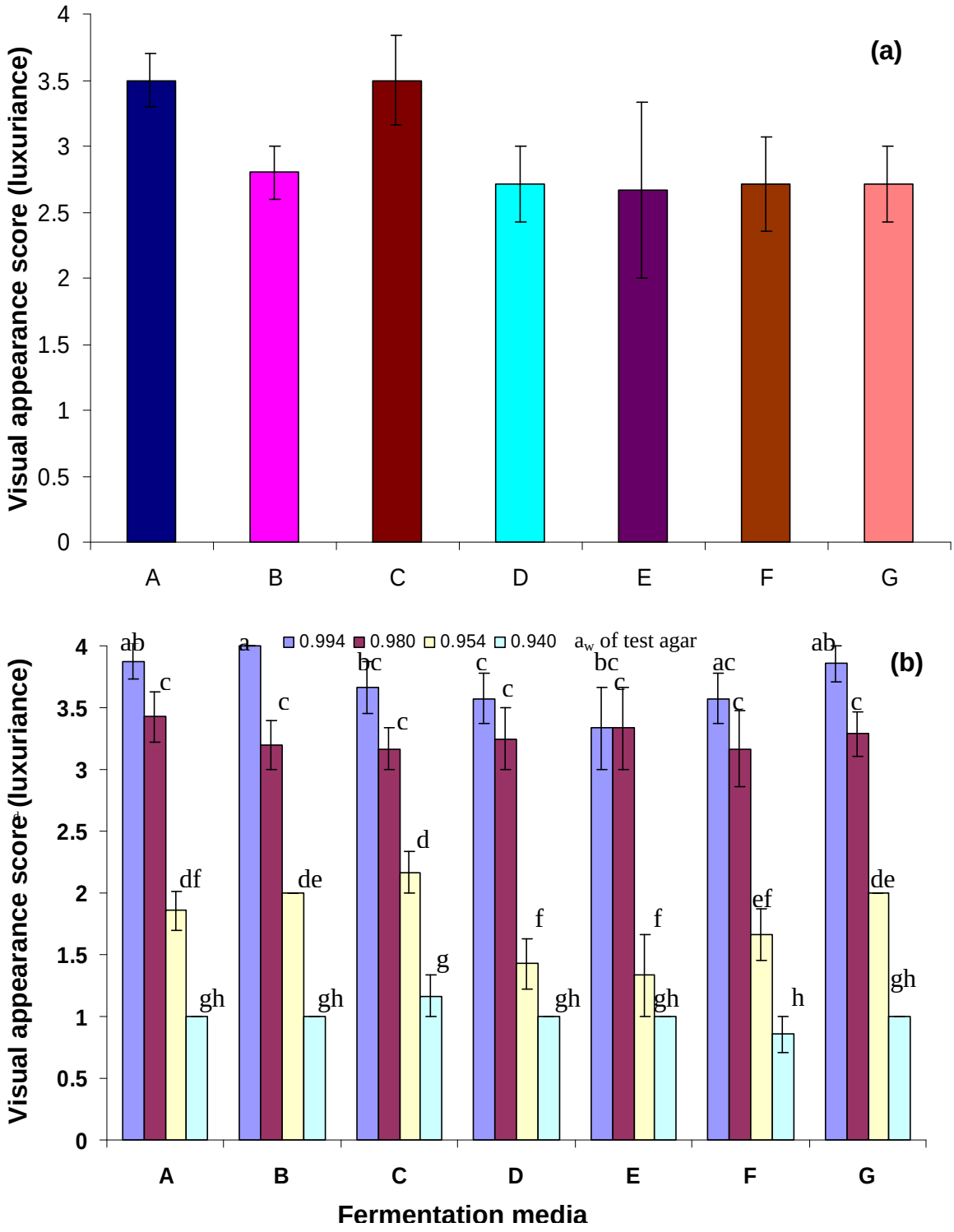


Fig. 4.6 Effect of nutrient modified fermentation media on spore adaptability, using a semi-quantitive score of visual appearance of growth on (a) wood agar, (b) MEA, a_w modified with glycerol.
Fermentation media based on malt extract broth:
A, rich medium, ME + peptone +Phostrogen®; B, ME + Phostrogen®; C, ME + peptone; D, simple medium, ME; E, ½ ME + peptone + Phostrogen®; F, ½ ME + peptone; G, ½ ME + Phostrogen®. Bars represent standard error of the means; different letters indicate statistical differences (*P* <0.01) between means.

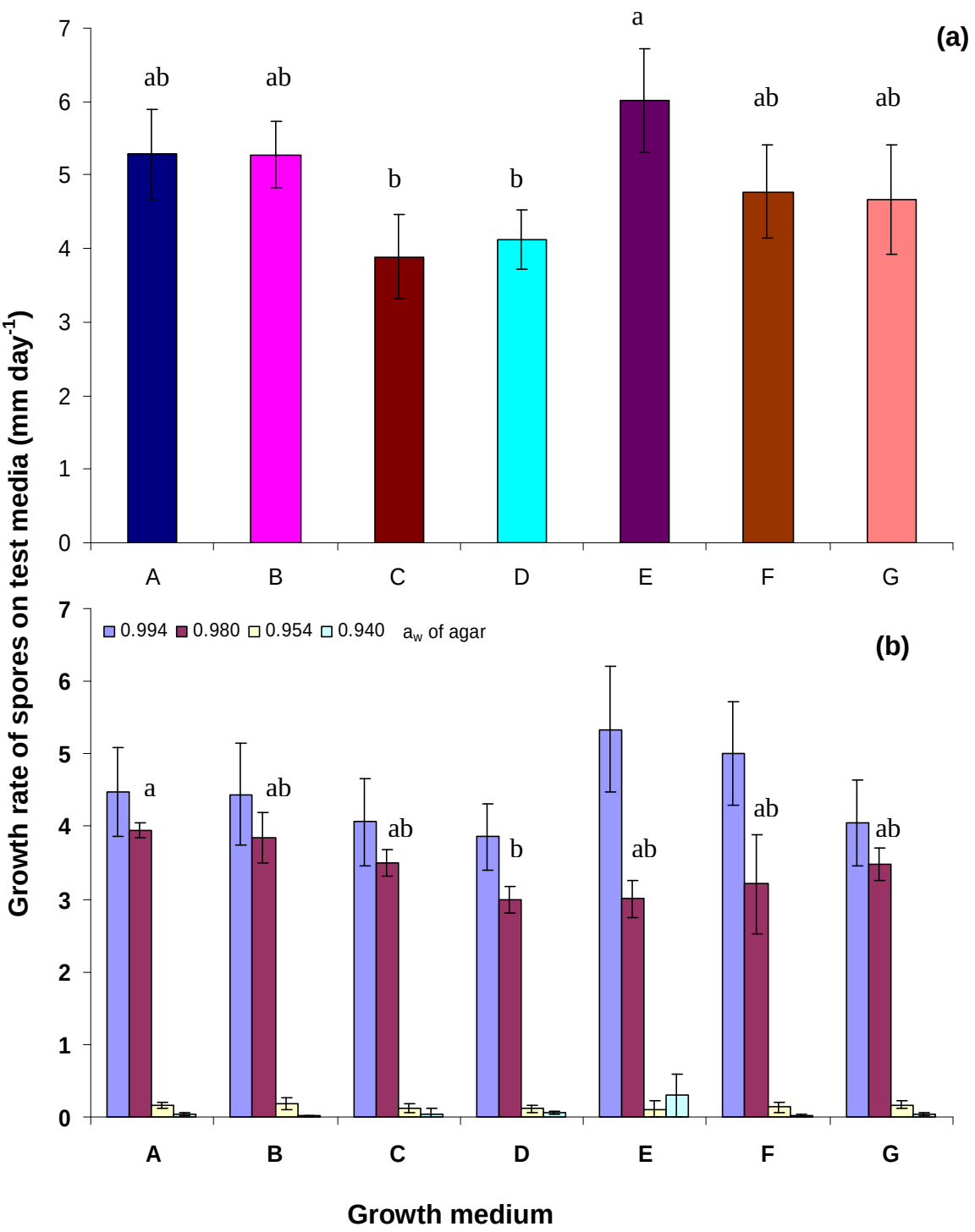


Fig. 4.7 Effect of nutrient modified fermentation media on spore production; growth rate of spores on (a) wood agar, (b) MEA modified with glycerol. Fermentation media based on malt extract broth:
A, rich medium, ME + peptone +Phostrogen®; B, ME + Phostrogen®; C, ME + peptone; D, simple medium, ME; E, ½ ME + peptone + Phostrogen®; F, ½ ME + peptone; G, ½ ME + Phostrogen®.
Bars represent standard errors of the means; there was no significant difference between treatments on growth rate at 0.994 a_w; different letters indicate statistical differences (P <0.01) between means.

4.2.4 Effect of reduced water availability on spore production

The effects of modification of fermentation broths by the addition of solutes to restrict the available water are shown in Figures 4.8 and 4.9. ANOVA of the effect of a_w was significant (P <0.01) at each day spores were counted. Figure 4.8 (a) shows the effect of using PEG 8000 to matrically modify a_w down to 0.978 a_w. When the available water was reduced to 0.978 a_w the production of spores was reduced to less than the control group when counted at each time interval. At 0.987 a_w the production was comparable with that of the control group (0.995 a_w) except on day 12 when there was great variation within the count of the control group. With the a_w slightly reduced to 0.991 a_w there was an increase in spore production, reaching a maximum of 6.8 $\log_{10}$ ml^{-1} on days 12 and 13 before slightly reducing to 6.7 $\log_{10}$ ml^{-1} on day 14. Figure 4.8 (b) shows the viability of the spores as measured by colony forming units (CFU) on MEA at 0.995 and 0.967 a_w, none of the spores formed CFU on MEA at 0.967 a_w. Of the 3 fermentation treatments that did grow, all produced a similar viability of just over 7 $\log_{10}$ CFU ml^{-1}. No CFU were counted from spores harvested from media of 0.978 a_w. A secondary line, counts of spores ml^{-1}, is included in the graph to show the discrepancy between the two counting methods.

Figure 4.9 shows the effect of using matric and solute modifications to reduce available water; this shows spore production (a) and viability as CFUs on MEA at 0.998 (b) and 0.988 a_w (c). As in the previous study, the greatest spore production at all time intervals was from the medium modified with PEG 8000 to 0.991 a_w, producing a maximum of 6.6 $\log_{10}$ ml^{-1} on day 12, and 6.5 $\log_{10}$ ml^{-1} on days 10 and 15. With a_w reduced to 0.987 a_w by PEG 8000 the maximum spore production was 6 $\log_{10}$ ml^{-1} on days 10 and 15. A 10-day incubation produced the maximum number of

spores from the control group, 6.2 $\log_{10}$ ml^{-1} and both groups with glycerol used to modify water availability, each at 6.3 $\log_{10}$ ml^{-1}. When sorbitol was used as the modifier at 0.994 a_w 6 $\log_{10}$ ml^{-1} spores were produced on days 10 and 15, but at 0.984 a_w this was reduced to 5.4 $\log_{10}$ ml^{-1}. ANOVA of the effect of treatment, fermentation time and two-way interaction, on spore production, showed treatment to be significant (P <0.01), but fermentation time and two-way interaction were not. Spore viability as measured by CFUs on 0.998 (b) and 0.988 a_w (c) showed that viable spores produced at 0.991 a_w with PEG 8000 were most numerous, 6.2 $\log_{10}$ CFU ml^{-1}, at 10-day growth on both test media. The control group, producing 5.7 $\log_{10}$ CFU ml^{-1}, was also out performed after 10 days of growth by both glycerol groups and the sorbitol 0.994 a_w group, producing 5.8 – 6 $\log_{10}$ CFU ml^{-1} on 0.998 a_w. On 0.988 a_w, 6 $\log_{10}$ CFU ml^{-1} were formed from spores produced in the sorbitol modified broth at 0.994 a_w. At all incubation times the least number of viable spores were produced at 0.984 a_w with sorbitol as the modifier. Interestingly, although the PEG 8000 treatment at 0.987 a_w had the lowest number of CFU at 10-day incubation, on both 0.998 and 0.998 a_w, by fifteen days it reached a maximum of 6 and 5.7 $\log_{10}$ CFU ml^{-1} respectively. ANOVA of effect of treatment, fermentation time and two-way interaction were each significant (P <0.01) on CFU at 0.998 and 0.988 a_w.

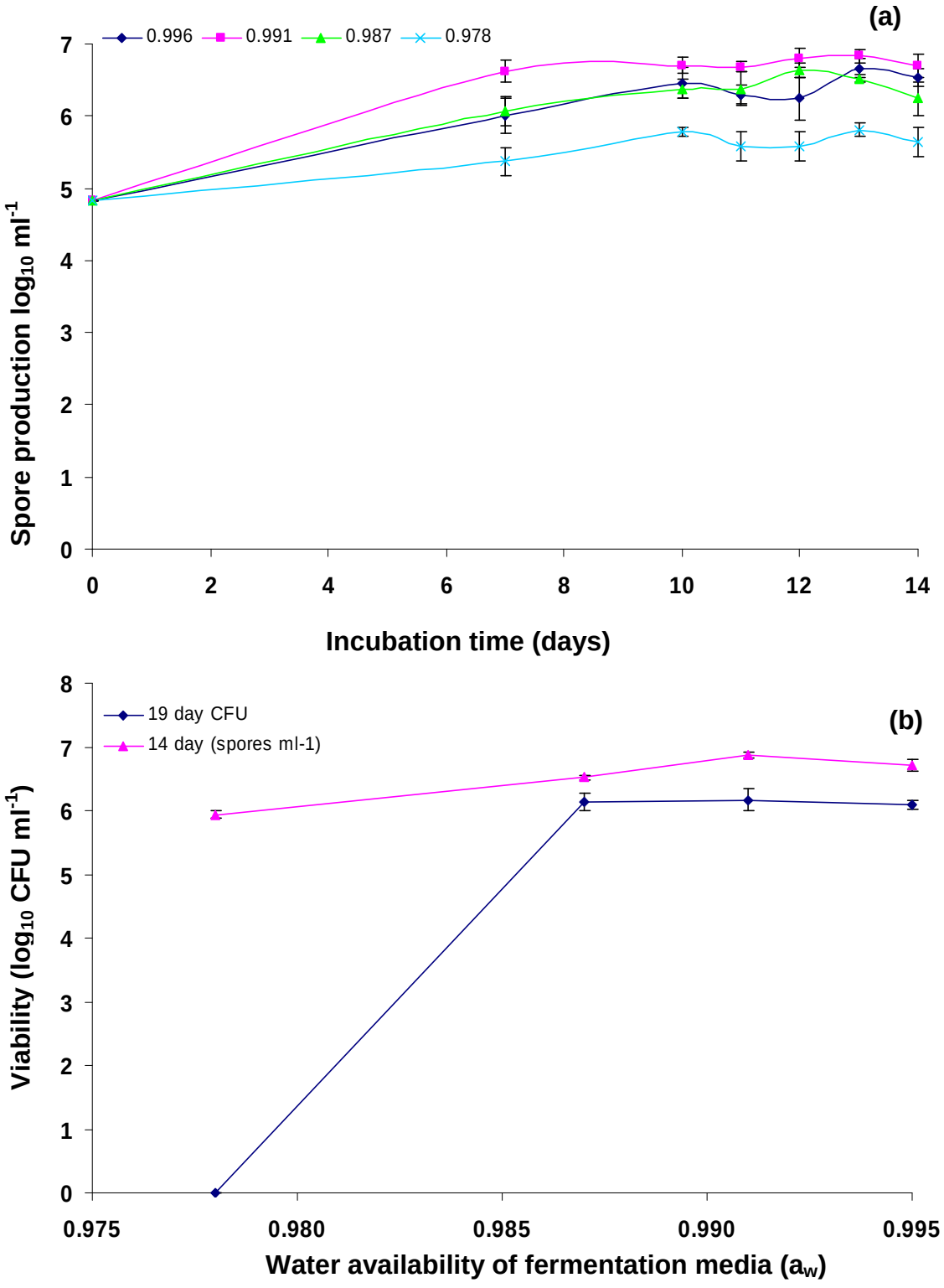


Fig. 4.8. Effect of restricted water availability, in fermentation media, on PG21 spore production. ME broth a_w modified with PEG 8000, incubated at 25 ºC at 150 rpm. Spore production at each a_w during incubation period (a), viability of spores from each a_w as CFU (19-day fermentation) on MEA at 0.995 a_w (b). Secondary line shows spore count from flasks, when fermented for 14 days.

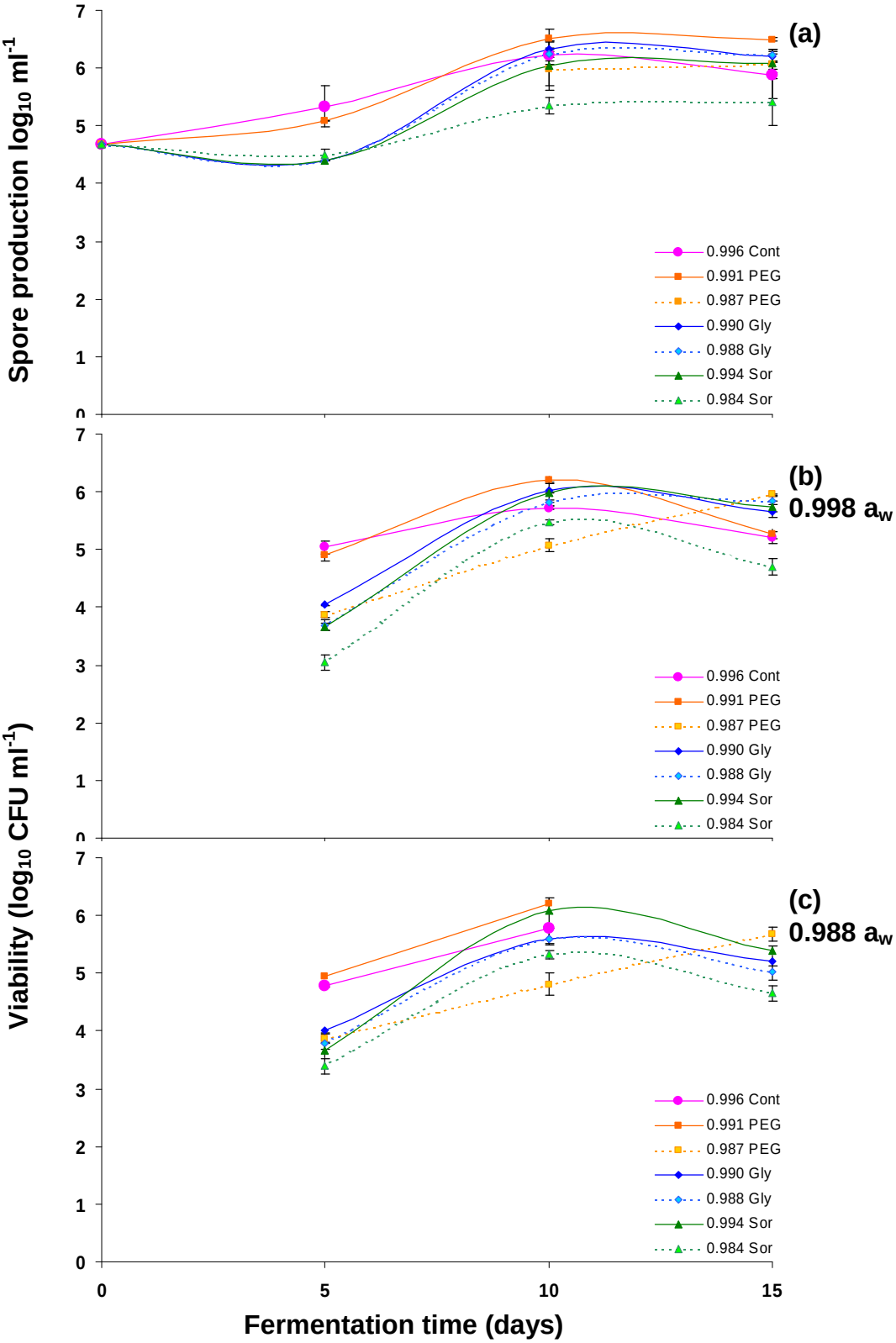


Fig. 4.9. Effect, on PG21 spore production and viability, of differing solutes to restrict water availability in fermentation media. ME broth (Control), a_w modified with PEG 8000 (PEG), glycerol (Gly) or sorbitol (Sor), incubated at 25 ºC on a shaker at 150 rpm. Spore counts of submerged conidia production (a), viability of spores from each a_w as CFU on MEA at 0.998 (b) and 0.988 a_w (c). Bars represent standard errors of the means.

4.2.5 Effect of supported growth at liquid/air interface on PG21 spore production

A primary harvest was taken from the ReacSyn^{TM} vessels after 10 days of incubation. From both the spent media and the washings from the slopes there were insufficient spores to count with a haemocytometer, however the results of CFUs are displayed in Figure 4.10 (a). From the secondary harvest, 10 days later, only 1 vessel had produced sufficient spores to be visible in the counting chamber; the CFU results are displayed in (b). In most cases more oidia were obtained from the slope surfaces than conidia from submerged culture, except for the 10-day harvest of the glycerol treatment group where more CFU were produced from the submerged conidia. Treatments of both PEG 8000 and sorbitol did not differ greatly from the control group, producing approximately 4 $\log_{10}$ CFU ml^{-1} viable oidia from the primary harvest and 3.6 – 3.9 $\log_{10}$ CFU ml^{-1} from the secondary harvest. With glycerol as the modifier the harvested oidia improved from 2 $\log_{10}$ CFU ml^{-1} in the primary to 3 $\log_{10}$ CFU ml^{-1} in the secondary harvest. Production of submerged conidia, in the control group, was only 3.3 $\log_{10}$ CFU ml^{-1} in the primary and reduced to 3 $\log_{10}$ CFU ml^{-1} in the secondary harvest. In the primary harvest this was slightly improved by glycerol at 3.7 $\log_{10}$ CFU ml^{-1} and in the secondary harvest the sorbitol treatment produced 3.8 $\log_{10}$ CFU ml^{-1}. ANOVA of the effect of media treatment, spore type, harvest time, a_w of CFU test agar, two, three and four-way interaction showed treatment, spore type, treatment x spore type, treatment x harvest time and treatment x spore type x harvest to be significant (P <0.01) all other factors and conditions were insignificant.

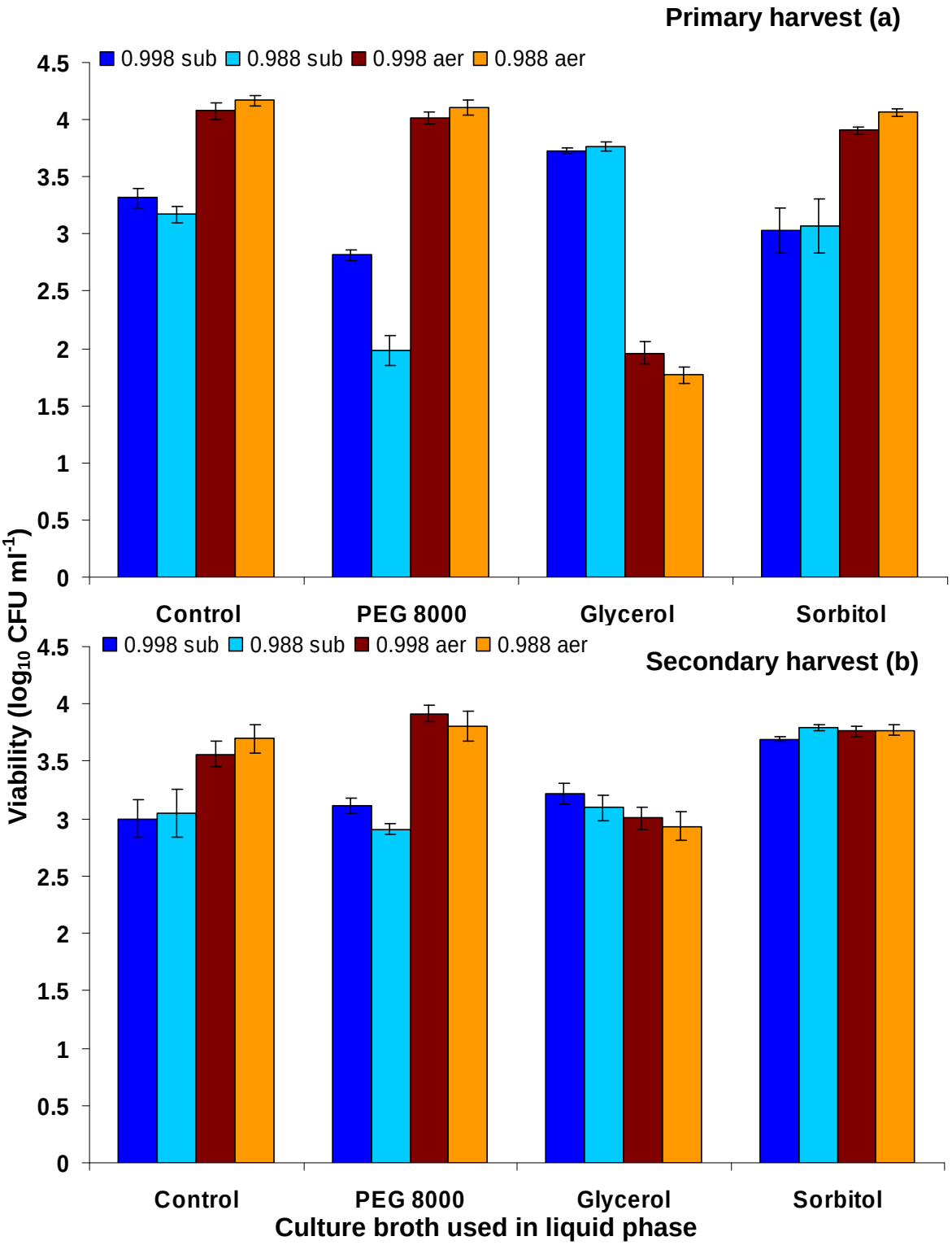


Fig. 4.10 Production of PG21 spores on a support at liquid/air interface using ReacSyn™ vessels. Culture broth was malt extract modified with: Control ME at 0.996 a_w, PEG 8000 at 0.991 a_w, glycerol at 0.990 a_w and sorbitol at 0.992 a_w. Incubation at 25 ºC, (a) primary harvest at 10 days, (b) secondary harvest with replenished broth. Viability assessed by growth of CFU on MEA at 0.998 a_w (dark columns) and 0.988 a_w (light columnns of each pair). Bars represent standard errors of means.

4.2.6 Effect of immobilisation of inoculum in alginate beads within a liquid medium, on PG21 spore production

A preliminary study was carried out with PG21 inoculated sodium alginate beads in malt extract broth as used for the control medium in previous experiments. A spore count was taken from all 6 flasks after 5 days of incubation on a rotary shaker at 25 ºC. Six days later all 6 flasks were already dense with germinating spores. The average number of spores counted on day 5 was 7.17 $\pm$ 0.05 $\log_{10}$ spores ml^{-1}.

Figure 4.11 shows the production of submerged conidia (a) and CFUs (b). In (a) it can be seen that from the liquid culture control group there was a rise from 4.8 $\log_{10}$ spores ml^{-1} at 3 days to 6.6 $\log_{10}$ spores ml^{-1} at 5 days, gradually rising to 7 $\log_{10}$ spores ml^{-1} by 10 days. The inoculum supported in beads showed a more rapid increase of spore production during the early days of incubation. By day 5 it had already produced 6 $\log_{10}$ spores ml^{-1} rising to 7 $\log_{10}$ spores ml^{-1} by day 5, staying at this level to day 10. Fig 4.11 (b) shows that no CFUs were formed from the 3-day samples taken from the beads, from the liquid culture flasks 3.7 $\log_{10}$ CFU ml^{-1} were produced. By day 5 there were 5 $\log_{10}$ CFU ml^{-1} and 6 $\log_{10}$ CFU ml^{-1}, respectively, and on day 7 they both produced 6.3 $\log_{10}$ CFU ml^{-1}. Over 10 and 15 days there were marginally more CFU from the beads than from the control broth, at 6.5 and 6.3 $\log_{10}$ CFU ml^{-1} respectively. ANOVA of the effect of treatment, fermentation time and two-way interaction were significant (P <0.01).

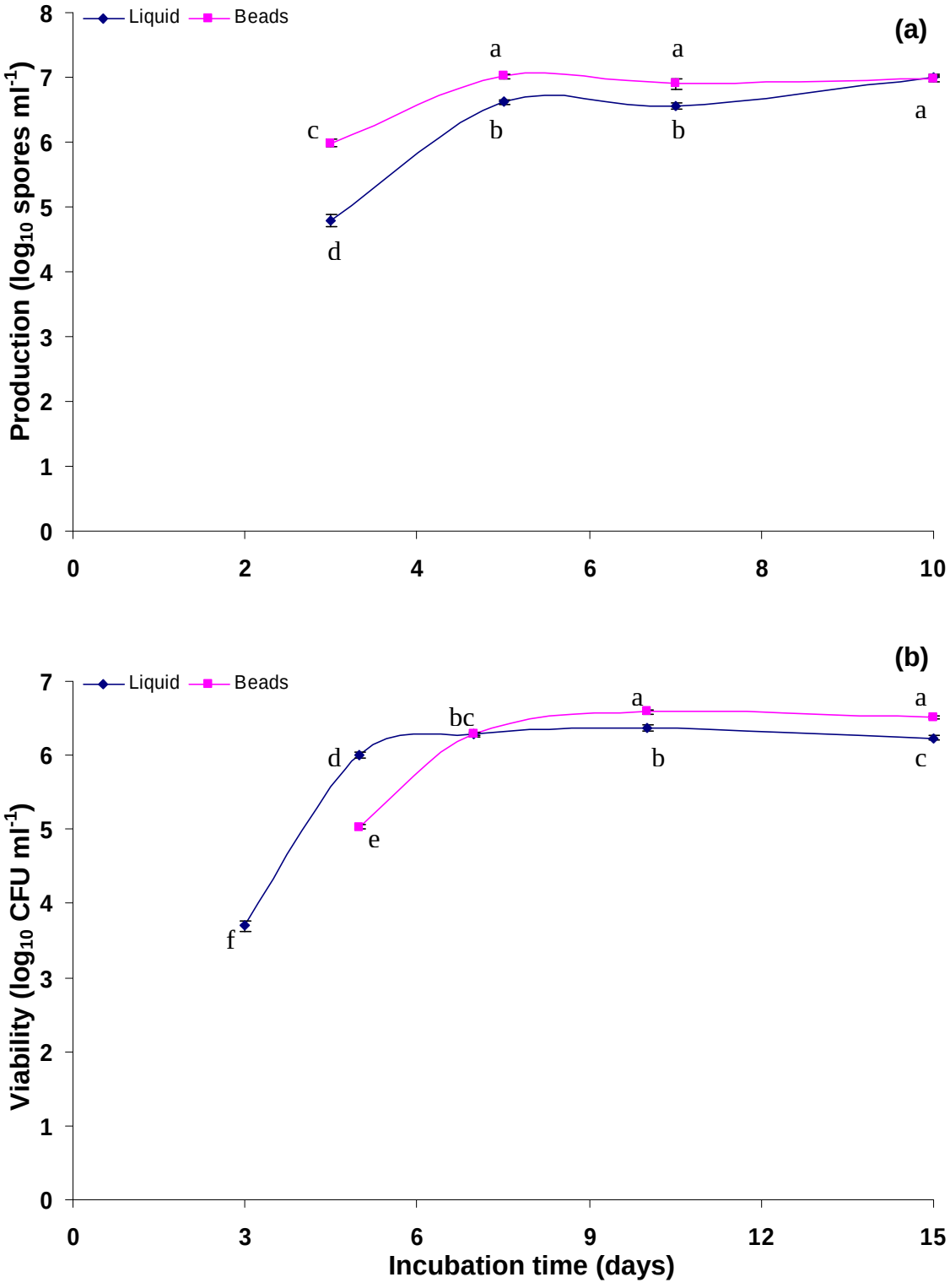


Fig. 4.11 Effect of support in sodium alginate beads in ME broth on production (a) and viability (b) of PG21 submerged conidia at 25 ºC on a shaker at 150 rpm. Bars represent standard errors of the means; different letters indicate statistical differences (*P* <0.01) between means.

4.3 Production of *Phlebiopsis gigantea* spores in solid substrate fermentation

4.3.1 Pilot studies on wood chips

The results from the first pilot study, using Pinewood chips as a solid substrate for production of PG21 oidia are shown in Table 4.4. This shows the relationship between the moisture content (MC) of wood chips (*Pinus* spp) and the number of PG21 spores produced after 8 weeks incubation, in humidity-controlled chambers, at 25 ºC. No CFU determinations were carried out in this study and it is possible that the result shown from the 0% MC was the remainder from the inoculum.

Table 4.4 Effect of moisture content on spore production from wood chips

Moisture content (MC) (%)	0	38	44	50
Production (log_{10} spores g^{-1} substrate)	6.1	7.4	7.7	7.9

A second pilot study was conducted, the results of which are displayed in Figure 4.12. When considering the production of oidia in (a) there were some spores visible in the haemocytometer from the 7 and 14% MC groups, but in (b) there were no CFUs present at these moisture contents; the wood chips were coarse and perhaps had not equilibrated, thus allowing growth at the start of incubation, but lack of moisture leading to early senescence. These show that there was very little growth below 29% MC.

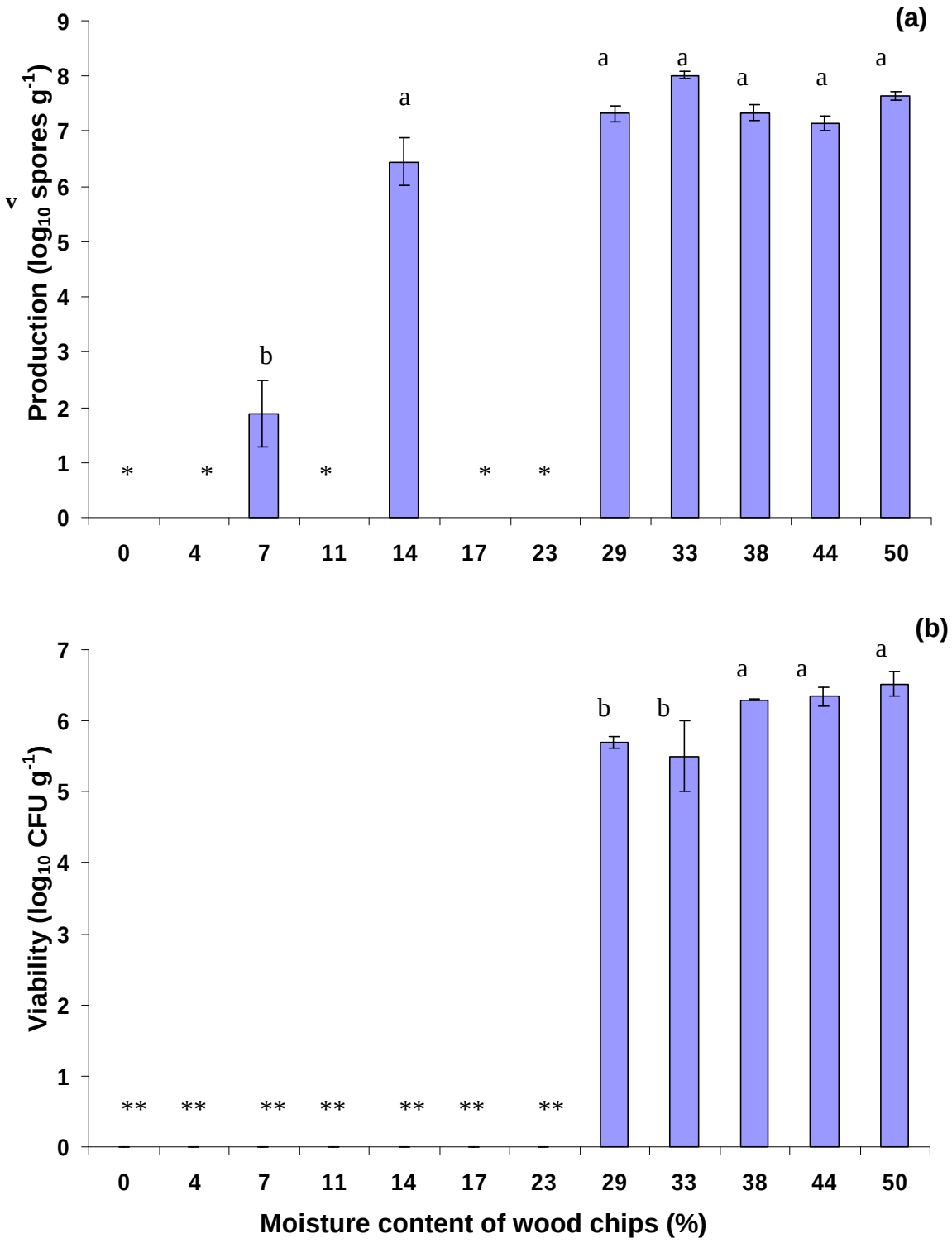


Fig. 4.12 Effect of moisture content (MC) of wood chips (*Pinus* spp.) on production of PG21 spores (oidia) (a) and viability as measured by CFU (b). Incubated in humidity-controlled chambers at 25 ºC for 4 months. Different letters between two treatments indicate significant differences (P <0.05).
*** No oidia production; ** no CFU production.**

4.3.2 Temporal study: the effect of moisture content on PG21 oidia production

Pinus sylvestris sawdust was used as the substrate in a temporal study to observe the effect of differing MC on PG21 oidia production; the results are displayed in Figure 4.13. With MC from 15 – 44% approximately 6 $\log_{10}$ spores g^{-1} were produced in both one and 2 months of incubation at 25 ºC. At 50 - 55% MC 7 $\log_{10}$ spores g^{-1} were produced at each time interval. Sub-samples of each spore suspension were spread-plated, on MEA 0.940 – 0.995 a_w to assess viability by CFU. From the 1 month harvest none of these plates showed any colonies and from the second harvest the only MC that formed CFU was 55% MC, producing 6 – 6.6 $\log_{10}$ CFU g^{-1} on 0.995, 0.980 and 0.970 a_w and 5.7 $\log_{10}$ CFU g^{-1} on 0.954 a_w. A shift in water availability was observed during the course of this study, as shown in Table 4.5. ANOVA of the effect of MC and incubation period showed MC to be significant (P <0.01); incubation time was not significant.

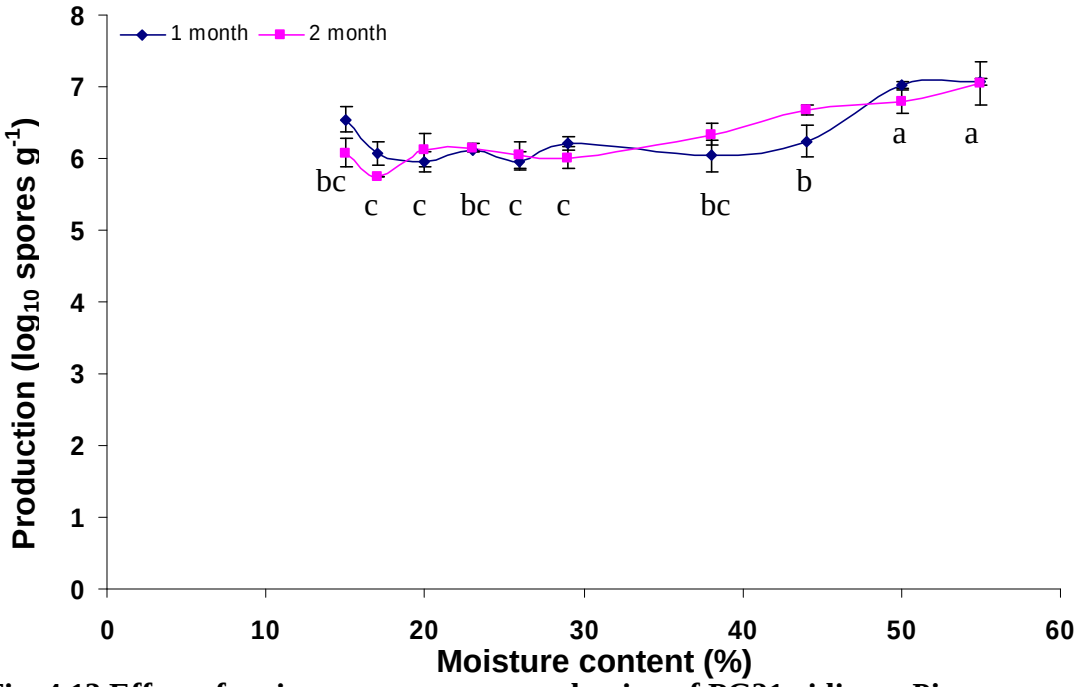


Fig. 4.13 Effect of moisture content on production of PG21 oidia on *Pinus sylvestris* sawdust incubated at 25 ºC for 1 and 2 months. Bars represent standard errors of the means; different letters between two means indicate significant difference (P <0.01).

Table 4.5 Drift of water availability over time when water added to 5 g oven dried sawdust prior to inoculation with PG21 and incubated at 25 ºC for 2 months

	Water activity ($a_{w)}$		
Water added (ml)	**Start**	**1 month**	**2 months**
0.875	0.888	0.826	0.607
0.875	0.859	0.825	0.589
0.875	0.869	0.755	0.603
1	0.930	0.707	0.657
1	0.907	0.686	0.646
1	0.931	0.686	0.618
1.25	0.954	0.835	0.705
1.25	0.928	0.772	0.731
1.25	0.926	0.744	0.758
1.5	0.980	0.689	0.669
1.5	0.987	0.616	0.664
1.5	0.982	0.635	0.637
1.75	0.987	0.736	0.615
1.75	0.980	0.726	0.631
1.75	0.988	0.764	0.629
2	0.993	0.880	0.729
2	0.995	0.990	0.724
2	0.997	0.895	0.761
3	0.989	0.923	0.864
3	0.991	0.985	0.816
3	0.989	0.938	0.768
4	0.996	0.986	0.937
4	0.989	0.992	0.928
4	0.997	0.988	0.936
5	0.998	0.991	0.937
5	1.000	0.992	0.974
5	0.996	0.994	0.993
6	1.000	0.992	0.995
6	1.000	0.995	0.993
6	1.000	0.994	0.987

4.3.3 Effect of moisture content on the small scale production of PG21 oidia, temporal study

A further temporal study was carried out with larger percentages of moisture content. Harvests were taken at 3, 8 and 12 weeks. Figure 4.14 (a) shows that at the time of the first harvest there was little difference between the treatment groups, all producing 7.5 – 7.6 $\log_{10}$ spores g^{-1}. By the 2nd harvest there was a slight increase in production, with 75 and 80% MC reaching 8 $\log_{10}$ spores g^{-1}. By 3 months of incubation treatments 53 and 67% MC produced 8.8 $\log_{10}$ spores g^{-1} and the three higher MCs 9

$\log_{10}$ spores g^{-1}. Figure 4.14 (b) and (c) show that at the first harvest, there was very little difference between the treatments in viability on both a_w test media, there was approximately 7.5 $\log_{10}$ CFU g^{-1} in all cases. The second harvest produced fewer CFUs from all treatments except the 75% MC which showed a viability of 7.6 $\log_{10}$ CFU g^{-1} on both a_w and this rose to 8 $\log_{10}$ CFU g^{-1} by the third harvest, while the other treatments showed lower viabilities. After the 3 month harvest had been carried out the remaining substrate was oven dried at 80 ºC for 48 hr and weighed. There was found to have been a mass loss of approximately 20% from the original dried sawdust. ANOVA of the effect of moisture content, incubation and two-way interaction were significant ($P < 0.01$); the a_w of test agar used for CFU was not.

4.3.4 Effect of water availability, addition of calcium chloride and scale up on PG21 oidia production

Figure 4.15 (a) shows results of the effects of water availability and calcium chloride addition when studied on a scale up of production from the previous 10 g substrate up to 50 g *P. sylvestris* sawdust. The control groups, at 75% MC showed no difference in number of spores harvested, whether from previous studies at 5 – 10 g, or between those harvested at 3 or 8 weeks of incubation at 25 ºC. The number of spores harvested was also unaffected by the addition of calcium chloride ($CaCl_2$) at either 20 or 40 mM, or by changes in substrate a_w modified with glycerol, production being 7.6 – 7.9 $\log_{10}$ spores g^{-1} in all cases. Viability, measured as CFU, displayed in Figure 4.15 (b) showed no difference between $CaCl_2$ treatments. When grown at 0.991,and 0.981 a_w there were 7 $\log_{10}$ CFU g^{-1}, but when grown with substrate at 0.974 a_w there were 6 – 6. 2 $\log_{10}$ CFU g^{-1}. These CFU were grown on test media at 0.998 and 0.988;

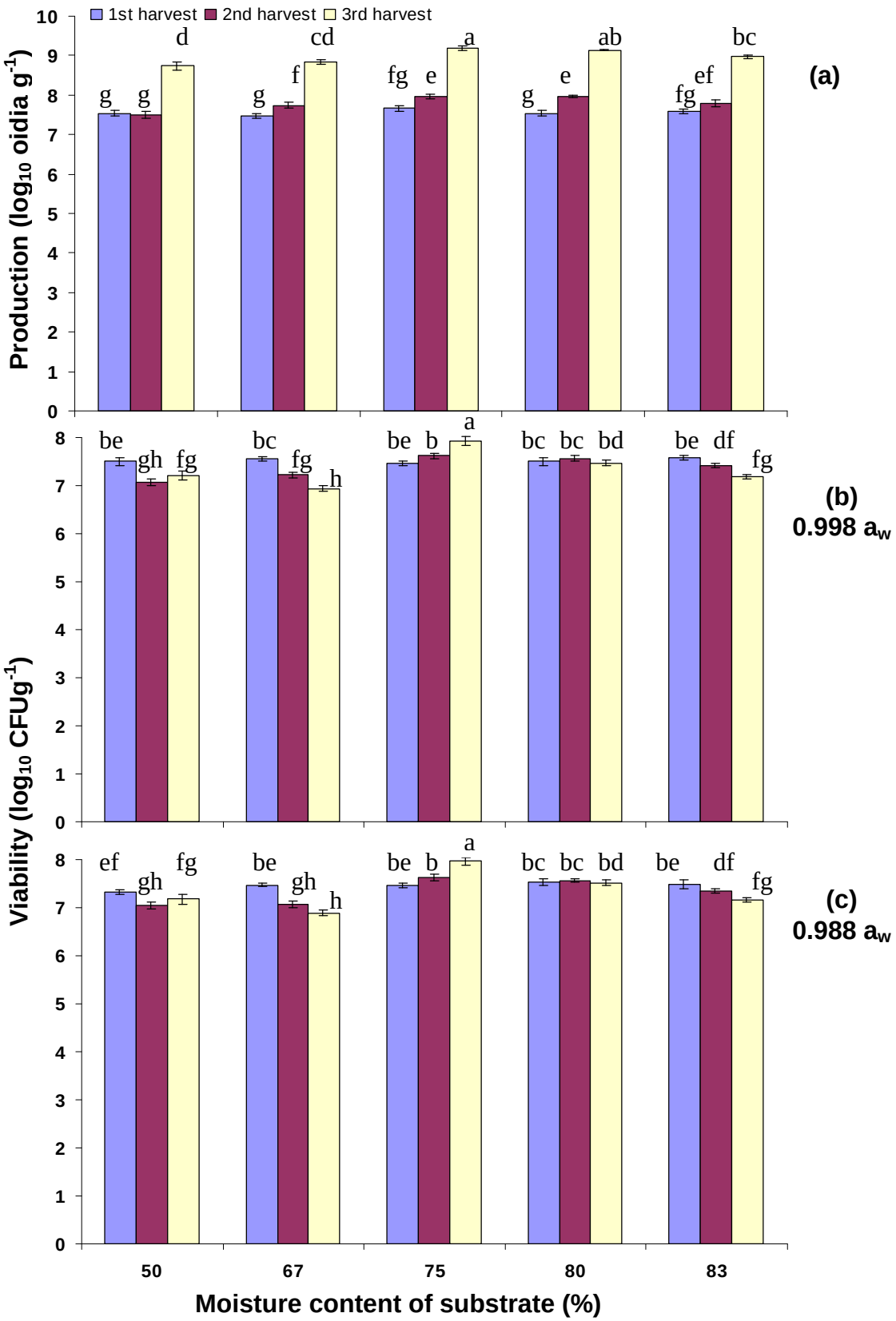


Fig. 4.14. Effect of substrate moisture content, on PG21 spore (oidia) production (a) and viability, as CFU on MEA at 0.998 (b) and 0.988 a_w (c), when grown on *Pinus sylvestris* sawdust, incubated at 25 ºC. Bars represent standard errors of the means; different letters between two treatments indicates significant differences (*P* <0.05).

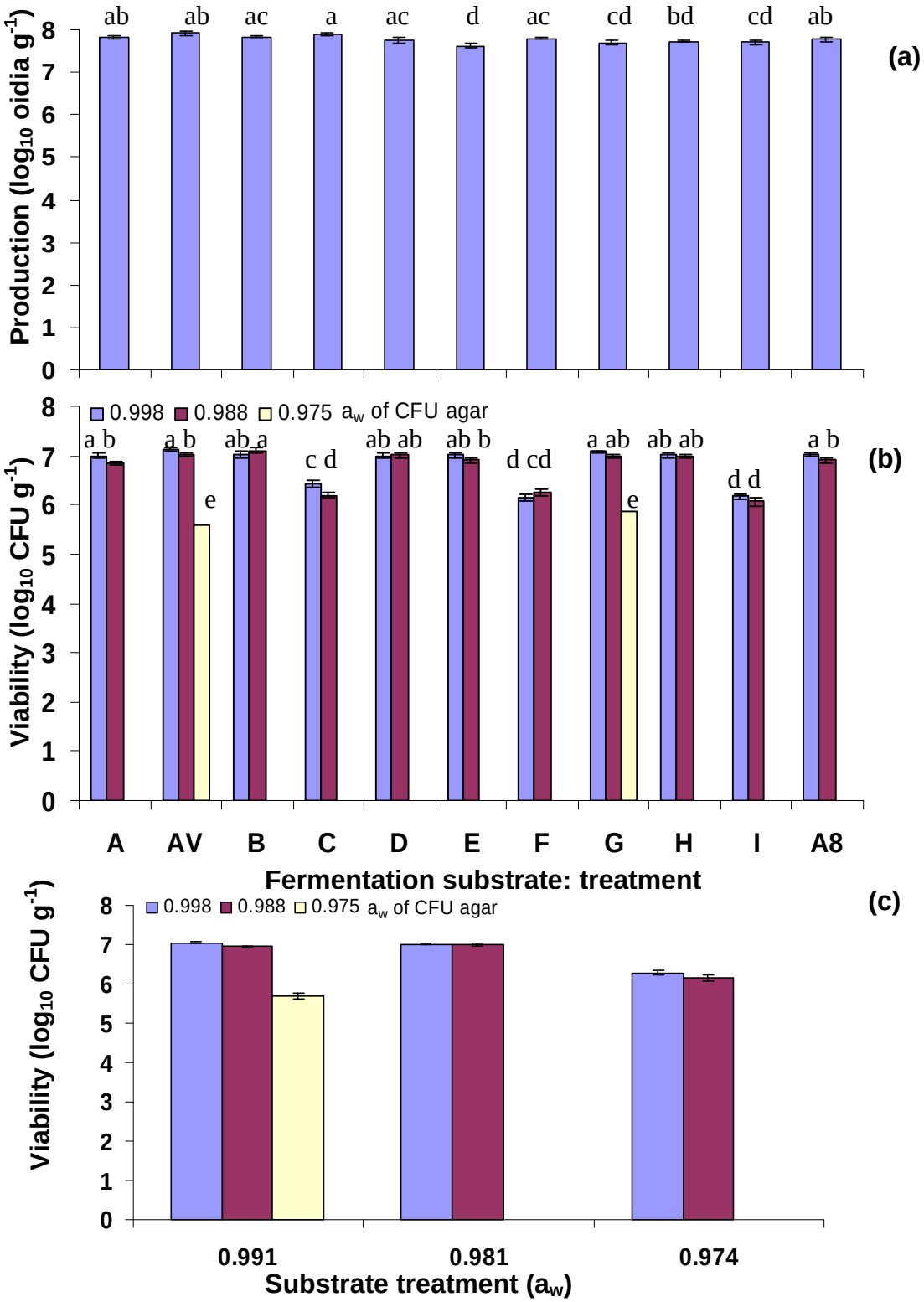


Fig. 4.15 Effect of water availability and $CaCl_2$ concentration on the production (a) and viability (b) and (c) of PG21 oidia when grown on *Pinus sylvestris* sawdust, incubated at 25 ºC for 3 weeks. Key to treatments: A control at 75% MC, AV = A ventilated and A8 = A incubated for 8 weeks, B 0.981 a_w, C 0.974, D 0.991 + $CaCl_2$ 20 mM, E 0.981 + $CaCl_2$ 20 mM, F 0.978 + $CaCl_2$ 20 mM, G 0.991 $CaCl_2$ 40 mM, H 0.981 + $CaCl_2$ 40 mM, I 0.974 a_w + $CaCl_2$ 40 mM. Water availability modified with glycerol, CFU on MEA at 0.998, 0.988 and 0.974 a_w. Bars represent standard errors of the means; different letters between two treatments indicate significant differences (P <0.05).

only spores from substrate at 0.991 a_w were able to produce CFU on medium at 0.975, producing 5.7 $\log_{10}$ CFU g^{-1} on 3 Petri dishes only. Figure 4.15 (c) shows the collected results of viability testing, disregarding the $CaCl_2$ treatments, where the data were re-grouped according to the available water of the growth substrate. ANOVA of the effect of a_w, $CaCl_2$ molarity, a_w of test agar for CFU and two and three-way interaction showed a_w of growth medium to be very significant (P <0.01), $CaCl_2$ molarity and two-way interaction to be significant (P <0.05), but test agar a_w to have no significance. ANOVA of the effect of ventilation and of incubation time showed there were no differences between treatments.

In a further run, treatments A, B and C were repeated with 6 replicates of each, the results are displayed in Figure 4.16. The number of spores was unaffected by a_w of the substrate, production being 7.4 – 7.5 $\log_{10}$ spores g^{-1} in all 3 treatments. Substrate treatments of 0.991 and 0.981 a_w made little difference to viability at 0.998 and 0.988 a_w, being 7 and 6.6 – 6.9 $\log_{10}$ CFU g^{-1} respectively, but when tested at 0.974 a_w viability was 6.6 and 6.2 $\log_{10}$ CFU g^{-1} respectively. However, viability of spores was affected by growth on the lower a_w treatment, at 0.974 a_w. From this group viability was 6 $\log_{10}$ CFU g^{-1} when water was freely available, down to 5.5 $\log_{10}$ CFU g^{-1} when water was restricted to 0.974 a_w on the test agar. ANOVA of the effect of substrate a showed no significant difference in spore production; CFU were affected by substrate a_w, CFU test media a_w and two-way interaction (P <0.01).

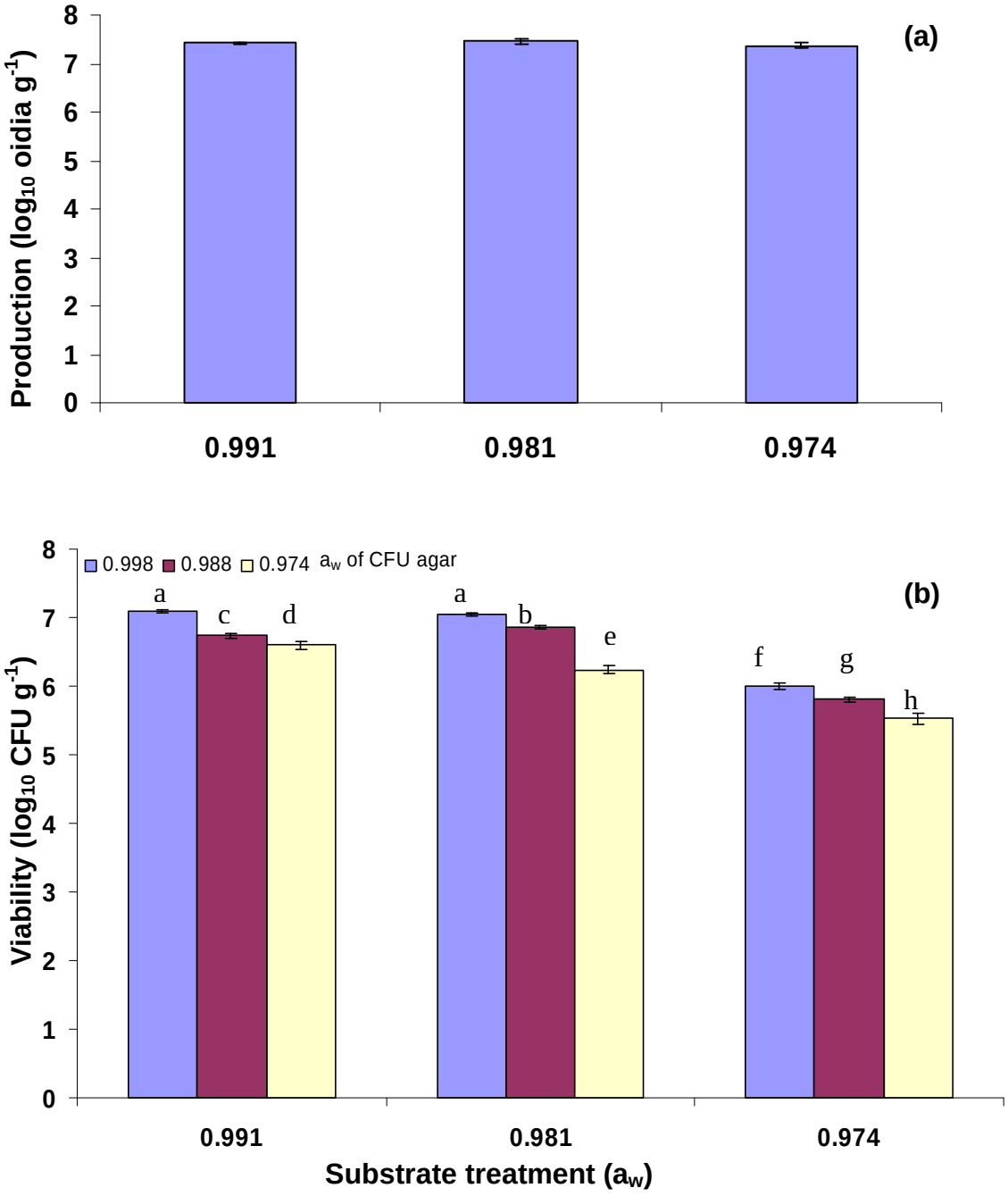


Fig. 4.16 Effect of water availability on the production (a) and viability (b) of PG21 oidia when grown on *Pinus sylvestris* sawdust, incubated at 25 ºC for 3 weeks. Water availability of solid substrate modified with glycerol. Viability assessed as CFU on MEA at 0.998, 0.988 and 0.974 a_w. Bars represent standard errors of the means: different letters between two treatments indicate significant differences (*P* <0.01).

4.3.5 Effect of further scale up of production on PG21 oidia production

When spore production was scaled up to 200g *P. sylvestris* sawdust per spawn bag the average production was 7.3 $\log_{10}$ oidia g^{-1} substrate. When tested for viability as CFUs on 0.998 and 0.988 a_w there were 7 $\log_{10}$ CFU g^{-1}, and on 0.981 a_w this was reduced to 6.7 $\log_{10}$ CFU g^{-1}, as can be seen in Figure 4.17. In a further run with substrate mass increased to 400 g per spawn bag and incubation increased to 6 weeks, there was an apparent increase in spore production to 7.7 $\log_{10}$ oidia g^{-1} substrate, but a decrease in viability to 6, 5.7 and 5.5 $\log_{10}$ CFU g^{-1} on 0.998, 0.988 and 0.981 a_w respectively. The oven-dried mass of the residual substrate was 370 g. Figure 4.17 also shows a comparison of the viabilities of PG21 oidia when produced on varied masses of *P. sylvestris* sawdust substrate at 75 % moisture content. This is a summary of data from the control groups of a range of studies and shows good comparability between the viabilities of the smaller scale productions and 200 g substrate group but that the 400 g substrate group had less viability. Production of viable oidia was reduced per g of substrate in the largest scale of production used in this study, 400 g. The growth was not even throughout the spawn bags and the mycelial front can be seen in Plate 4.1.

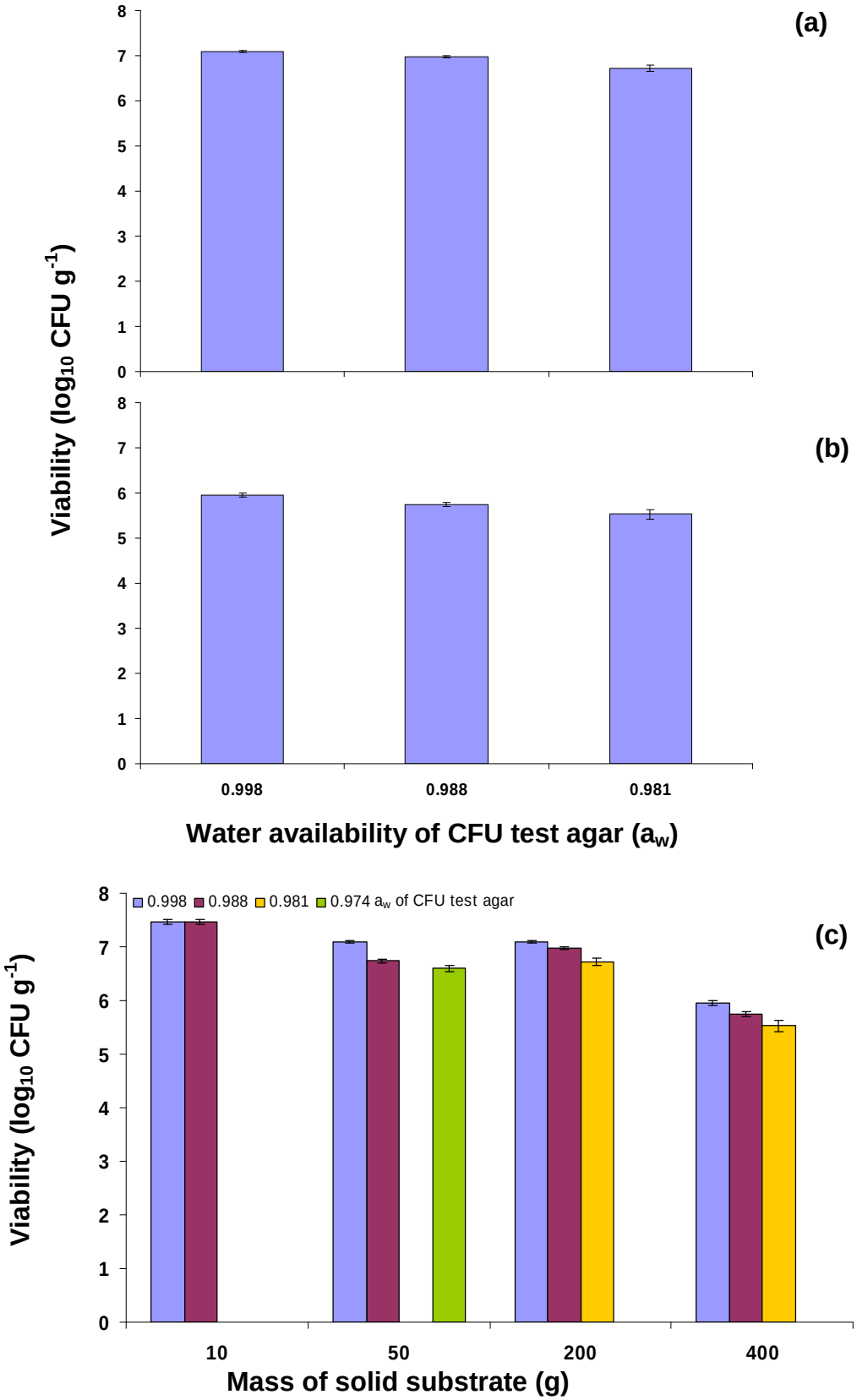


Fig. 4.17 Viability of PG21 oidia when scale of production increased to 200 g (a) 400 g substrate (b) and comparison of the viabilities across the range of production from 10 – 400 g (c); *Pinus sylvestris* sawdust at 75 % MC, incubated at 25 ºC for 3 – 6 weeks. Viability assessed as CFU on MEA at 0.998, 0.988 and 0.981 or 0.974 a_w. Bars represent standard errors of the means.

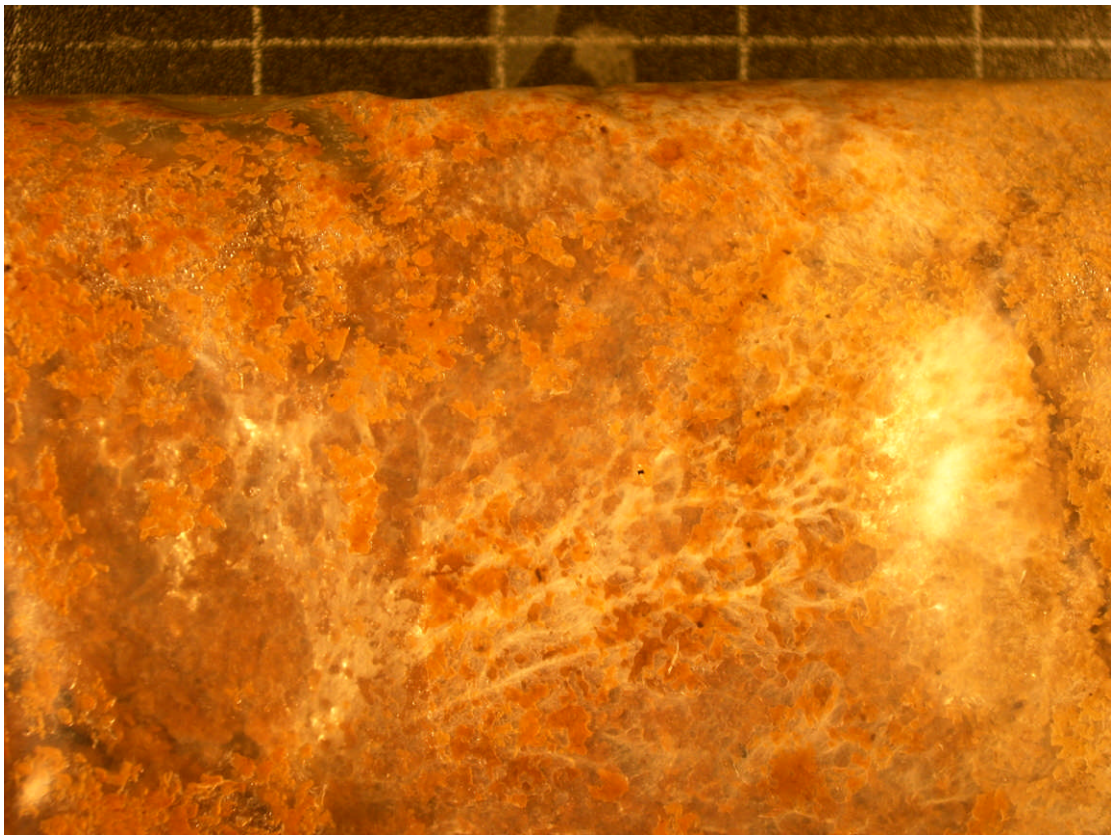


Plate. 4.1 Advancing mycelial front and rhizomorphs of PG21 on *P. sylvestris* substrate, in commercial spawn bags, at 25 ºC for 3 weeks.

4.4 Endogenous reserves: polyol content of *P. gigantea* and *H. annosum* with restricted water availability

Figure 4.18 shows the effect of restricted water availability on the production of endogenous reserves, as measured by polyol detection. Antagonist and pathogen isolates were grown on MEA with a_w modified with glycerol.

Figure 4.19 and 4.20 show the effect of matric versus solute restrictions to water availability on the endogenous alcohols of mycelial pellets and submerged conidia.

When *Pinus sylvestris* sawdust was used as a solid substrate for PG21 spore production with the addition of $CaCl_2$ and glycerol to some treatment groups, it was found that the $CaCl_2$ made little difference to the growth pattern so the results have been grouped as glycerol treatments to modify a_w. The effect of a_w modification on endogenous reserves is shown in Figure 4.21. Total polyol content was not significantly different, 1.6 – 2.4 mg g^{-1} when grown at either 0.991, 0.981 or0.974 a_w. Mannitol was increased as a proportion of the total alcohols in the lowest a_w treatment group, but was least in the intermediate a_w treatment (P <0.05). The 3 remaining alcohols measured did not show a discernable pattern. Figure 4.22 shows the total alcohols and proportional alcohols from PG21 spores produced on sawdust at the same 3 water availabilities, with 6 replicates in each group. This shows that total alcohols were significantly higher than in the previous experiment, but in concordance with previous results, did not differ between the treatments; of the proportional alcohols only mannitol was affected by a_w of the substrate (P <0.05).

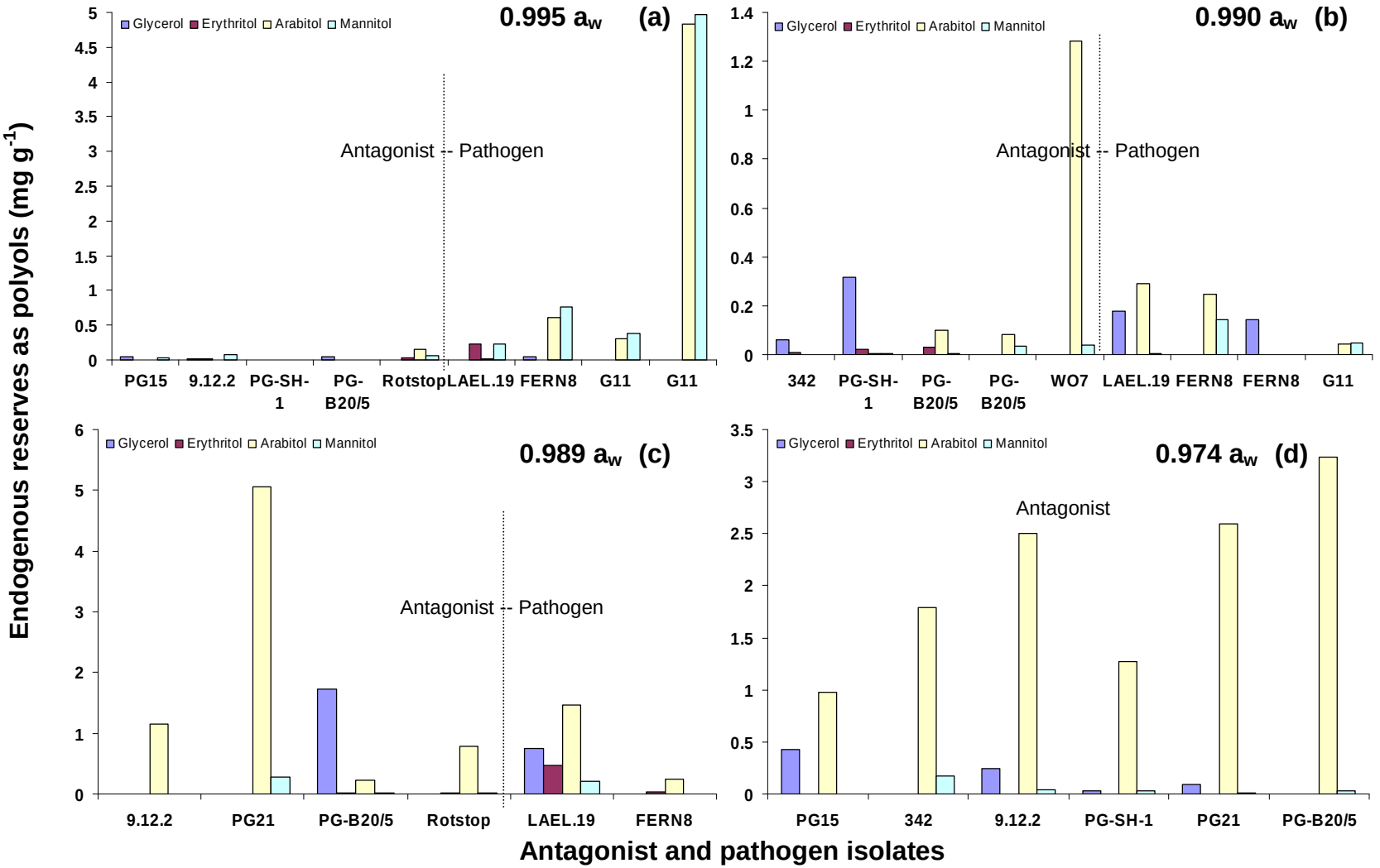


Fig. 4.18 Endogenous reserves of antagonist, *Phlebiopsis gigantea*, and pathogen, *Heterobasidion annosum*, isolates cultured on MEA at (a) 0.995, (b) 0.990, (c) 0.982 and (d) 0.974 a_w at 25 ºC for 46 (a) and (c), and 68 (b) and (d) days.

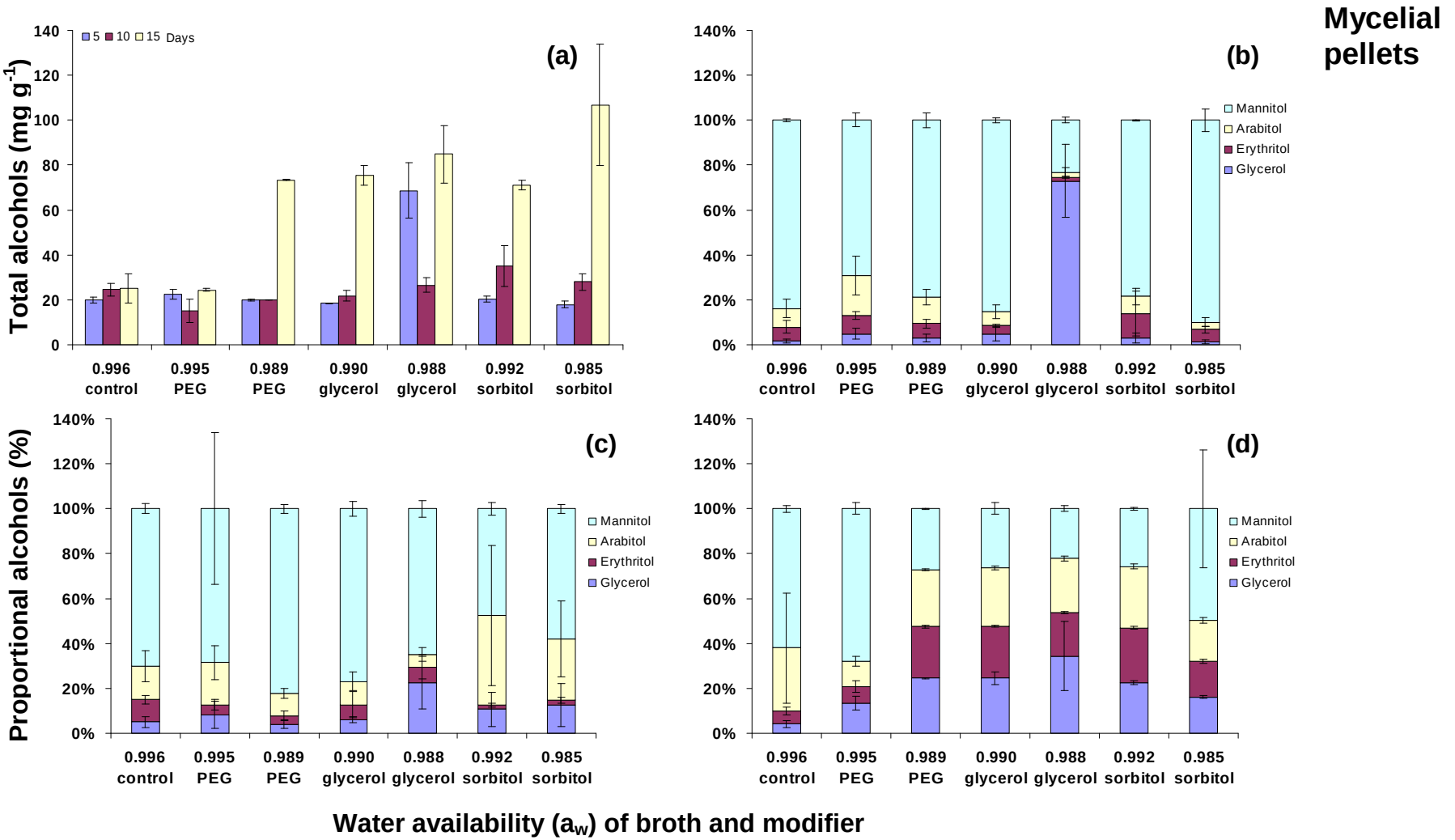


Fig. 4.19 Effect of matric and solute stress on production of endogenous sugar alcohols (a) within PG21 mycelial pellets produced in liquid fermentation of malt extract broth with restricted water availability, incubated at 25 ºC for 5 (b), 10 (c) and 15 (d) days.

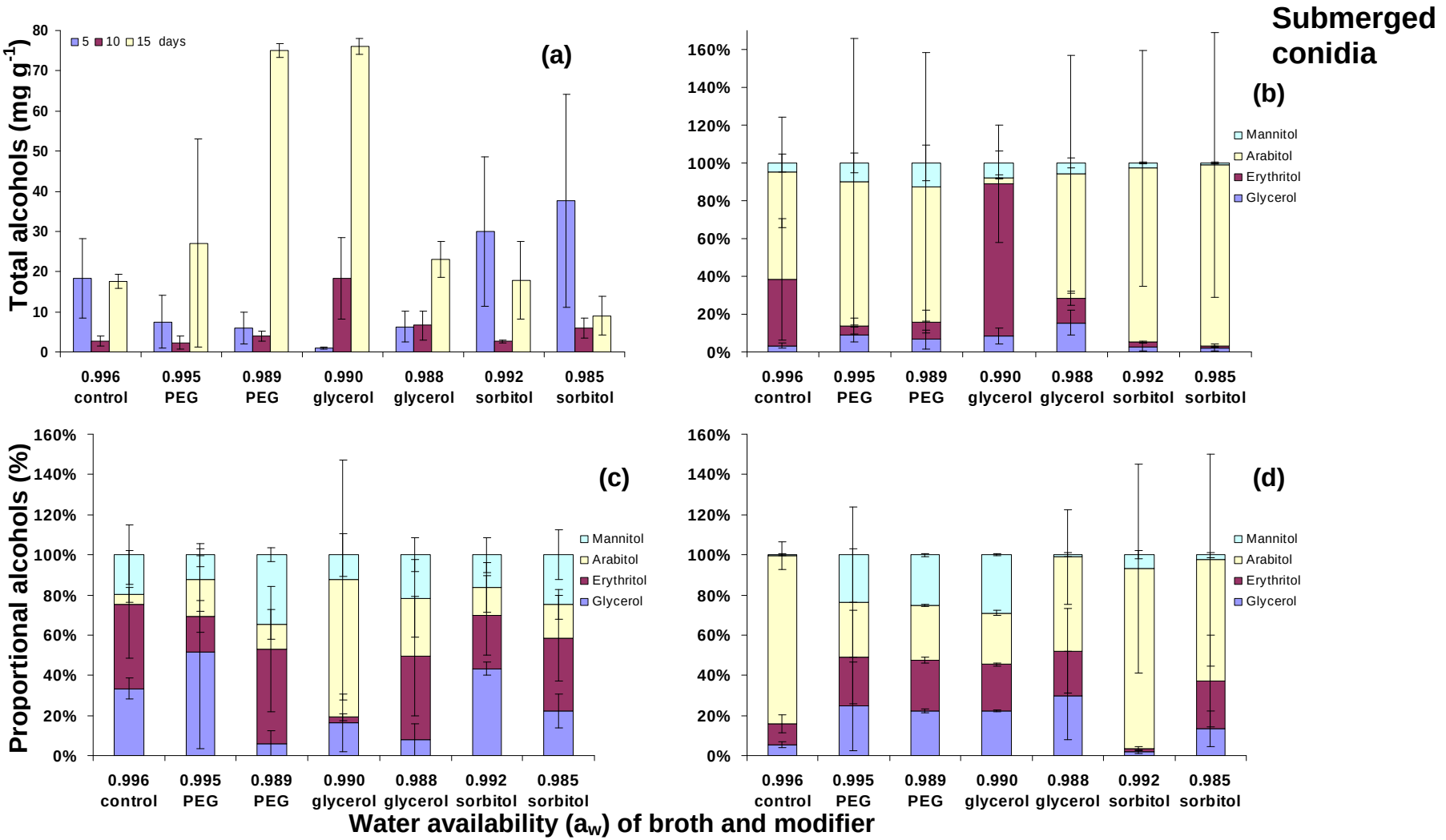


Fig. 4.20 Effect of matric and solute stress on production of endogenous sugar alcohols (a) within PG21 submerged conidia produced in liquid fermentation of malt extract broth with restricted water availability, incubated at 25 ºC for 5 (b), 10 (c) and 15 (d) days.

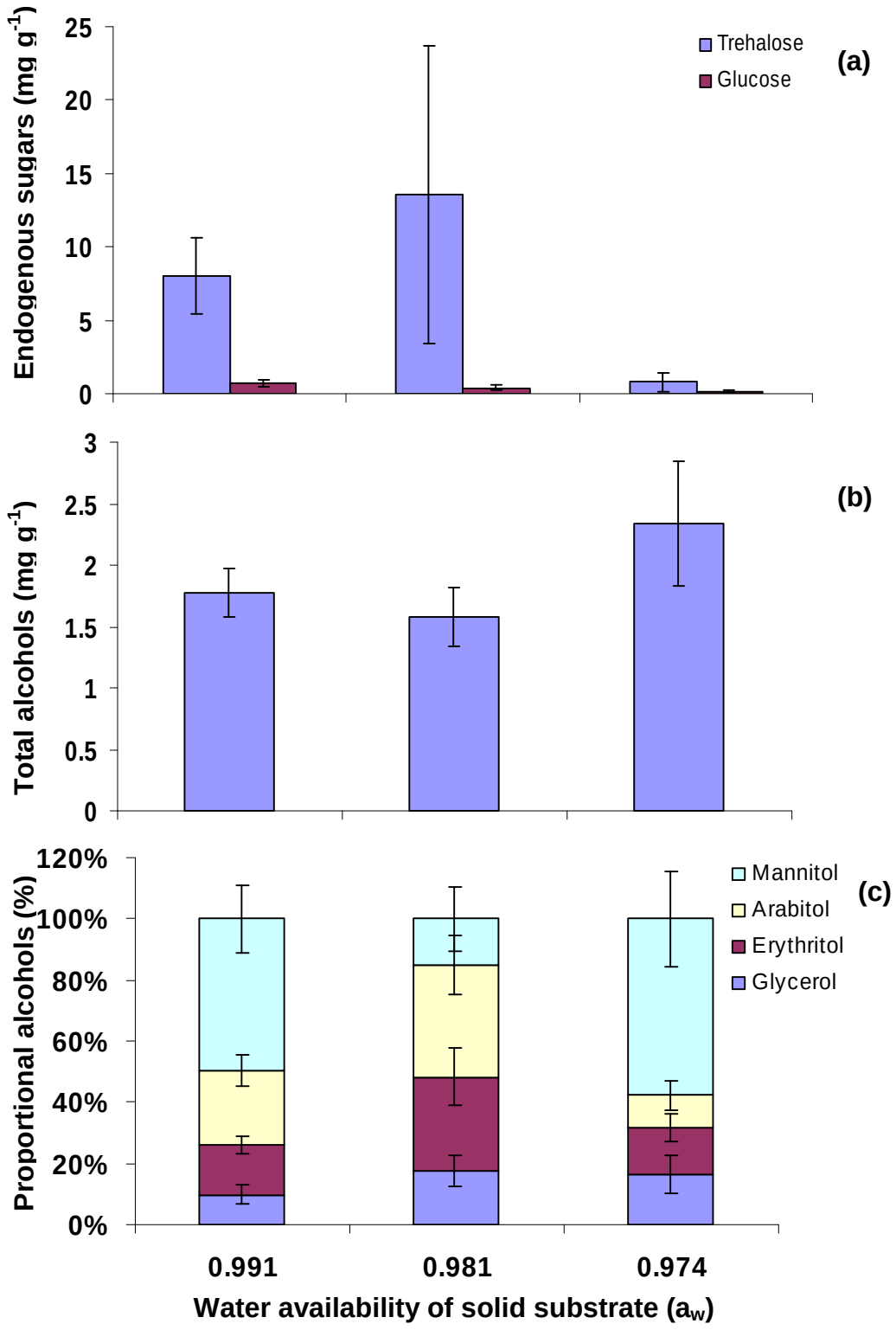


Fig. 4.21 Endogenous reserves of PG21 oidia produced on a solid substrate of *Pinus sylvestris* sawdust, at 25 ºC for three weeks, water availability modified with glycerol. NB sub- groups had additions of 20 and 40 mM $CaCl_2$. (a) endogenous sugars, (b) total sugar alcohols and (c) proportional sugar alcohols.

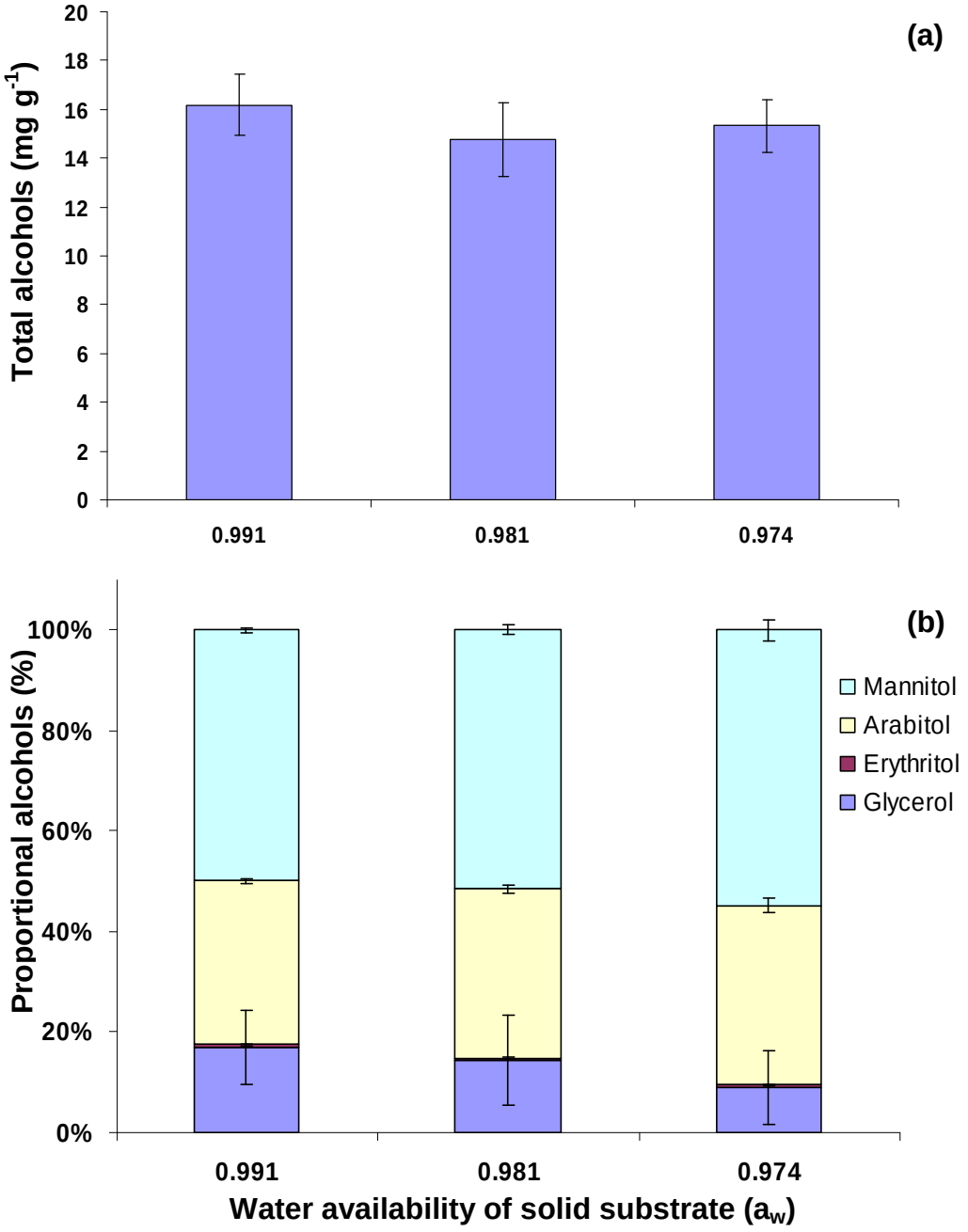


Fig. 4.22 Endogenous reserves of PG21 oidia produced on a solid substrate of *Pinus sylvestris* sawdust, water availability modified with glycerol, (a) total sugar alcohols and (b) proportional sugar alcohols. Incubation at 25 ºC for 3 weeks

4.5 Evaluation of fluidised bed drying as a means of preservation of the biocontrol agent *P. gigantea*

Pilot studies were conducted in which oidia were harvested from 4 Petri dishes of MEA and processed as described in 2.16 to the blotted pellet stage were dried in the pre-warmed uplift tube of a fluidised bed reactor at 50 ºC for 10 min. The resultant dried fragments were placed on MEA and the plates were incubated at 25 ºC. No *P. gigantea* growth occurred on MEA, but weed species were seen.

Further studies were undertaken where harvests of a larger bulk of spores, from solid substrate fermentations, were used. Spores were washed in isotonic KCl solution. Starting mass of fresh material was 1 g per sample. Table 4.6 shows the effects of temperatures from 30 – 60 ºC at 10 or 20 min drying time. The viability of re-hydrated samples was tested immediately after the drying process and was not a measure of shelf life. The results presented in each part of the table are from different trials and show that the treatment does not give predictable results. Further studies were carried out in which the wet paste was mixed 50:50 by weight with dried milk powder as a desiccant proctectant. In all cases, at all temperatures tested the resultant pellet formed into a hardened mass that would not flow in the updraft to allow the passage of air through the sample to dry it. This resulted in insufficiently dried pellets, e.g. 0.904 a_w when dried at 60 ºC for 10 min and 0.677 a_w at 20 min. With longer drying times, extremely hard pellets were obtained from which it was difficult to separate sub-samples to test for viability.

Table 4.6 Effect of fluidised bed drying at different times and temperatures on the survival of *P. gigantea* oidia. (a) Trial 1, (b) Trial 2.

(a)

Temperature (ºC)	30		40		50		60	
Time (min)	10	20	10	20	10	20	10	20
a_w	0.954	0.823	0.538	0.807	0.505	0.631	0.466	0.513
Dry mass (g)	0.1825	0.1753	0.1448	0.1797	0.1295	0.1756	0.1452	0.1419
PG growth (√) (dried sample)	√	√	√	√	√	√	√	√
Viability re-hydrated (CFU g^{-1} wet paste)	1.8 $x10^6$	8 $x10^5$	3 $x10^5$	7 $x10^5$		1 $x10^5$	1 $x10^5$	5 $x10^5$

(b)

Temperature (ºC)		40		50		60	
Time (min)		10	20	10	20	10	20
a_w		0.629	0.420	0.395	0.392	0.403	0.333
Dry mass (g)		0.1671	0.1401	0.1636	0.1774	0.1584	0.1724
Viability re-hydrated (CFU g^{-1} wet paste)	0.998 a_w	$1.4x10^7$	$1.6x10^7$	$2 x10^7$	$1.8x10^7$	$4 x10^7$	$1.2x10^7$
	0.988 a_w	$1.8x10^7$	$2.9x10^7$	$4.8x10^7$	$4 x10^7$	$6 x10^7$	$2 x10^7$
	0.981 a_w	$7.8x10^6$	$8.9x10^6$	$5.6x10^6$	$1.9x10^7$	$1.6x10^7$	$2.6x10^7$
Viability fresh paste		1.2 x 10^8 CFU g^{-1} on 0.988 and 0.981					

Chapter 5

Results: volatile fingerprints of *Phlebiopsis gigantea* and *Heterobasidion annosum*

5.1 Sensor replication within AlphaM.O.S. system

Volatile fingerprints of *P. gigantea* and *H. annosum* were analysed using an αFOX sensor array system (SAS). A typical sensor response, from one of the sets of data, is displayed in Figure 5.1, showing the replication between samples within one run. Each set of data was transformed, using discriminate factorial analysis (DFA) by the AlphaM.O.S. software, and groups within the results were analysed. All results within this chapter are displayed as paired DFA charts, at one and two weeks of culture incubation.

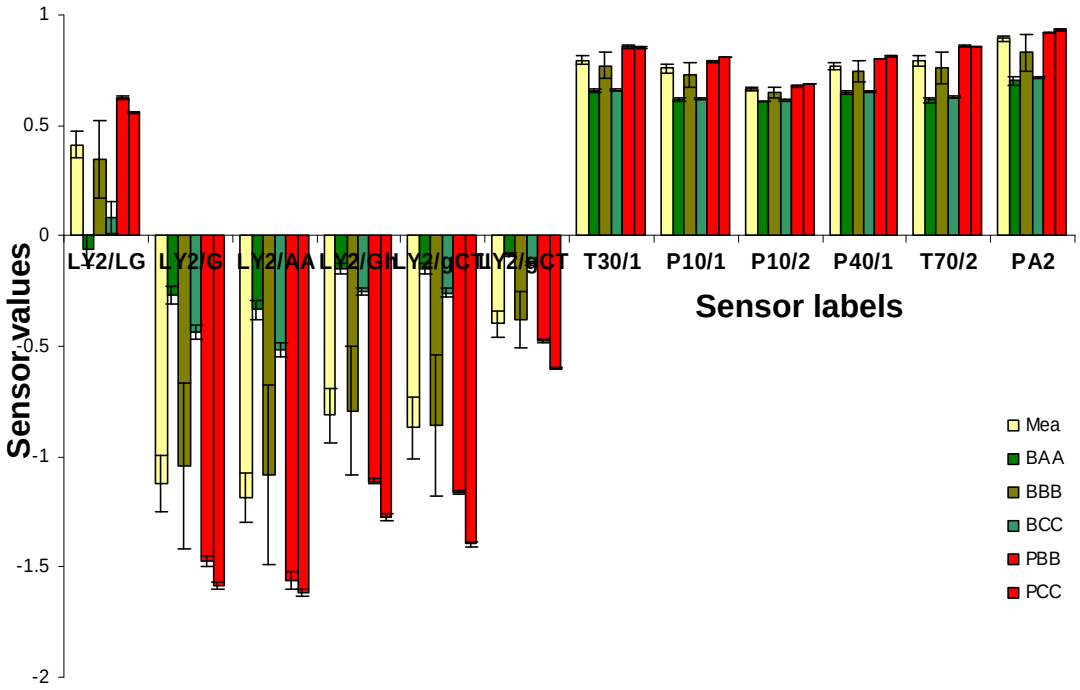


Fig. 5.1 AlphaMOS sensor response of pathogen and antagonist isolates grown on MEA.
Key: MEA agar, prefix B = biocontrol agent (antagonist), prefix P = pathogen isolate. Bars represent standard errors of the means between sample replicates.

5.2 Volatile fingerprints of *P. gigantea* and *H. annosum* on sawdust substrate

A preliminary study was undertaken with pure cultures of pathogen and antagonist isolates grown on a solid substrate of *Pinus sylvestris* sawdust. Figure 5.2 shows differentiation between the volatile fingerprints of the isolates of the pathogen compared with those of the antagonist. When sampled after 1 week of incubation on sawdust substrate the separation between species was visible on the DFA, with 94% of the information represented by component 1 and 2 of the multivariate analysis. At 2 weeks of incubation the separation was further accentuated, with the first 2 components accounting for 99% of the data.

Key to codes:

• SS	sterile sawdust	substrate	yellow
• BA	PG21	antagonist	green
• BB	PG-B20/5	antagonist	green
• BC	Rotstop	antagonist	green
• PA	LAEL.19	pathogen	red
• PB	FERN8	pathogen	red
• PC	G11	pathogen	red

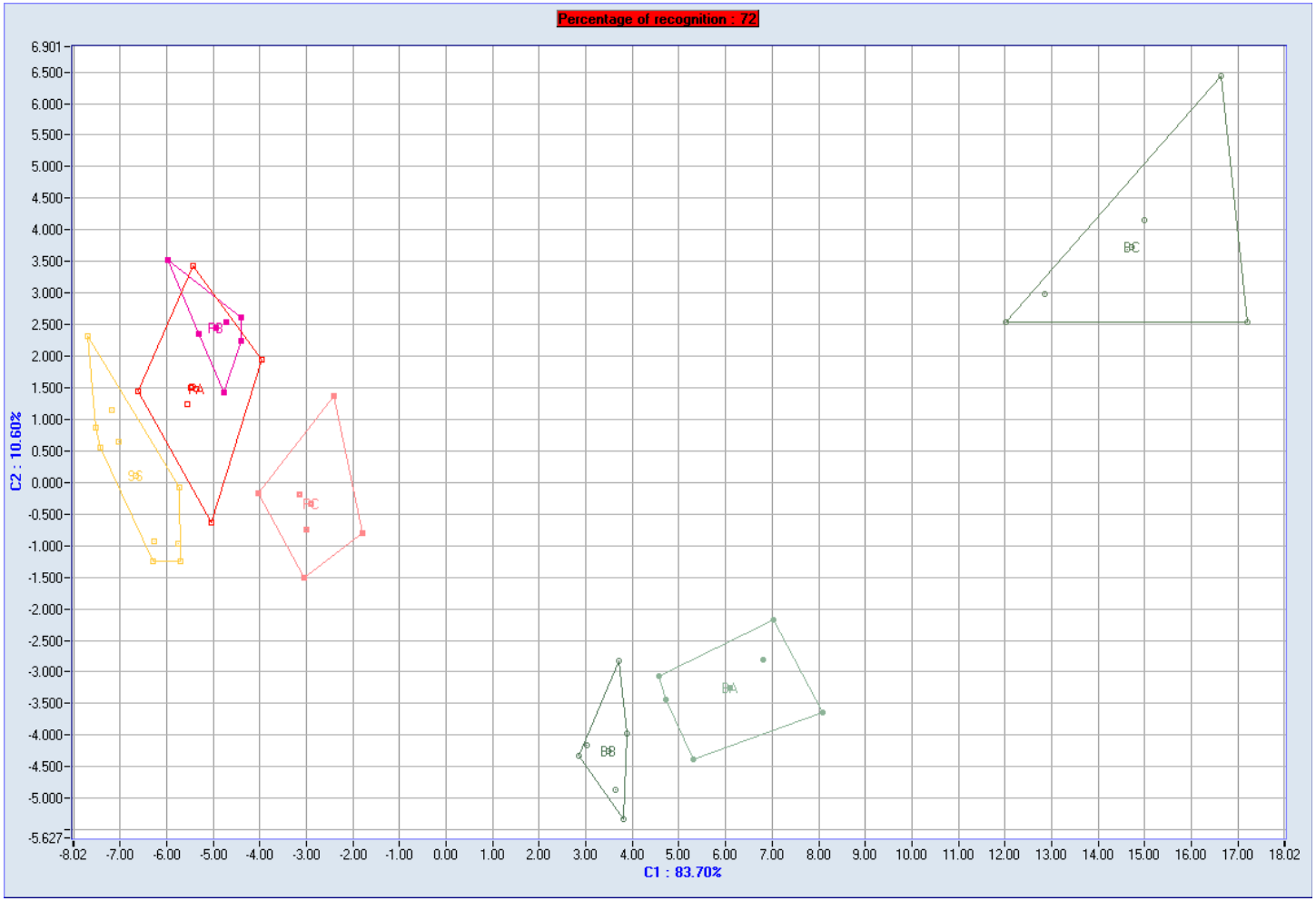
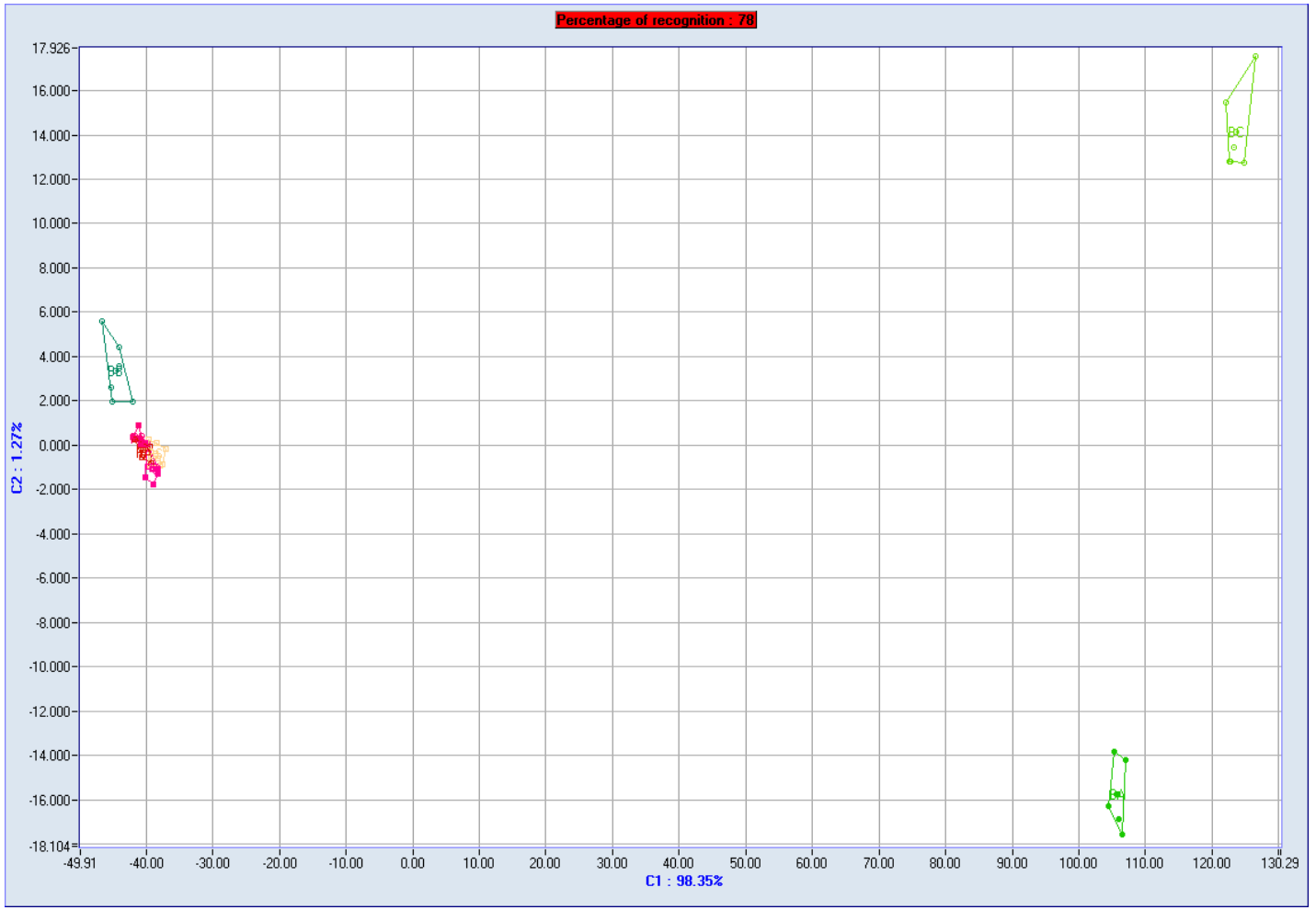


Fig. 5.2 Volatile fingerprints of pathogen and antagonist isolates grown as separate cultures on *Pinus sylvestris* sawdust, at 25 ºC for 1 and 2 weeks. Displayed as differential functional analyses (DFA); colour codes used within the charts: yellow denotes growth substrate; green, pure cultures of antagonist; red, pure cultures of known pathogen isolates; **Continued on next page**



2 Weeks

Fig. 5.2 Continued from previous page.
pinks and blues, various treatments e.g. interactions as explained for each study. Key: antagonist isolates green, BA PG21, BB PG-B20/5, BC Rotstop; pathogen isolates red, PA Lael.19, PB FERN8, PC G11; yellow, SS sterile sawdust.

5.3 Volatile fingerprints of *P. gigantea* and *H. annosum* on MEA

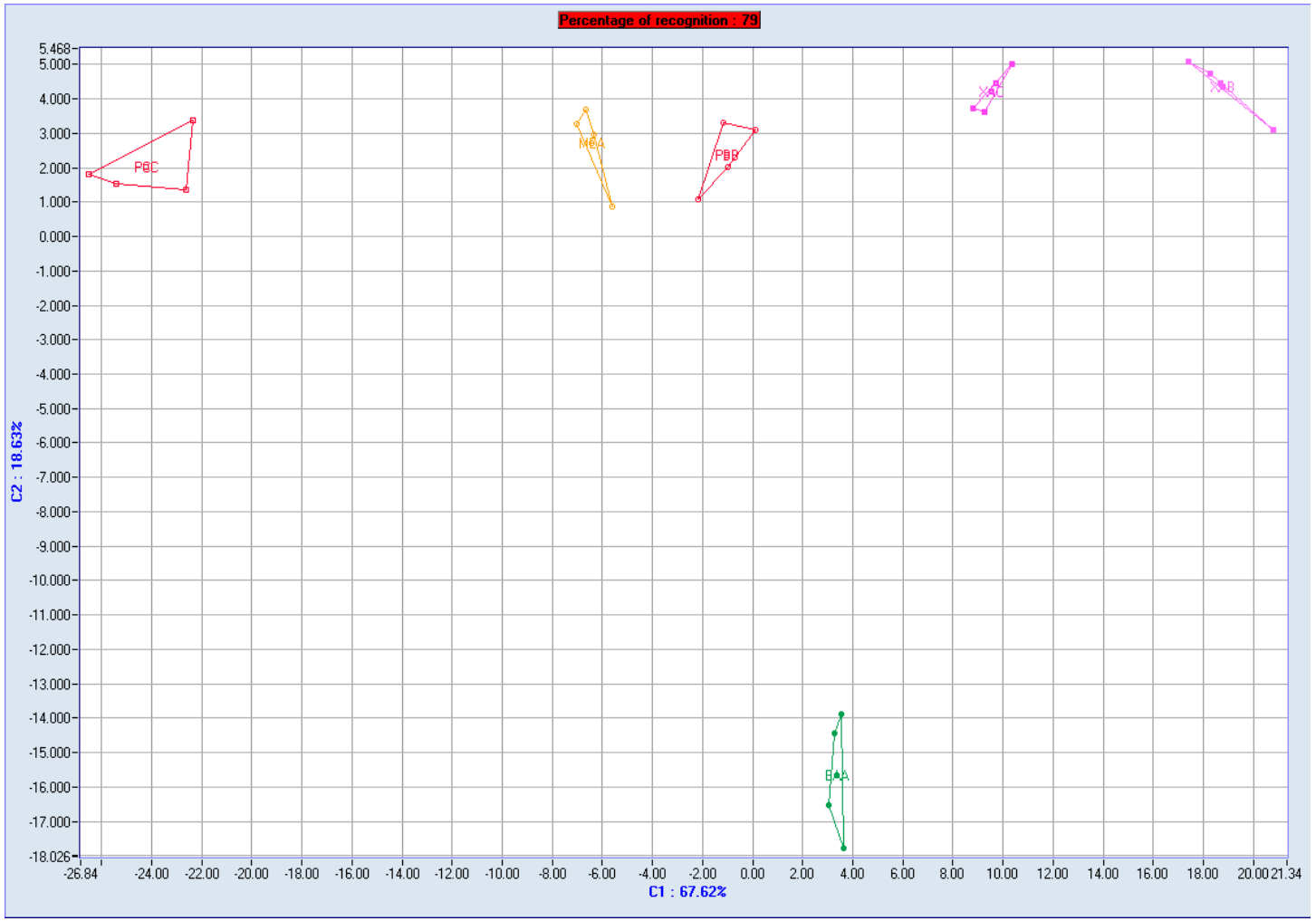
A study was undertaken with pure cultures of pathogen and antagonist isolates grown on malt extract agar (MEA) for 1 and 2 weeks. At the time of sampling four 1 cm discs of agar of each pure culture were placed into each sample vessel; amalgamations were paired, 1 antagonist and 1 pathogen, into sample vessels.

Key to codes:

• MEA	malt extract agar	substrate	yellow
• BAA	PG21	antagonist	green
• BBB	PG-B20/5	antagonist	green
• BCC	Rotstop	antagonist	green
• PBB	FERN8	pathogen	red
• PCC	G11	pathogen	red
• XAB	added BAA and PBB	pink	
• XAC	added BAA and PCC	pink	
• XBB	added BBB and PBB	pink	
• XBC	added BBB and PCC	pink	
• XCB	added BCC and PBB	pink	
• XCC	added BCC and PCC	pink	

Each of Figures 5.3 – 5.5 show differentiation between the volatile fingerprints of 1 antagonist isolate and 2 isolates of the pathogen compared with those of the amalgamations. Each figure displays the DFA after 1 week of incubation followed by 2 weeks. In each case, when sampled after 1 week the separation between species and mixtures was visible in the DFA and at 2 weeks the separation was further accentuated. The first 2 multivariate components, of each DFA, accounted for 84 - 96% of the data.

Fig. 5.3 Volatile fingerprints of pathogen and antagonist isolates grown as separate cultures on MEA, at 25 ºC for 1 and 2 weeks. Displayed as differential functional analyses (DFA). Key: antagonist isolate, green BAA, PG21; pathogen isolates, red PBB, FERN8; PCC, G11; **Continued on next page**



2 Weeks

Fig. 5.3 Continued from previous page.
pink XAB and XAC antagonist and pathogen samples mixed prior to sampling; yellow, MEA.

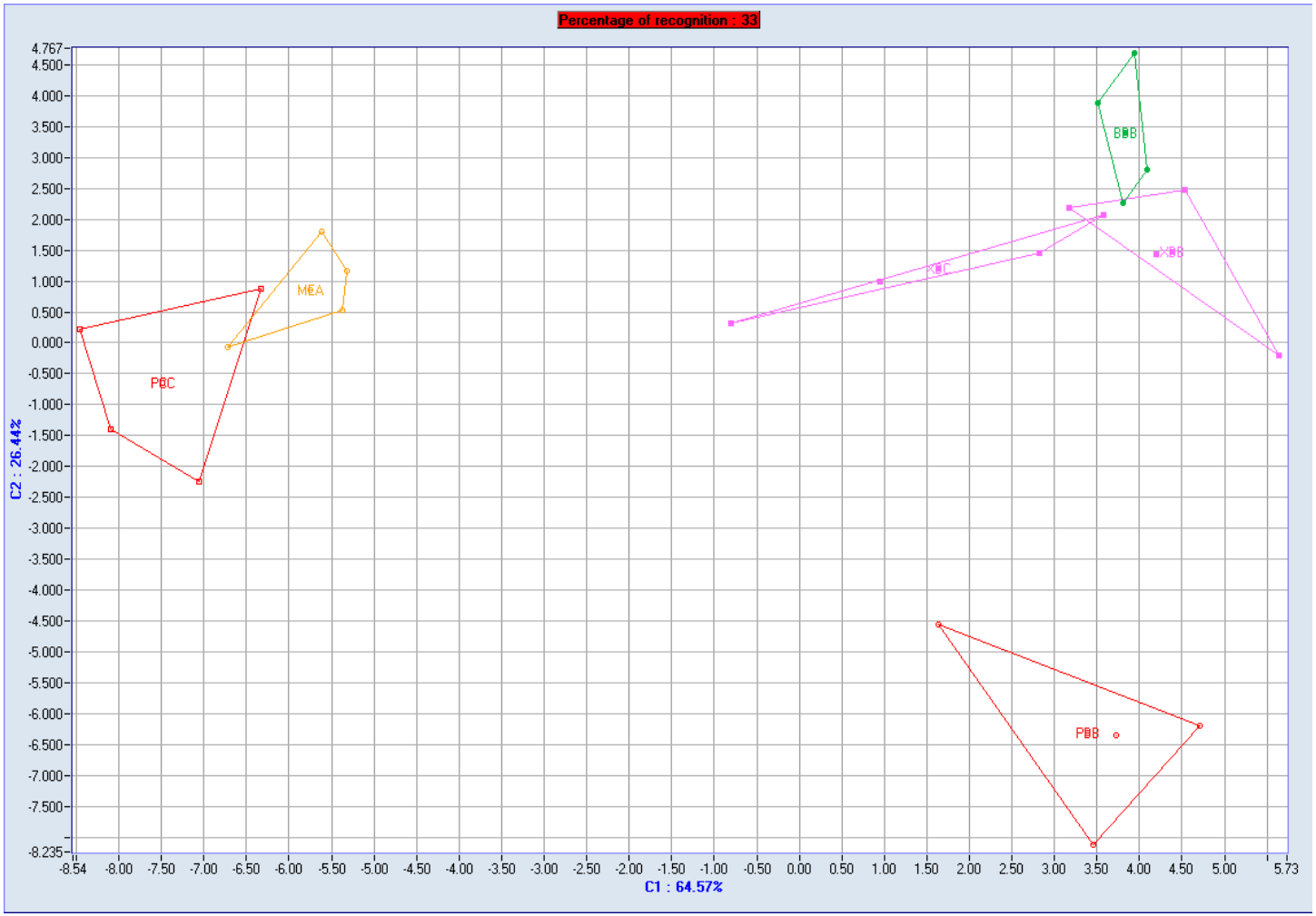
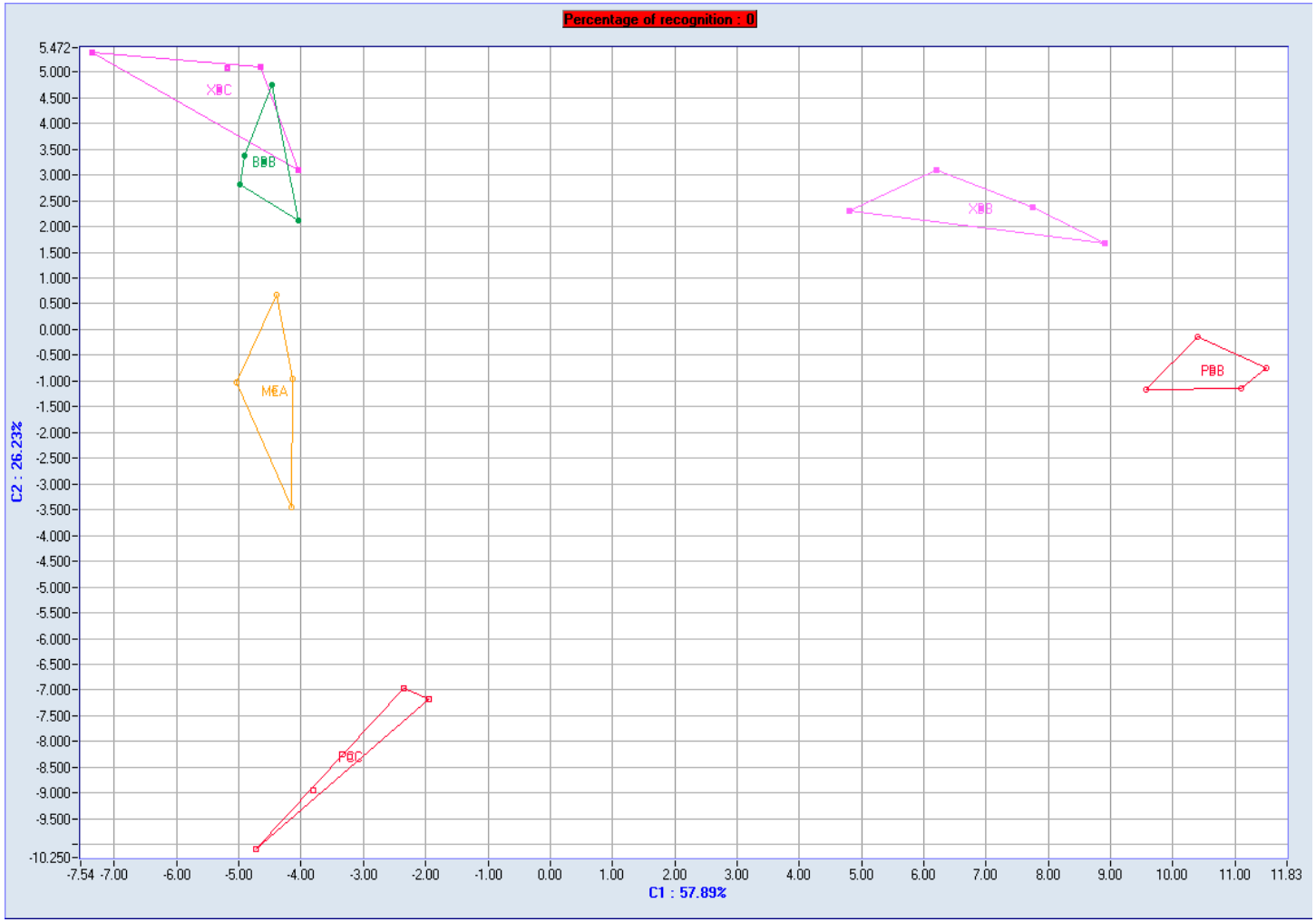
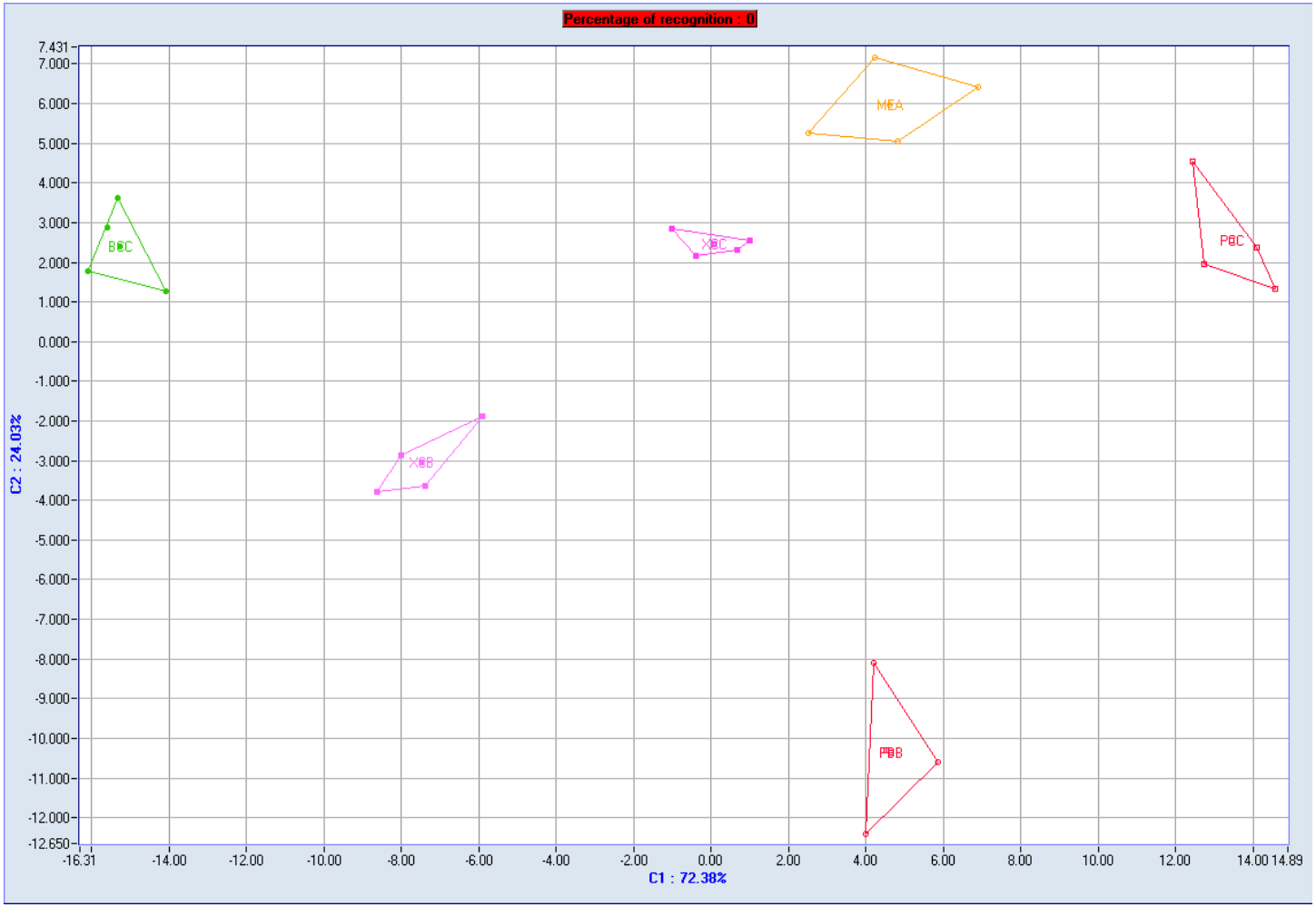


Fig. 5.4 Volatile fingerprints of pathogen and antagonist isolates grown as separate cultures on MEA, at 25 ºC for 1 and 2 weeks. Displayed as differential functional analyses (DFA). Key: antagonist isolate, green BBB, PG-B20/5; pathogen isolates, red PBB, FERN8; PCC, G11; **Continued on next page**



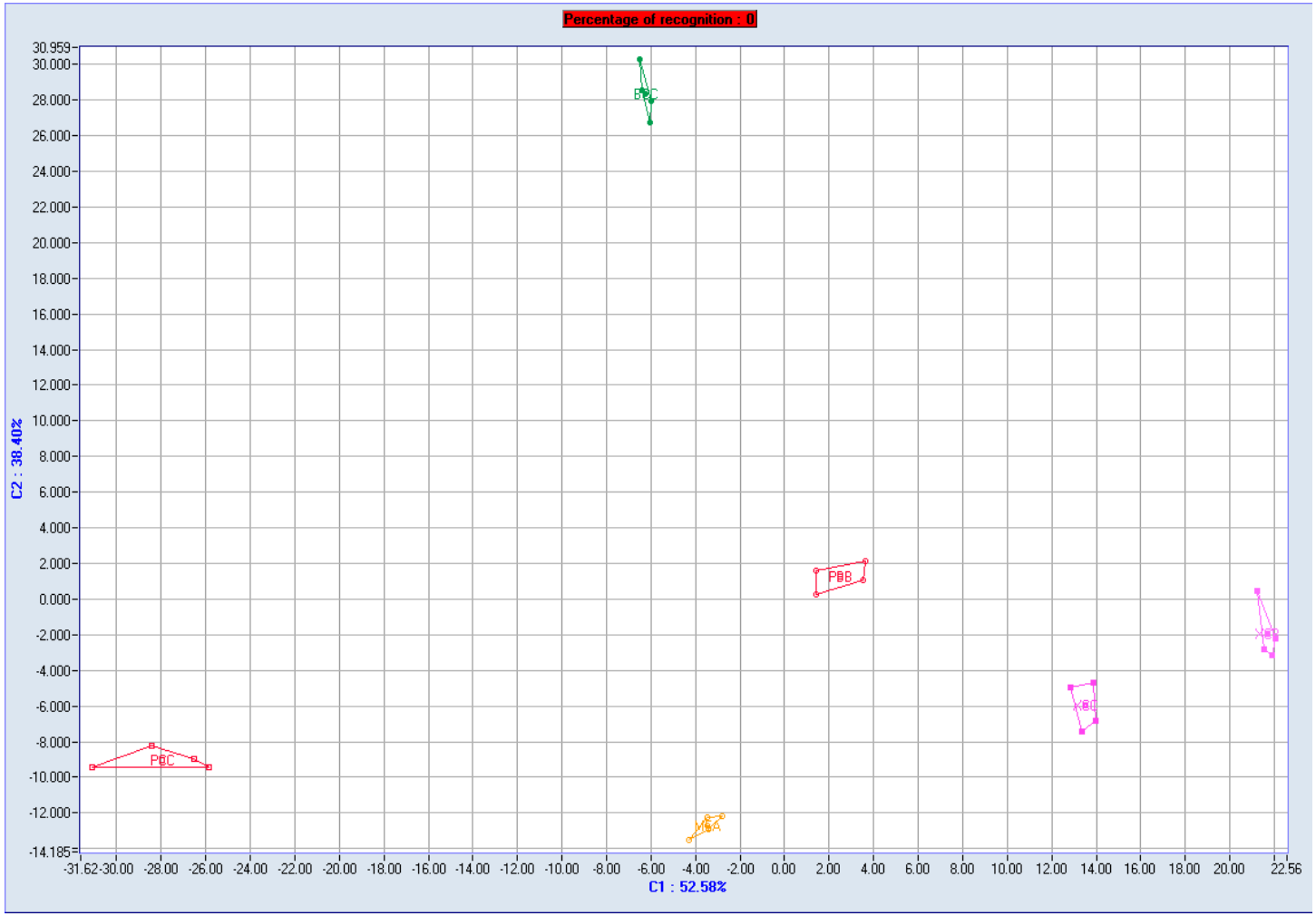
2 Weeks

Fig. 5.4 Continued from previous page
pink XBB and XBC antagonist and pathogen samples mixed prior to sampling; yellow, MEA.



1 Week

Fig. 5.5 Volatile fingerprints of pathogen and antagonist isolates grown as separate cultures on MEA, at 25 ºC for 1 and 2 weeks. Displayed as differential functional analyses (DFA). Key: antagonist isolate, green BCC, Rotstop; pathogen isolates, red PBB, FERN8; PCC, G11; **Continued on next page**



2 Weeks

Fig. 5.5 Continued from previous page
pink XCB and XCC antagonist and pathogen samples mixed prior to sampling; yellow, MEA.

5.4 Volatile fingerprints of interactions between antagonists and pathogen strains on MEA

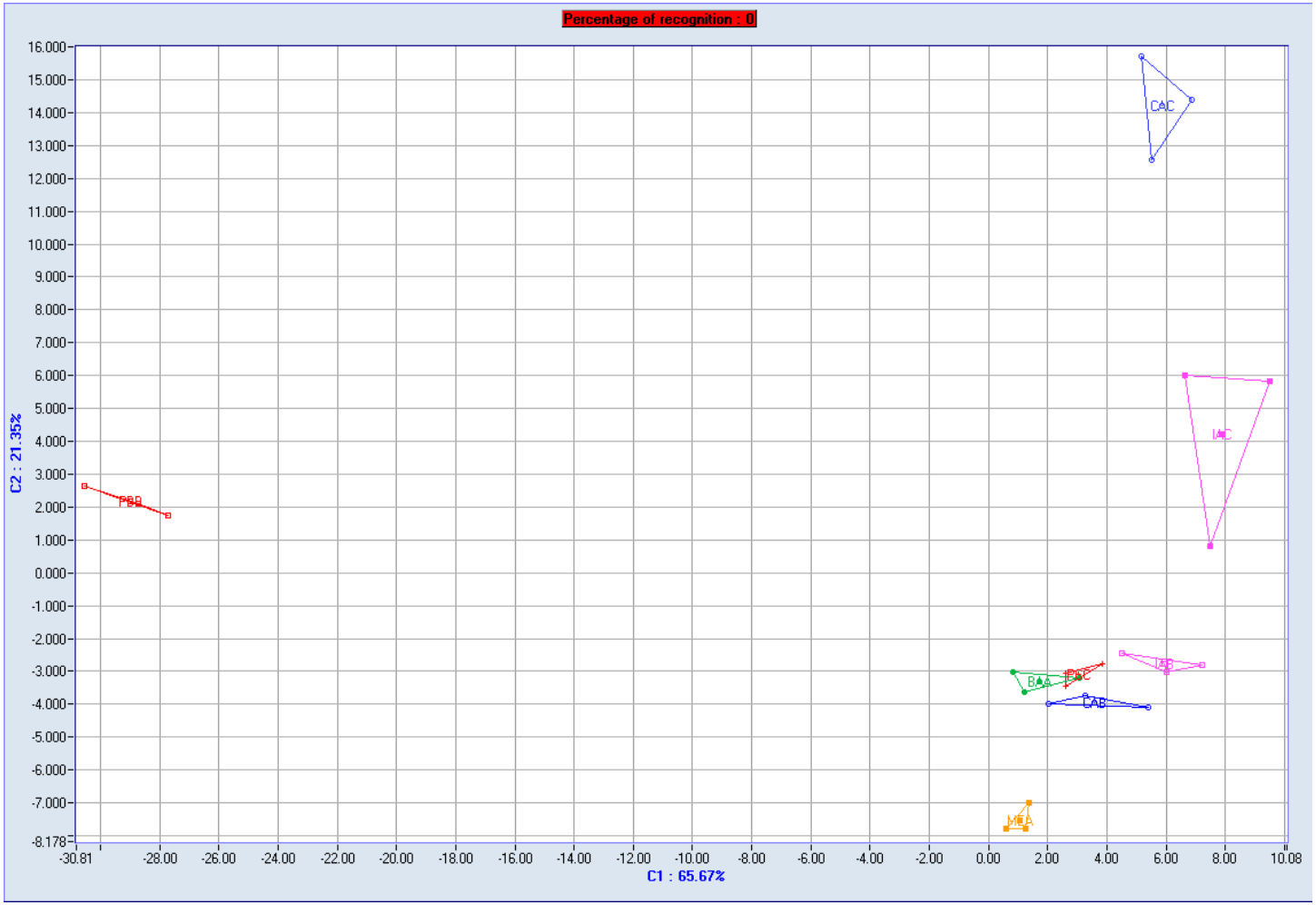
A further study was undertaken using the same isolates of pathogen and antagonist as used previously described in section 5.3. These were grown as pure cultures and paired in competition, as co-inoculated mixed lawns and as interactions from point inoculations.

Key to codes:

• MEA	malt extract agar	substrate	yellow
• BAA	PG21	antagonist	green
• BBB	PG-B20/5	antagonist	green
• BCC	Rotstop	antagonist	green
• PBB	FERN8	pathogen	red
• PCC	G11	pathogen	red
• IAB	interaction zone between BAA and PBB		pink
• IAC	interaction zone between BAA and PCC		pink
• CAB	co-inoculated lawn of BAA and PBB		blue
• CAC	co-inoculated lawn of BAA and PCC		blue
• IBB	interaction zone between BBB and PBB		pink
• IBC	interaction zone between BBB and PCC		pink
• CBB	co-inoculated lawn of BBB and PBB		blue
• CBC	co-inoculated lawn of BBB and PCC		blue
• ICB	interaction zone between BCC and PBB		pink
• ICC	interaction zone between BCC and PCC		pink
• CCB	co-inoculated lawn of BCC and PBB		blue
• CCC	co-inoculated lawn of BCC and PCC		blue

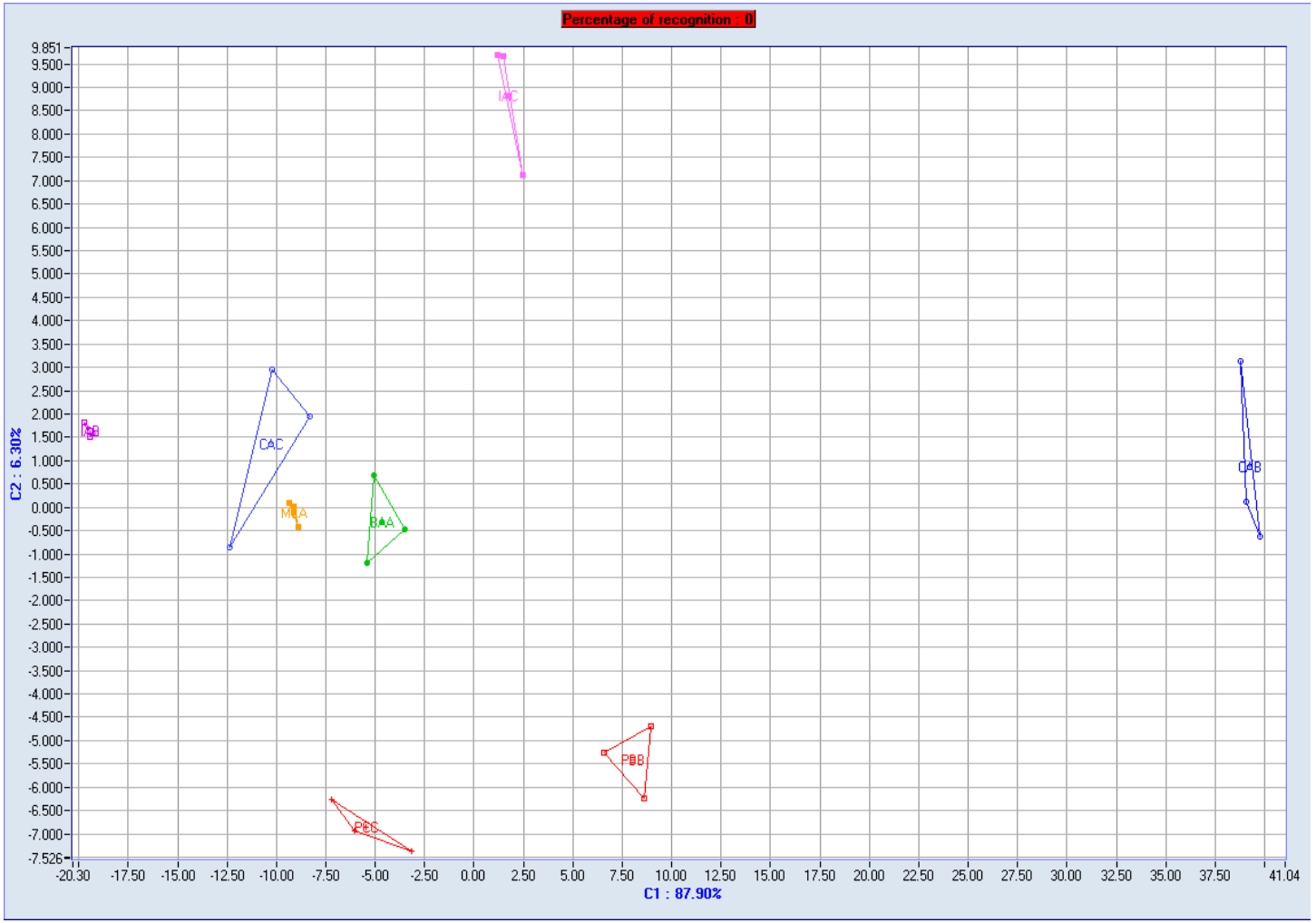
Each of Figures 5.6 – 5.8 show clearer separation of volatile fingerprints of the samples by 2 weeks of incubation. The first 2 multivariate components of each DFA accounted for 90 - 94% of the data. Figures 5.6 -5.8 show the volatile fingerprints of antagonists PG21, PG-B20/5 and Rotstop, respectively, in interaction with the

pathogens FERN8 and G11. With both PG21 and Rotstop (Figures 5.6 and 5.8) there is clear separation between species and both forms of interactions. In Figure 5.8 the fingerprints of the lawns and of the interaction zone with the pathogen G11 are near to the fingerprint of the antagonist Rotstop but the fingerprints of both pathogens are distant. Interestingly, in Figure 5.7, the fingerprints of both lawns are co-incidental with that of the antagonist, PG-B20/5, and those of the interactions are close, meanwhile those of the pathogen isolates are distant.



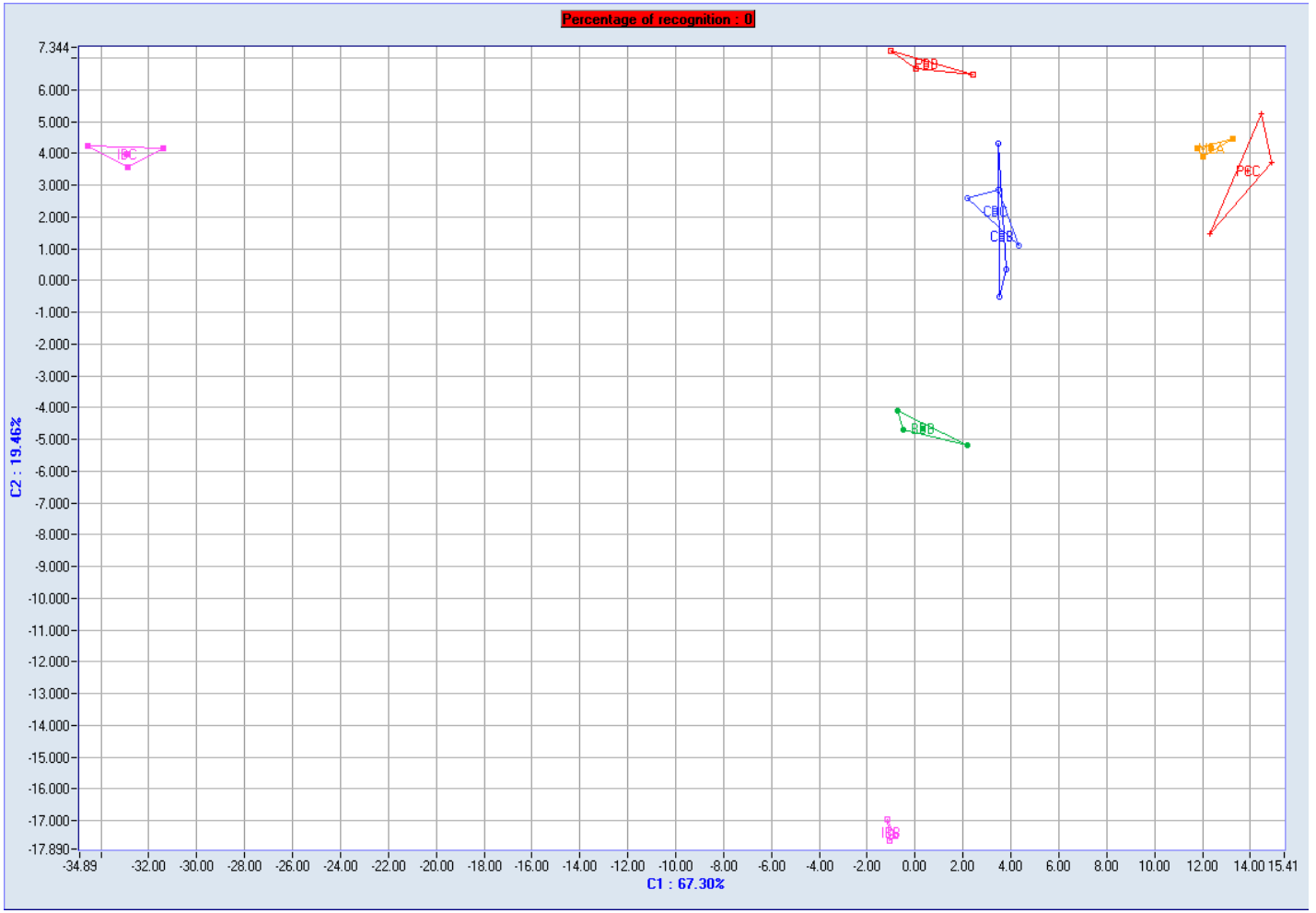
1 Week

Fig. 5.6 Volatile fingerprints of pathogen and antagonist isolates grown as separate cultures, as mixed lawns and as interactions on MEA, at 25 ºC for 1 and 2 weeks. Displayed as differential functional analyses (DFA). Key: antagonist isolate, green BAA, PG21; pathogen isolates, red PBB, FERN8; PCC, G11; pink IAB and IAC antagonist and **Continued on next page**



2 Weeks

Fig. 5.6 Continued from previous page
pathogen interaction zones; blue antagonist and pathogen co-inoculated lawns CAB and CAC; yellow, MEA



1 Week

Fig. 5.7 Volatile fingerprints of pathogen and antagonist isolates grown as separate cultures, as mixed lawns and as interactions on MEA, at 25 ºC for 1 and 2 weeks. Displayed as differential functional analyses (DFA). Key: antagonist isolate, green BBB, PG-B20/5; pathogen isolates, red PBB, FERN8; PCC, G11; **Continued on next page**

Fig. 5.7 Continued from previous page
pink IBB and IBC, antagonist and pathogen interaction zones; blue antagonist and pathogen co-inoculated lawns CBB and CCC; yellow, MEA

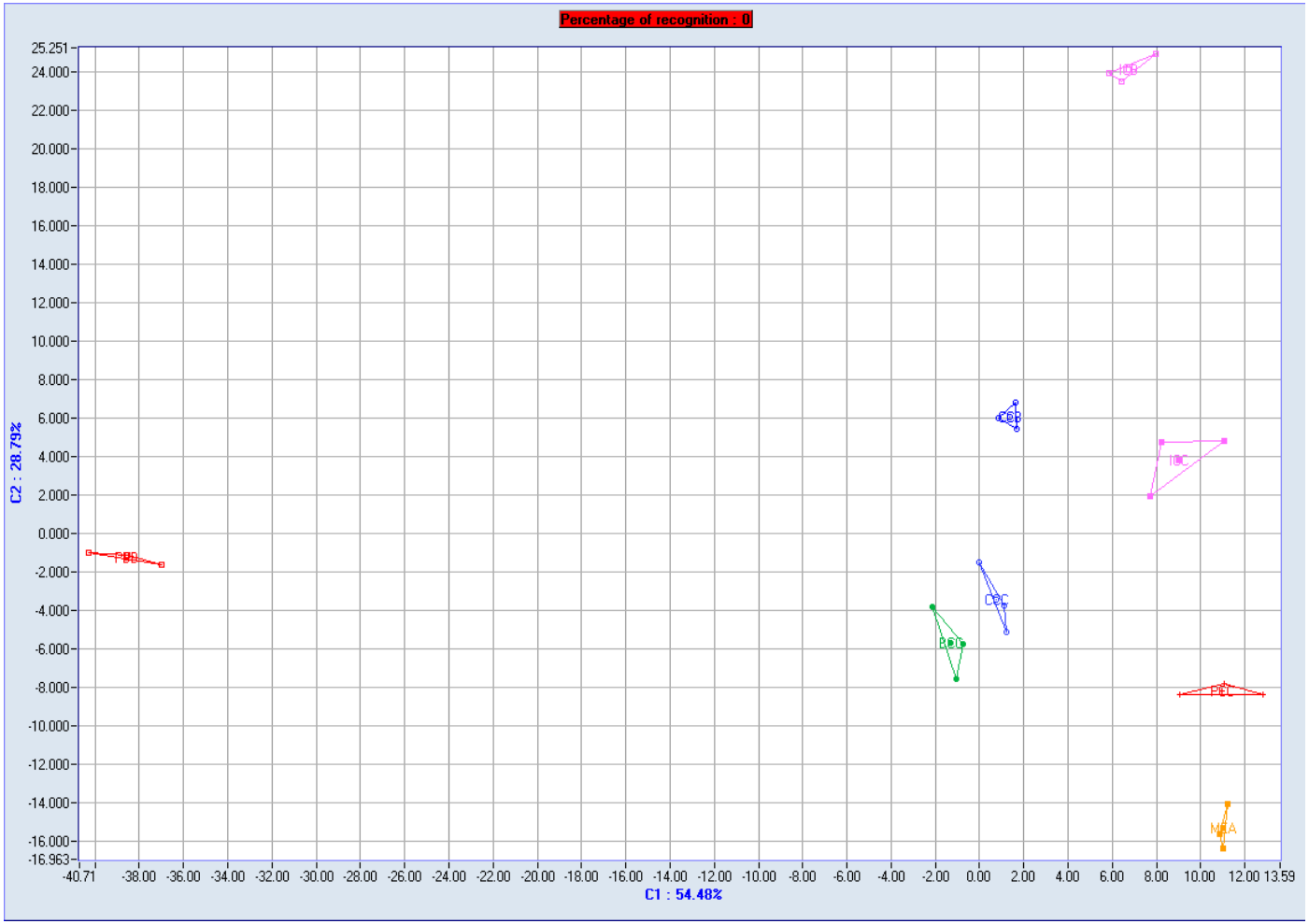


Fig. 5.8 Volatile fingerprints of pathogen and antagonist isolates grown as separate cultures, as mixed lawns and as interactions on MEA, at 25 ºC for 1 and 2 weeks. Displayed as differential functional analyses (DFA). Key: antagonist isolate, green BCC, Rotstop; pathogen isolates, red PBB, FERN8, PCC, G11; **Continued on next page**

2 Weeks

Fig. 5.8 Continued from previous page
pink antagonist and pathogen interaction zones ICB and ICC; blue antagonist and pathogen co-inoculated lawns CCB and CCC; yellow, MEA

5.5 Volatile fingerprints of isolates of *P. gigantea* and *H. annosum* on MEA

A further study was undertaken with cultures of antagonist and pathogen isolates grown on malt extract agar (MEA) for 1 and 2 weeks to see if they would be grouped with isolates used in previous studies.

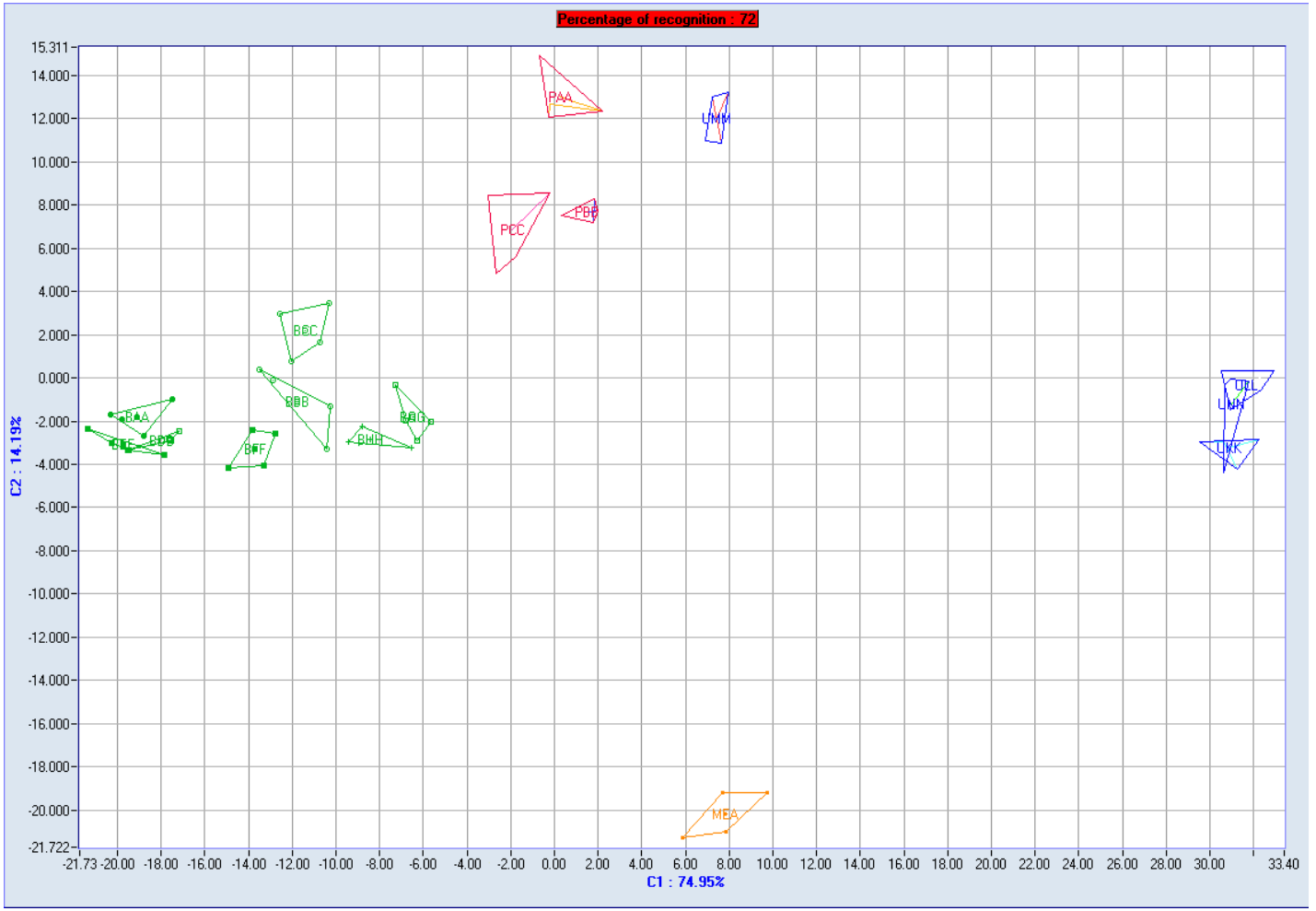
Key to codes:

• MEA	malt extract agar	substrate	yellow
• BAA	PG21	antagonist	green
• BBB	PG-B20/5	antagonist	green
• BCC	Rotstop	antagonist	green
• BDD	342	antagonist	green
• BEE	WO7	antagonist	green
• BFF	PG15	antagonist	green
• BGG	BU4	antagonist	green
• BHH	PG-SH-1	antagonist	green
• PAA	LAEL.19	pathogen	red
• PBB	FERN8	pathogen	red
• PCC	G11	pathogen	red
• UKK	T3,6 Fb2or3	pathogen	blue
• ULL	T13,12 Fb31	pathogen	blue
• UMM	Plot H	pathogen	blue
• UNN	T5,5 Sp8	pathogen	blue

Figure 5.9 shows clusters of fingerprints that are not clearly defined after 1 week incubation. However, after 2 weeks there was clear clustering of fingerprints of antagonist isolates. Volatile fingerprints of pathogen isolates formed 2 clusters, those from Bennachie Forest, north-east Scotland i.e. T3,6 Fb2or3, T13,12 Fb31 and T5,5 Sp8 in one group and Plot H from Castle Archdale Forest, Northern Ireland, joining the cluster of LAEL.19, FERN8 and G11 from Grace, Scotland. The first 2 multivariate components, of the 2-week DFA, accounted for 89% of the data.

1 Week

Fig. 5.9 Further identification of isolates. Volatile fingerprints of pathogen and antagonist isolates grown as separate cultures on MEA, at 25 ºC for 1 and 2 weeks. Displayed as differential functional analyses (DFA) Key: antagonist isolates green, BAA PG21, BBB PG-B20/5, BCC Rotstop, BDD 342, BEE W07, BFF PG15, BGG BU4, BHH PG-SH-1 **Continued on next page**



2 Weeks

Fig. 5.9 Continued from previous page;
pathogen isolates red, PAA LAEL.19, PBB FERN8, PCC G11; further pathogen isolates blue, UKK T3,6 Fb2or3, ULL T13,12 Fb31, UMM Plot H, UNN T5,5 Sp8; yellow, MEA

5.6 Interactions between antagonist and pathogen

A further study was undertaken using some of the antagonist isolates examined in section 5.5. These were grown as co-inoculated mixed lawns with pathogen isolates.

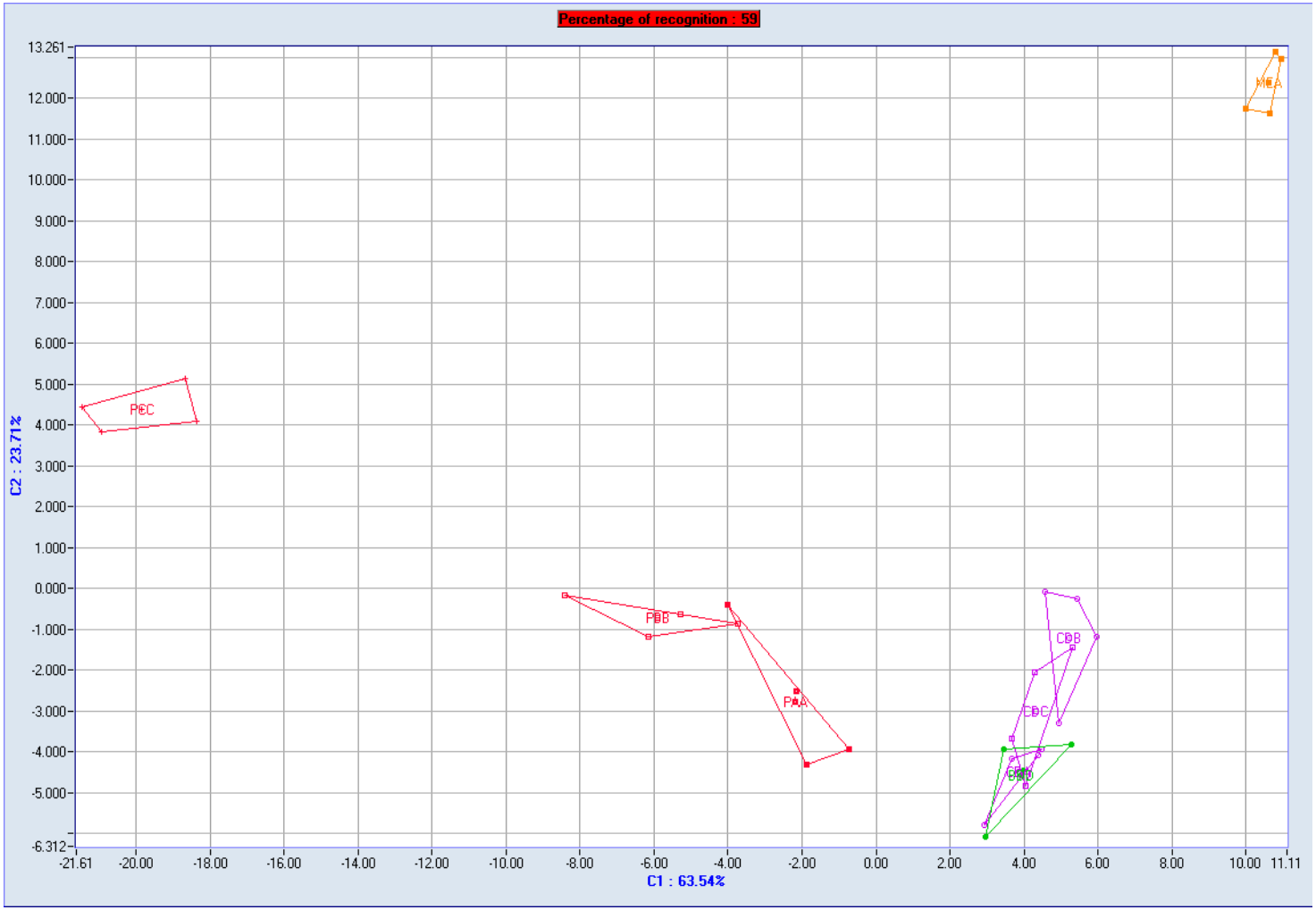
Key to codes:

- MEA malt extract agar substrate yellow
- BDD 342 antagonist green
- BEE WO7 antagonist green
- BFF PG15 antagonist green
- PAA LAEL.19 pathogen red
- PBB FERN8 pathogen red
- PCC G11 pathogen red
- CDA co-inoculated lawn of BDD and PAA pink
- CDB co-inoculated lawn of BDD and PBB pink
- CDC co-inoculated lawn of BDD and PCC pink
- CEA co-inoculated lawn of BEE and PAA pink
- CEB co-inoculated lawn of BEE and PBB pink
- CEC co-inoculated lawn of BEE and PCC pink
- CFA co-inoculated lawn of BFF and PAA pink
- CFB co-inoculated lawn of BFF and PBB pink
- CFC co-inoculated lawn of BFF and PCC pink

Figure 5.10 shows the 7 d volatile fingerprints of the treatments are close to that of the antagonist, 342, and closer or co-incidental by 14 days, meanwhile those of the pathogen isolates were more distant. In Figure 5.11 there was a grouping of fingerprints of the mixed inoculum and the antagonist WO7; the pathogen fingerprints were distant. In Figure 5.12 the antagonist PG15 strongly overlapped with interaction treatments after 7 days. The first 2 multivariate components, of each DFA, accounted for 87 - 98% of the data.

1 Week

Fig. 5.10 Volatile fingerprints of pathogen and antagonist isolates grown as separate cultures and as mixed lawns on MEA, at 25 ºC for 1 and 2 weeks. Displayed as differential functional analyses (DFA). Key: antagonist isolate, green BDD 342; pathogen isolates red, PAA LAEL.19, PBB FERN8, PCC G11; **Continued on next page**



2 Weeks

Fig. 5.10 Continued from previous page;
pink antagonist and pathogen co-inoculated lawns CDA, CDB and CDC; yellow MEA.

Fig. 5.11 Volatile fingerprints of pathogen and antagonist isolates grown as separate cultures and as mixed lawns on MEA, at 25 ºC for 1 and 2 weeks. Displayed as differential functional analyses (DFA). Key: antagonist isolate, green BEE W07; pathogen isolates red, PAA LAEL.19, PBB FERN8, PCC G11; **Continued on next page**

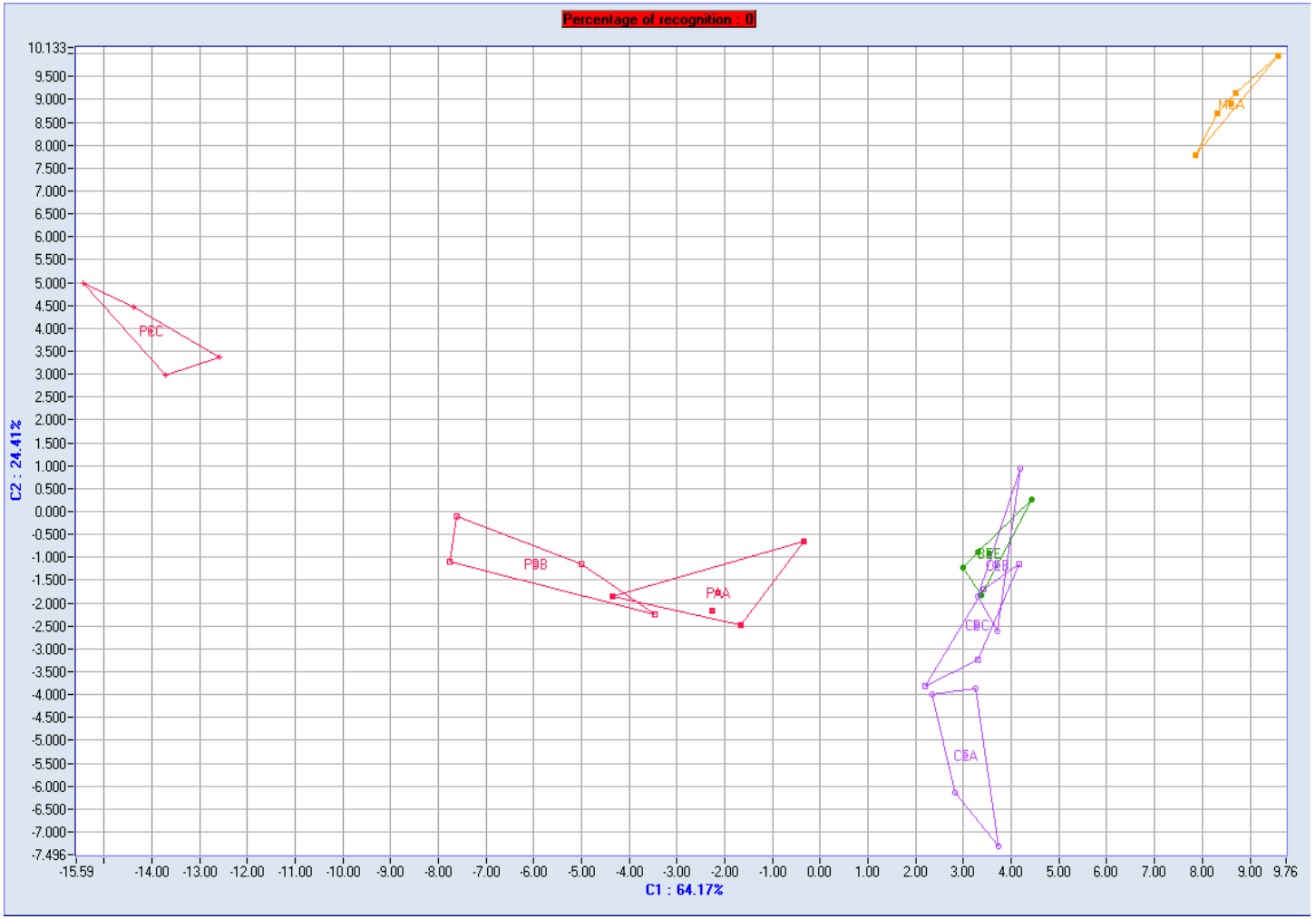


Fig. 5.11 Continued from previous page;
pink antagonist and pathogen co-inoculated lawns CEA, CEB and CEC; yellow MEA.

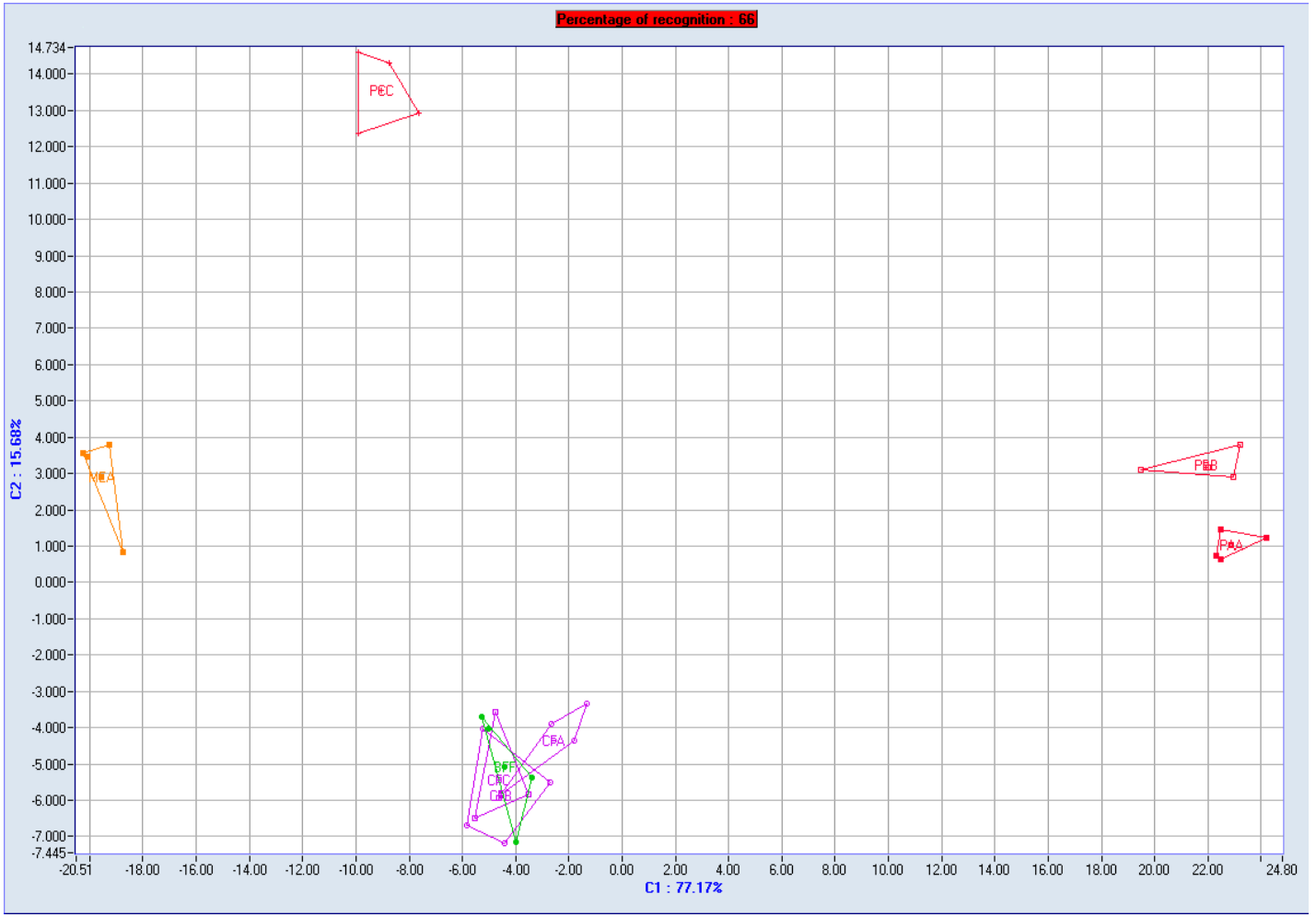


Fig. 5.12 Volatile fingerprints of pathogen and antagonist isolates grown as separate cultures and as mixed lawns on MEA, at 25 ºC for 1 and 2 weeks. Displayed as differential functional analyses (DFA). Key: antagonist isolate, green BFF PG15; pathogen isolates red, PAA LAEL.19, PBB FERN8, PCC G11; **Continued on next page**

2 Weeks

Fig. 5.12 Continued from previous page
pink antagonist and pathogen co-inoculated lawns CFA, CFB and CFC; yellow MEA.

Chapter 6

Discussion

6.1 Water availability in the surface of tree stumps

Little quantitative information was available previously on the temporal rates of water availability changes in freshly cut *Pinus sylvestris* tree stumps prone to infection by *H. annosum*. There is a reduction in moisture content in tissues as wood slowly dies after felling. This reduction in water content, along with other changes that occur during seasoning has an effect on the invasion of and colonisation by different microorganisms (Bendz-Heldren & Stenlid, 1998; Murray & Woodward, 2007; Schoeman *et al*., 1999). During the present study, it was observed that the available water in the surface tissue of stumps over a one month period changed rapidly. There was a rapid reduction of 0.01 a_w within the first three days, and during the course of the study a_w remained in the region of 0.967 – 0.985 a_w. Of course this still allows a range of fungi to colonise the stumps but a narrower range of a_w in the wetter range is necessary for both antagonist and pathogen establishment. During this monitoring period it was cold and inclement with some precipitation of both rain and snow. Thus under warmer, drier periods of time water loss from the stumps would probably be more rapid further restricting the window of opportunity for effective establishment of either antagonist or pathogen, as during this study, each grew very slowly at less than 0.74 a_w.

6.2 Effect of water availability and solute type on the growth of isolates of *P. gigantea* and *H. annosum*

A preliminary experiment was carried out to deduce the appropriate range of water activities and suitable solute to use in further studies. For both fungi growth was

observed at the lowest a_w when using glycerol as the modifying agent; this is non-ionic and does not severely disrupt cell metabolism. KCl, being a small, strongly ionic molecule, is usually more toxic at higher concentrations against a range of fungi (Magan, 1997). *P. gigantea* was found to be particularly sensitive to KCl, and growth was inhibited by about 70% at 0.984 a_w. *H. annosum* was more tolerant, with a reduction of about 50% in growth at the same a_w. Glucose, a small solute molecule that produces strong osmotic pressure, can be utilised as a food source. Thus both fungi had a similar tolerance to glucose when compared with KCL. Glycerol was chosen as a suitable solute as it allowed growth at the lowest water activities of the solutes tested, with relatively consistent effects on relative growth with freely available water. Previous studies on *Agaricus bisporus*, the cultivated mushroom by, Beecher *et al.* (2000) found that this species was also more sensitive to ionic (KCl) than non-ionic (glycerol) water stress.

This study compared the response of the antagonist and pathogen strains to solute and matric stress. There was a large variation in the response to these water stress factors. When water was freely available the *P. gigantea* isolates all grew at a faster rate than the *H. annosum* ones. However, as a_w stress was imposed the *H. annosum* isolates were more tolerant and able to grow more quickly than the antagonist isolates. This could have considerable practical implications for use of *P. gigantea* as a biocontrol agent in the forest during spells of dry weather, with the pathogen having the ecological advantage.

The *P. gigantea* isolates PG21 and Rotstop are the two isolates currently used as a biocontrol agent. It is interesting to note that each of these species grew rapidly when

water was freely available but were outperformed by the pathogen isolates under conditions of water stress. However, the antagonist isolates PG-B20/5 and 9.12.2 were less affected by water stress and deserve further investigation as possible isolates for use in the biocontrol formulation.

On matrically-modified media the antagonist and pathogen isolates grew significantly slower than on solute modified media at the same a_w levels. This knowledge has not been previously available but fits in with the patterns for other basidiomycetes such as *Agaricus*, *Pleurotus* and wood decaying *Trametes* species, which were all significantly more sensitive to matric than osmotic stress (Magan *et al*., 1995; Mswaka & Magan, 1999, Lee *et al*., 2000).

6.3 Determination of colonisation of wood-based agar by *P. gigantea* and *H. annosum*

Both fungi (*H. annosum* and *P. gigantea)* usually grow on wood, as a parasite and a saprophyte, respectively. The Forest Research Institute in Finland regularly carries out experiments to monitor the efficacy of the *P. gigantea* biocontrol preparation in use. As it is expensive and time consuming to carry out the experiments directly on stumps in the forest they simulated stumps by using freshly cut logs to monitor growth of the pathogen and antagonist in the laboratory (Korhonen, 2001). Thus the use of wood based agar was chosen as a compromise between the artificial MEA medium and the use of fresh wood discs. The latter had been used in an experiment, but being non-sterilised produced growth from fungi other than those with which they had been inoculated. Wood is mainly a mixture of cellulose, hemicellulose and lignin giving a

high percentage of complex carbohydrates and a low percentage of protein (Browning, 1963). MEA is rich sugar (maltose) and protein (peptone) medium. The particular recipe in use by the production company is also enriched with Phostrogen®, a source of readily available nitrogen compounds and other minerals. The additives used in this experiment were chosen to enhance the availability of both simple and complex carbon and nitrogen sources.

PG21 grew more slowly in the presence of the readily utilisable food source glucose. However, this may not be an accurate measure of the amount of growth as there were morphological differences between the mycelia. Growth on the glucose-enriched medium was very dense, i.e. a feeding form characterised by penetrative hyphae, and that on the technical agar was very sparse, although with more rapid radial extension. On the former the mycelium was able to utilise nutrients easily and produce oidia, but on the latter medium the mycelium growth was effuse as foraging hyphae explored for new food sources.

Webber & Thorpe (2002a) compared the colonising ability of UK and Scandinavian isolates including PG21 and Rotstop (the isolates currently in use as BCA) on Scots pine, Norway and Sitka spruce. On Scots pine colonisation of 50 cm^2, but on spruce the largest colonisation regions were 20 cm^2 on Norway and 30 cm^2 on Sitka.

6.4 Effect of temperature and medium on the growth rate of *P. gigantea* and *H. annosum* isolates.

A decrease in incubation temperature, from 25 to 20 ºC, significantly reduced the growth rates of all tested isolates of the antagonist on MEA, and on wood agar there was a further reduction, of growth rates, when temperature was decreased to 15 ºC. Although growth rates of the pathogen isolates were also reduced with temperature, at 15 ºC, two of the pathogen isolates were able to grow more quickly than 2 of the antagonist isolates, on wood agar. It is interesting to note the relative effect, on growth rates, of a change in the growth medium under different environmental conditions and wood agar is an easy to use compromise between the commonly used MEA and natural wood. As the pathogen is ubiquitous in the environment it is essential that any antagonist isolate used in the biocontrol formulation must be able to rapidly colonise the substrate and become established. It is thus critical that choice of effective isolates with the relevant environmental tolerances are used especially under UK weather conditions, which seldom go >25 ºC except for short periods in the summer.

6.5 Effect of pairing in competition on growth rates

Isolates of pathogen and antagonist were grown in pairs in competition at 2 temperatures and 2 water availability conditions. It was shown that at 25 ºC, with water freely available, the antagonist isolates showed optimum growth. Under the same conditions the pathogen grew more slowly than the antagonist. However, a small decrease in available water resulted in a more rapid establishment of the pathogen than the antagonist at 25 ºC and 0.973 a_w. The pathogen isolate FERN8 had

a similar growth rate to the commercial antagonist Rotstop at the lower temperature and 0.995 a_w. This was found to be faster than the other potential antagonist and pathogen isolates. In a direct comparison between growth rates in axenic-culture and paired in competition it was shown that the growth rate of antagonist PG21 was reduced when in competition with the pathogen FERN8, whilst that of the latter was slightly increased. At the lower a_w this had the effect of reducing the growth rate of the antagonist to one quarter that of the pathogen. This indicated that water availability had a more pronounced influence on the relative growth rates of the pathogen and antagonist than temperature when grown in competition.

6.6 Interactions between *P. gigantea* and *H. annosum*

6.6.1 Initial screening for interactions: *in vitro*

When individual isolates of *P. gigantea* were grown against a panel of three *H. annosum* isolates in interaction experiments, the isolate PG-B20/5 was most effective against all three pathogen isolates. The individual pathogen isolates were affected differently by the antagonist isolates, e.g. *P. gigantea* isolate PG 342 had greatest control over the pathogen LAEL.19 although the biocontrol agent PG-B20/5 was the better overall antagonist. This experiment was carried out as a pre-screening method at 25 °C and 0.995 a_w. The effects of fluctuating environmental conditions are probably needed to obtain more information on the ecological niche of both antagonist and pathogen, to more closely mimic the natural environment.

6.6.2 Photomicrography of hyphae

The photographs presented in section 3.7.2 of developing hyphae of both the pathogen and the antagonist indicated that at 25 ºC and 0.995 a_w, the antagonist was able to produce propagules more rapidly than the pathogen. Thus under ideal growing conditions the antagonist should be able to more effectively colonise new territories than the pathogen.

After some development of the method (Magan & Lacey, 1984a), damaged hyphae were stained red/orange with methyl red solution. This compares well with results obtained by Rishbeth (1963), and showed that the method of biocontrol of *H. annosum* by *P. gigantea* is to lyse the cells on contact, protoplasm becoming disorganised and membrane integrity being affected. It was noted in interaction studies that the *P. gigantea* growth was denser as it over grew the area that had been previously colonised by the *H. annosum*. This is possibly due to a release of nutrients from the lysed *H. annosum* hyphae. As part of the system of retention of a primary resource, the production of secondary metabolites to prevent or reduce the ability of other organisms to occupy the same niche has been found to give a competitive advantage in grain storage fungi (Cairns *et al*., 2003).

6.6.3 Quantification of interactions with Index of Dominance (I_D)

When individual isolates of the antagonist *P. gigantea* were grown in competition with single pathogen isolates under differing environmental conditions the biocontrol isolates PG21 and Rotstop were effective at controlling the pathogen when water was freely available at each of the temperatures used. However, the isolate PG-B20/5 was

only partially effective. When water was restricted to 0.973 a_w the *P. gigantea* isolates were only able to mutually inhibit the pathogen isolates G11 and Fern8 at 25°C. At 15 °C the only pathogen isolate that was mutually inhibited was G11, whilst the isolates LAEL.19 and FERN8 were able to grow over the antagonist isolates. Under the experimental conditions used the isolate that proved to be the most dominant, according to the I_D calculated, was the pathogen FERN.8. This was particularly competitive under drier conditions when it was able to dominate the antagonist strains tested. Thus the antagonist was shown to be effective against the pathogen on MEA, when the conditions were warm and wet, but under cooler drier conditions it was ineffective. The influence of environmental factors and nutrition on relative outcome of interactions and the I_D has been previously shown for other ecological systems, e.g. stored grain (Magan & Lacey, 1984a). Alteration of a_w or temperature was also found to alter the interspecific interactions between grain fungi which altered the pattern of dominance or mutual inhibition (Hope, 2004; Magan *et al*., 2003). Marin *et al.* (1998) also found the I_D of *Fusarium* species in maize grain to vary with environmental conditions. From the results of these interaction experiments it can be concluded that any screening system for examining the competitiveness of *P.gigantea* isolates as a biocontrol agent (BCA), should include environmental factors to ensure that isolates with the required ecological competence can be obtained (Magan, 2006).

6.6.4 Interactions *in situ*: on wood discs

In field trials conducted to compare effectiveness of the antagonists *P. gigantea* and *Phaeotheca dimorphospora* against *H. annosum*, Roy *et al.*, (2003) found that results did not reflect those that had been obtained in *in vitro* assays. They concluded that relevant field simulations and field studies were required to obtain effective results.

Thus the study of interactions, *in situ*, on wood discs (*Pinus sylvestris* and *Picea abies*), was set up to attempt to partially replicate field conditions by using a spread of temperatures that could be encountered during the seasons in the UK. The most important feature of these results was that the antagonist isolates were efficient at excluding the pathogen at 25 ºC, but that efficacy decreased with temperature. At 25 ºC there were only minor differences between the performances of the antagonist isolates tested, but as temperature was reduced the isolate PG-B20/5 was the most effective at excluding the pathogen from both species of wood. The antagonist isolate PG21 is in use as PG suspension on East Anglian plantations but was found to be less effective on spruce. The *P.gigantea* isolate Rotstop, is in use in Finland where it is effective against *H. annosum* infection of Scots pine stumps (Korhonen *et al.,* 1994), but it has less efficacy on Norway spruce (Lipponen, 1991; Korhonen *et al.,* 1994). Nicolotti *et al.* (1999), working in Italy, found that *P. gigantea* reduced *H. annosum* infection by 85% on Norway spruce. Under the conditions used in this study the antagonist isolate PG-B20/5 outperformed both commercial isolates on both pine and Sitka spruce, particularly at the lower temperatures, and would warrant further investigations.

6.7 Production of *Phlebiopsis gigantea* spores:

6.7.1 Simulated trial of existing methods of production of PG suspension

The existing method of production of *P. gigantea* oidia is very labour intensive and does not necessarily produce sufficient viable spores to be used in the field as a successful bio-control agent, as 3.5 x 10^6 CFU ml^{-1} suspension is required for rapid

confluent growth, to achieve adequate control (Pratt, 1999). Although the number of spores harvested from each plate could be sufficient, the subsequent handling stages reduce the numbers and viability. There is a considerable mechanical stress on the cells due to the requirement to separate them from each other and from the parent mycelium. This is followed by osmotic shock when the cells are placed in the sugar solution (0.864 a_w) and the bulk syrup solution (0.819 a_w). Viability is checked for each batch of PG suspension produced by counting CFU ml^{-1} of serial dilutions of the suspension after 4 and 7 days incubation at room temperature in the dark (Pratt, 1999b). Quality control of the product is not merely a matter of ensuring sufficient viable oidia in the concentrate, but also a check on purity and to ensure that human pathogens are not present. Samples of PG suspension are also checked partway through their shelf-life to ensure sufficient viable spores will still be applied in the field (Pratt *et al.*, 2000). Hutcheson *et al*. (1988) used a rapid viablue staining method that distinguished between viable and dead cells of the yeast *Saccharomyces cerevisiae* when viewed with a fluorescence microscope. In the current study this method was tried with *P. gigantea* spores, but did not distinguish between viable and heat-killed oidia.

Although in the commercial production of PG suspension from agar plates the oidia are harvested when the colonies of *P. gigantea* (PG21) are approximately 21 days old, the present study has shown the production of oidia peaks at 8 $\log_{10}$ $plate^{-1}$ at day 13 before gradually declining.

6.8 Production of *P. gigantea* spores: fermentation

6.8.1 Investigation of effects of inoculum size

A modern method of bulking up production of micro-organisms is to use liquid culture. Thus a series of experiments were carried out to assess the feasibility of using shake flasks to produce suitable quantities of viable spores. The Finnish product, Rotstop, is produced from a shake flask inoculum that is then transferred into a solid-state fermentation system (Pratt *et al.*, 2000). In the present study at this scale there was no significant difference in the quantity of spores produced, 5 – 7.5 $\log_{10}$ ml^{-1}, or in the dried bio-mass, by using inocula of different sizes. However, the differences in mycelial pellet size were significant. If the pellet grows too large there is a problem with mass transfer of nutrients and oxygen into the centre and waste products out of it. This can lead to morbidity and necrosis in the centre of the pellet. Humphreys *et al.* (1990) inoculated liquid cultures with a conidial inoculum of 5 x 10^5 spores ml^{-1} *Paecilomyces farinosus* or blastospores of *Beauveria bassiana*. This was similar to one treatment used in the present study. They obtained a maximum yield of 8 for the former and 8.8 $\log_{10}$ spores ml^{-1} the latter, in batch culture.

6.8.2 Effect of inoculum age, fermentation time and nitrogen source

The age of the inoculum culture made very little difference to the number of spores produced (approx. 6.5 $\log_{10}$ spores ml^{-1}), regardless of treatment. The flasks inoculated from 13-day cultures produced the least amount of spores (by a factor of 10) although the most biomass. This could have been due to the higher inoculum level producing a greater number of smaller mycelial pellets than in the other flasks. The remaining dried biomass, after harvesting of spores, steadily increased from 8 mg ml^{-1}

from 5 day inoculum to 10.5 mg ml^{-1} after 13 days. However, this quantity decreased to 7 when the inoculum was 15 days old, before a second increase to 9.6 mg ml^{-1} when using the 20-day inoculum. The medium used for the 15 day inoculum was pre-prepared, thus it was autoclaved twice and could have affected the production of biomass. When the effect of fermentation time was considered it was noted that as the production of spores increased over time, from 5.3 at 4 days to 6.6 log_{10} spores ml^{-1} by 13 days, the mycelial biomass decreased, peaking at 11 mg on day 5 and declining to 6.6 mg ml^{-1} by day 13. This suggests that conversion of mycelial biomass to spores occurred.

The commercial method of production of PG suspension uses MEA + Phostrogen® as the growth medium. This formulation contains peptone as an organic nitrogen source and an inorganic N source in Phostrogen®. Thus to develop a suitable liquid growth medium malt extract, peptone and Phostrogen®, in the same proportion, was used as the control food source. Some studies have found that nitrogen exhaustion induces some fungi to sporulate profusely (Morton, 1961; Trinci & Collinge, 1974; Crandall *et al*., 1977). In a preliminary study on manipulation of C and N sources the rich, control broth produced 6.8 and media not containing peptone 7.4 – 7.7 log_{10} spores ml^{-1}, although this was not statistically significant. In a more detailed study it was found that the richest broth, containing malt extract (ME), peptone and Phostrogen® and that retaining the same levels of peptone and ME produced 6.3 – 6.6 log_{10} spores ml^{-1}. Using a visual appearance score, as a semi-quantitive measure of vitality of spores produced in liquid fermentation, the densest growth was observed on wood-based agar media. A reduction in ME, or removal of peptone from the broth resulted in between 5.5 – 5.9 log_{10} spores ml^{-1} being produced. Spore quality was also assessed

by growth rates, on wood agar. The slowest growth was from spores grown without N or with just ME and Phostrogen®, at 4 mm day^{-1}, and the fastest was with reduced C as 50% ME but with full peptone and Phostrogen® at 6 mm day^{-1}. Other treatments gave growth rates in the range 4.5 – 6.5 mm day^{-1}. When tested on restricted a_w media, low C treatments grew best at 0.995 and slowest at 0.980 a_w. These results contrast with the findings of Inch *et al.* (1986) who found *P. fumosoroseus* blastospore production to not be appreciably different whether the medium was C or N limited. However, they did find an appreciable increase in glycogen content of N limited blastospores, which may increase their longevity in storage.

6.8.3 Effect of reduced water availability on spore production

When grown in a matrically modified nutrient broth at 0.991 a_w the PG21 spore production was slightly increased above that of the control at 0.995 a_w. As the solute used was PEG 8000 the broth was slightly viscous and perhaps formed a microscopic support for the growing mycelia, thus allowing earlier maturation and production of spores. The viability of the spores was not affected by a_w of the fermentation broth, except those fermented at 0.978 a_w which did not produce any CFUs at the dilutions used.

When matric and solute modifiers were compared in the fermentation broth, maximum spore production, 6.6 $\log_{10}$ spores ml^{-1}, was again from broth at 0.991 a_w modified with PEG 8000, after 10 days fermentation. At the same a_w, with glycerol as solute, production was marginally above that of the control, but with sorbitol the production rate was decreased. At the lower a_w treatments tested the lowest number of

spores produced was from sorbitol treatment modified to 0.984 a_w. Interestingly, there was no decrease in viability at either a_w treatment.

6.8.4 Effect of supported growth on PG21 spore production

Harvest from ReacSyn™ slopes was greatly reduced when compared with that from pure liquid fermentation. However, when viability was measured by CFUs there were differences between the treatment groups. Both those treated with sorbitol and PEG8000 produced a similar number of CFUs to the control group at 0.996 a_w from the aerial oidia, whereas this was decreased by >50 % for oidia produced in the glycerol treatments. For the submerged conidia this pattern was reversed. In a secondary harvest the production was still much lower than that produced by shake flask fermentation systems and it was concluded that this system was not suitable for production of PG suspension. The ReacSyn™ slopes are produced for use predominantly as fermentation vessels for the production of secondary metabolites.

When mycelial growth was supported in sodium alginate beads within a liquid broth, both production of spores and their viability were comparable with those produced with PEG 8000 modification of the broth, i.e. marginally above that of the control group of normal malt extract broth, at 6.6 and 6.2 $\log_{10}$ CFU ml^{-1}, respectively

In summary; production of PG21 spores in submerged fermentation gave a maximum viability of 6.6 $\log_{10}$ spores ml^{-1} medium. This maximum was only reached when the developing mycelium had some support within the medium i.e. that of the sodium alginate beads or with the solute PEG 8000.

6.9 Production of *P. gigantea* spores in solid substrate fermentation

6.9.1 Temporal study: the effect of moisture content on PG21 oidia production

Initial pilot studies carried out on *Pinus* spp. wood chips showed a similar production of spores to that from the best conditions of submerged fermentation, when the chips were moist. The greatest viability came from growth at 44 and 50 % moisture content (MC). There was very little growth below 29 % MC which correlates well with known information that wood is rarely decayed by fungi at MC lower than 20 %, due to protection afforded by the presence of lignin (Dinwoodie, 2000). In an isolated group at 14 % MC there were countable spores but these did not show viability. This could be accounted for by incomplete equilibration of the water through the course wood chips of the substrate and growth of spores being followed by early senescence.

In the long-term study the effect of MC on spore production was observed. Although there were countable spores in all groups, only those at 55 % MC showed viability after 2 months incubation. There was a downward shift of a_w during the course of this study, possibly due to the small mass of substrate in the fermentation vessels being dried by the air-conditioning unit in the controlled temperature chamber. It was decided to abandon this study at the 2-month stage.

In further studies with higher MC there was little difference in oidia production between the MC treatments between 50 and 83 % MC after one month incubation. However, after 2 months the moist groups produced marginally more spores. At 3

months incubation the number of spores harvested had increased by ten-fold, with the 75 % MC reaching 9 $\log_{10}$ spores g^{-1} substrate. This group was also the most viable on water stress agar, of 0.988 a_w, producing 8 $\log_{10}$ CFU g^{-1} substrate. The oven-dried mass was calculated for the remaining substrate after the 3-month harvest. This showed that there were still unused reserves within the substrate as there was a mass loss of 20 % from the original. A loss in mass is characteristic of fungal attack in wood and timber can lose up to 80% of its initial air-dried mass (Dinwoodie, 2000).

6.9.2 Effects of a_w modification and addition of $CaCl_2$ on PG21 spore production

The addition of $CaCl_2$ to the substrate made no discernable difference in either production quantity or viability. This contrasts with other workers who found an increase in sporulation of *Ulocladium atrum* in the presence of $CaCl_2$ (Frey & Magan, 2001). In contrast, the addition of glycerol to modify a_w of the substrate to 0.974 a_w reduced the viability of the resultant oidia by a factor of 10. The production of oidia was unaffected by the decrease in available water. When water restricted media were used to assess viability at 0.975 a_w the only CFU produced were from the un-modified substrate groups, with or without the addition of $CaCl_2$.

When the glycerol modified treatment groups were repeated in a further run of the same experiment there was a marginal decrease in the overall production of oidia, but the viability remained high from the first 2 treatment groups, 0.991 and 0.981 a_w when assessed as CFU on agar at 0.998 a_w, with a small reduction in viability when assessed on 0.981 a_w. When assessed under greater water stress, at 0.974 a_w, the oidia from the unmodified substrate produced the greater number of CFU. Oidia produced

on substrate with more water stress, at 0.974 a_w, were less viable than the other groups, producing less CFU on each of the test agar. This contrasts with expected findings, as other workers in the field have found that production of propagules under conditions of water stress have increased their ability to survive adverse conditions and have improved their potential as BCAs (Frey & Magan, 1998; Magan, 2001).

6.9.3 Effects of scale up on PG21 spore production

Studies were made of the possibility of scaling up the production of PG21 oidia grown on sawdust substrate. The highest viability was produced from 10 g samples of *Pinus sylvestris* sawdust fermented within vented Magenta pots. When scaled up to 50 g, within vented 1 l Duran bottles and 200 g in porous spawn bags there was only a marginal loss of viable spore production, but when the mass of substrate was doubled to 400g in spawn bags the production was reduced by a factor of 10. In the case of the latter group, it was noted at the time of inoculation that the substrate was wetter at one end. With the larger bulk of substrate mixing was difficult and homogeneity was not achieved. Under such conditions the inoculum had first established at one end of the bag, taking several weeks for the mycelial front to progress through the substrate. This study shows the feasibility of scaling up of production into commercially available fermentation vessels, but suggests that good gas exchange is necessary to prevent oxygen limitation.

6.10 Endogenous reserves: polyol content of *P. gigantea* and *H. annosum* with restricted water availability

Under conditions of water stress during submerged fermentation there was an increase in total polyol content of mycelial pellets, with time. There was a change in the proportions of intracellular sugar alcohols, chiefly consisting of mannitol with lesser amounts of arabitol and erythritol and very little glycerol, except for the group where the medium had been moderated with glycerol to 0.988 a_w. This latter group had more than 70 % glycerol in the sample. Glycerol is a small solute and able to cross the cell membrane. Other workers have found that glycerol is transported into the cell and as a compatible solute is used as a protective to balance osmotic pressure, thus allowing the hypha to remain turgid (Hallsworth & Magan, 1996). Xerotolerant species, e.g. *Penicillium* and *Aspergillus* species, have been shown to produce low molecular weight sugar alcohols as secondary metabolites, in particular glycerol and erythritol. As mentioned, these are osmotic protectants that allow functioning of metabolic enzymes (Magan, 1997; Neschi *et al*., 2004). Abadias *et al*. (2001) showed an improvement in stress tolerance in the yeast *Candida sake* with a high correlation between intracellular water potential and accumulation of polyols. This study shows concordance with these findings in the case of polyol distribution within the mycelial pellets; however, the endogenous reserves in the submerged conidia do not show this pattern.

The effect of a_w of solid substrate, *P. sylvestris* sawdust, on endogenous reserves was studied. Total sugar alcohols were not significantly different between the groups, on either run of the experiment, but were different between each run, 1.5 – 2.5 in the

former and 15 – 16 mg g^{-1} in the latter. Interestingly, the proportions of the polyols also differed between the runs. In the first, the proportions of glycerol, erythritol and arabitol increased at the expense of mannitol when a_w reduced to 0.981, but mannitol regained its proportion of 55 % at 0.974 a_w. In contrast, on the repeat run the proportion of mannitol did not differ between the treatment groups; glycerol content reduced from 18–10 % from 0.991–0.974 a_w, as arabitol increased to the same amount. In all 3 treatment groups the amount of erythritol was insignificant. The absolute concentrations of the sugar alcohols did not change significantly between the treatments.

6.11 Evaluation of fluidised bed drying as a means of preservation of the BCA *P. gigantea*

Frey (2000) found 75– 98% viability of fluidised bed dried spores when compared with fresh *Ulocladium atrum* spores. After unsuccessful trials, this study showed that *P. gigantea* oidia are able to survive the drying process, at up to 60 ºC, with a loss of viability from 1.2 x 10^8 g^{-1} from fresh paste down to 1.2 – 1.4 x 10^8 g^{-1} after drying. Sample size was very small; fresh paste was 1 g in each case, and was insufficient to flow freely in the updraft of the fluidised bed reactor; this led to inconsistency in the drying and unpredictable a_w. In some preservation studies additives, e.g. glycerol, various sugars, trehalose or polyols, have been used as desiccants or freeze protectants to protect membrane integrity during the process (Carpenter & Crowe, 1988; Crowe *et al*., 1984; Hoekstra *et al*., 1997). Dried milk powder has also been used successfully as a protectant to air-dry blastospores of *Metarhizium anisopliae*, an entomopathogenic fungus (Stephan & Zimmermann, 1998). In this study dried skim

milk was added, however it formed a hardened lump as it dried, not allowing the hot air current through it and drying was unsuccessful.

6.12 Volatile fingerprints of *P. gigantea* and *H. annosum*

Fungi produce aromatic secondary metabolites; this study was designed to investigate the possibility of detecting changes in cultures, using an electronic nose, to signify competition between the pathogen and the antagonist. Electronic nose technology has previously been used for detecting off flavours in water due to microorganisms (Canhoto & Magan, 2004), and for detection of fungal contamination of library paper (Canhoto *et al*., 2004). Needham *et al.*, (2005) and Magan & Evans (2000) investigated the possibility of using this technology to detect early spoilage of bakery products and stored grain. Magan *et al*. (2002) found detection of and discrimination of species of spoilage fungi and isolates of the same species, in stored grain and cereal products, *in vitro* and *in situ*. In this study the AlphaM.O.S. software was used to process sensor information to produce DFAs. These indicated that the system could be used to identify between the species, grouping data points of different isolates of the same species close to each other and those of the other species more distant. Separation was clearly shown on both *P. sylvestris* sawdust substrate and on MEA. When samples of each species were mixed immediately prior to sampling the DFA showed the fingerprints of the mixtures in different locations to either of the pure cultures. When grown as interactions, each of the antagonist isolates showed different separations in the DFAs. Both PG21 and Rotstop showed clear separation between the species and the interactions, whilst the DFA of PG-B20/5 showed the fingerprints of the co-inoculated mixed lawns with each pathogen to be co-incidental with the

fingerprint of the antagonist. When a large number of different isolates of pathogen and antagonist were compared the electronic nose produced fingerprints of antagonist isolates clustered together and pathogen isolates in 2 distinct groups. In a further study the volatile finger prints of the interaction treatments were shown to be close to or co-incidental with the antagonist isolates, 342, WO7 and PG15. In each case the separation between the fingerprints was clearer after 14 day incubation than 7 day, but in some cases there was already clear separation by 7 days e.g. PG15. This novel study has shown the possibility of using electronic nose technology as a rapid screening method for detection of potential isolates to be used in BCA formulations to protect against a range of pathogen isolates. As the separation of fingerprints was clear on sawdust the author of this report would suggest that further studies be made of interactions *in situ*.

Chapter 7

Conclusions and further work

The antagonist *Phlebiopsis gigantea* has a competitive edge over the pathogen *Heterobasidion annosum* when incubated in optimal conditions i.e. when warm, 25 ºC, and wet, with water freely available. However the antagonist is more susceptible to environmental stress than is the pathogen, when either the temperature or available water is sub-optimal the growth rate and dominance are adversely affected. Water availability had a greater effect on competition than temperature within the ranges tested. When both conditions are sub-optimal the effect is synergistic and the antagonist is so badly affected that the pathogen is given a competitive advantage. The pine stumps that were observed in this study had reduced water availability in the surface tissues despite the inclement weather; it could be presumed that under milder conditions the tissue would dry more rapidly, thus becoming a less hospitable substrate for the antagonist. Isolates of the antagonist have different responses to environmental stress factors and are worthy of further investigation into the possibility of incorporation into the bio-control formulation for use in different weather conditions. The first part of this study on ecology of the species showed that under warm wet conditions the antagonist has an advantage and out-competes the pathogen, but that under cooler dry conditions it is ineffective. However, there were differences in performance between antagonist isolates and further studies would show if it would be beneficial to use different isolates in the BCA formulation according to the season.

Isolates of the pathogen displayed differing vigour, thus a panel of pathogen isolates should be used in any test of efficacy of the antagonist preparations. Although initial screening on MEA is useful, further work on surface sterilised wood samples would

be beneficial to the decision-making process when choice of isolate for use in the formulation is being considered.

Production techniques for the BCA need improving; the current method of production of PG suspension is too labour intensive. This study has shown the feasibility of production of viable propagules either in submerged fermentation or on a sawdust solid substrate. In liquid culture spore production was increased in a viscous medium or by the use of impregnated sodium alginate beads in the nutrient broth. Liquid fermentation has the advantage of scale-up of batch-culture, and possibly to large scale production using automated continuous fermentation and automated harvesting. Solid substrate fermentation on sawdust, being a more natural substrate, produced viable oidia that were easy to harvest in bulk. Care would be needed in an industrial scale-up of production to ensure sufficient aeration to prevent oxygen limitation. The system of supported growth on a platform in liquid culture was not suitable in this study. Oidia produced from solid substrate fermentation had greater viability on water stress agar than did the blastospores produced in submerged fermentation. Oidia are small rectangular spores, but blastospores were large, irregular and sometimes branched; such irregular shapes may be more fragile, or could clump, leading to the possibility that application equipment, the Oregon spray bar, could be blocked if the latter were used in the formulation. Studies of polyol accumulation in propagules was inconclusive, although correlation could be made of accumulation of compatible solutes and a_w of broth in the submerged fermentation. It has been shown that the antagonist spores are able to survive the rigour of fluidised-bed drying, although in this study shelf-life studies were not carried out. It is suggested that larger samples of wet paste be used in the uplift tubes in further investigation of preservation.

In this study a novel application was made of electronic nose technology to assess volatile compounds emitted by the pathogen and antagonist isolates. This showed that differentiation was made between isolates of the same species, whilst grouping their volatile fingerprints together. A greater differentiation was made between species and when grown in competition fingerprints of some interactions were co-incidental with that of the antagonist, suggesting that the pathogen was dominated under these conditions. It is suggested that these studies be carried forward with interactions between the species under a range of environmental conditions as a rapid pre-screening of potential antagonist isolates.

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APPENDIX I

Tables of statistics

Table 1. Analysis of variance of the effect of time since felling, visual appearance group and two-way interactions on tree stump water availability of freshly felled *Pinus sylvestris*, when monitored over a period of 4 weeks after felling.

Effect	Univariate Results for Each DV (Spreadsheet1 in tree stump data) Sigma-restricted parameterization Effective hypothesis decomposition				
	Degr. of Freedom	Water activity SS	Water activity MS	Water activity F	Water activity p
Intercept	1	40.08631	40.08631	1746499	0.000000
Stump appearance	1	0.00007	0.00007	3	0.082059
Time (d)	6	0.00089	0.00015	6	0.000225
Stump appearance*Time (d)	6	0.00027	0.00005	2	0.102110
Error	28	0.00064	0.00002		
Total	41	0.00188			

Table 2. Analysis of variance (a) and least significant difference test (b) of the effect of water activity, modifier and two and three-way interactions on growth rates of *Phlebiopsis gigantea* and *Heterobasidion annosum* when grown on malt extract agar. Water activity modified with glucose, glycerol and potassium chloride.

(a)

Effect	Univariate Results for Each DV (Spreadsheet4) Sigma-restricted parameterization Effective hypothesis decomposition				
	Degr. of Freedom	Growth rate SS	Growth rate MS	Growth rate F	Growth rate p
Intercept	1	77.8193	77.81931	1442.356	0.000000
Organism	1	0.0000	0.00000	0.000	1.000000
Modifier	2	7.7733	3.88666	72.038	0.000000
Water activity group	4	165.5683	41.39206	767.188	0.000000
Organism*Modifier	2	0.1398	0.06989	1.295	0.280782
Organism*Water activity group	4	0.1624	0.04059	0.752	0.560094
Modifier*Water activity group	8	13.2558	1.65698	30.712	0.000000
Organism*Modifier*Water activity group	8	0.2070	0.02588	0.480	0.866292
Error	65	3.5069	0.05395		
Total	94	209.7676			

(b)

Cell No.	LSD test; variable Growth rate (Spreadsheet4) Homogenous Groups, alpha = .05000 (Non-Exhaustive Search) Error: Between MS = .05395, df = 65.000											
	Organism	Modifier	Water activity group	Growth rate Mean	1	2	3	4	5	6	7	8
3	Ha	glucose	d	-0.000000	****							
14	Ha	KCl	e	-0.000000	****							
24	Pg	glycerol	e	0.000000	****							
4	Ha	glucose	e	0.000000	****							
5	Ha	glucose	f	0.000000	****							
29	Pg	KCl	e	0.000000	****							
20	Pg	glucose	f	0.000000	****							
15	Ha	KCl	f	0.000000	****							
25	Pg	glycerol	f	0.000000	****							
30	Pg	KCl	f	0.000000	****							
18	Pg	glucose	d	0.004369	****							
10	Ha	glycerol	f	0.004693	****							
19	Pg	glucose	e	0.007039	****							
9	Ha	glycerol	e	0.008434	****							
28	Pg	KCl	d	0.016100	****							
13	Ha	KCl	d	0.033414	****							
23	Pg	glycerol	d	0.050000	****							
8	Ha	glycerol	d	0.090129	****							
27	Pg	KCl	c	0.577766		****						
12	Ha	KCl	c	0.853236		****	****					
2	Ha	glucose	c	0.981392			****					
17	Pg	glucose	c	1.118204			****					
22	Pg	glycerol	c	1.938180				****				
7	Ha	glycerol	c	2.155502				****	****			
1	Ha	glucose	b	2.393651					****	****		
26	Pg	KCl	b	2.676519						****	****	
16	Pg	glucose	b	2.768864						****	****	
11	Ha	KCl	b	2.794160							****	
6	Ha	glycerol	b	4.393607								****
21	Pg	glycerol	b	4.538325								****

Table 3. Analysis of variance (a) and least significant difference test (b) of the effect of osmotic vs. matric stress, water activity, isolate and two and three-way interaction on growth rates of *P. gigantea* and *H. annosum* MEA modified with glycerol and, malt extract broth modified with PEG 8000, at 25 ºC

(a)

Effect	Univariate Results for Each DV (expt 2 gr rt data) Sigma-restricted parameterization Effective hypothesis decomposition				
	Degr. of Freedom	Growth rate SS	Growth rate MS	Growth rate F	Growth rate p
Intercept	1	2411.920	2411.920	10745.38	0.00
Isolate	10	56.555	5.655	25.20	0.00
Modifier	1	812.228	812.228	3618.57	0.00
a_w	3	589.304	196.435	875.14	0.00
Isolate*Modifier	10	36.985	3.699	16.48	0.00
Isolate* a_w	30	109.977	3.666	16.33	0.00
Modifier* a_w	3	101.252	33.751	150.36	0.00
Isolate*Modifier* a_w	30	59.385	1.979	8.82	0.00
Error	176	39.505	0.224		
Total	263	1805.192			

(b)

LSD test; variable Growth rate (Spreadsheet5 in Workbook2)
Homogenous Groups, alpha = .05000 (Non-Exhaustive Search)
Error: Between MS = .18255, df = 220.00

Cell No.	Isolate	Modifier	a_w	Growth rate Mean	1	2	3	4	5	6	7	8	9	10	11	12	13	14	1
49	PG-B20/5	Glycerol	945	0.000000	****														
1	PG15	Glycerol	945	0.000000	****														
25	PG-SH-1	Glycerol	945	0.000000	****														
93	LAEL.19	PEG	974	0.000000	****														
37	PG21	Glycerol	945	0.000000	****														
33	PG-SH-1	PEG	974	0.000000	****														
121	342	Glycerol	945	0.000000	****														
61	WO7	Glycerol	945	0.000000	****														
13	9.12.2	Glycerol	945	0.011111	****														
73	Rotstop	Glycerol	945	0.035859	****	****													
35	PG-SH-1	PEG	990	0.048504	****	****													
71	WO7	PEG	990	0.069092	****	****	****												
85	LAEL.19	Glycerol	945	0.078914	****	****	****	****											
59	PG-B20/5	PEG	990	0.088256	****	****	****	****											
109	G11	Glycerol	945	0.089216	****	****	****	****											
47	PG21	PEG	990	0.093316	****	****	****	****											
95	LAEL.19	PEG	990	0.099869	****	****	****	****											
23	9.12.2	PEG	990	0.103820	****	****	****	****											
83	Rotstop	PEG	990	0.127350	****	****	****	****											
9	PG15	PEG	974	0.147140	****	****	****	****											
129	342	PEG	974	0.169941	****	****	****	****											
97	FERN 8	Glycerol	945	0.191954	****	****	****	****											
131	342	PEG	990	0.217647	****	****	****	****											
10	PG15	PEG	982	0.254760	****	****	****	****	****										
21	9.12.2	PEG	974	0.270689	****	****	****	****	****										
11	PG15	PEG	990	0.300543	****	****	****	****	****	****									
45	PG21	PEG	974	0.303982	****	****	****	****	****	****									
34	PG-SH-1	PEG	982	0.318395	****	****	****	****	****	****									
26	PG-SH-1	Glycerol	957	0.328620	****	****	****	****	****	****									
57	PG-B20/5	PEG	974	0.340287	****	****	****	****	****	****									
122	342	Glycerol	957	0.343233	****	****	****	****	****	****									
117	G11	PEG	974	0.361128	****	****	****	****	****	****									
70	WO7	PEG	982	0.367794	****	****	****	****	****	****									
81	Rotstop	PEG	974	0.380291	****	****	****	****	****	****									
69	WO7	PEG	974	0.395436	****	****	****	****	****	****									
58	PG-B20/5	PEG	982	0.401536	****	****	****	****	****	****									
74	Rotstop	Glycerol	957	0.461594	****	****	****	****	****	****	****								
2	PG15	Glycerol	957	0.479018	****	****	****	****	****	****	****								
62	WO7	Glycerol	957	0.486791	****	****	****	****	****	****	****								
94	LAEL.19	PEG	982	0.507602	****	****	****	****	****	****	****								
22	9.12.2	PEG	982	0.534061	****	****	****	****	****	****	****	****							
130	342	PEG	982	0.534790	****	****	****	****	****	****	****	****							
110	G11	Glycerol	957	0.561800	****	****	****	****	****	****	****	****							
105	FERN 8	PEG	974	0.570305	****	****	****	****	****	****	****	****							
38	PG21	Glycerol	957	0.583437	****	****	****	****	****	****	****	****							
82	Rotstop	PEG	982	0.665082	****	****	****	****	****	****	****	****							
118	G11	PEG	982	0.708268		****	****	****	****	****	****	****							
50	PG-B20/5	Glycerol	957	0.755359			****	****	****	****	****	****							
14	9.12.2	Glycerol	957	0.759777				****	****	****	****	****							
98	FERN 8	Glycerol	957	0.934907					****	****	****	****	****						
123	342	Glycerol	974	0.965639						****	****	****	****	****					

Table 4. Analysis of variance (a) and least significant difference test (b) of the effect of species, growth medium and two-way interaction on growth rates of *P. gigantea* and *H. annosum* grown on MEA, wood agar and wood agar modified with carbon and nitrogen sources, at 25 ºC

(a)

Effect	Univariate Results for Each DV (wood agar expt 1 data) Sigma-restricted parameterization Effective hypothesis decomposition				
	Degr. of Freedom	Growth rate SS	Growth rate MS	Growth rate F	Growth rate p
Intercept	1	663.3969	663.3969	6015.119	0.000000
Species	1	45.5873	45.5873	413.347	0.000000
Medium	6	3.9354	0.6559	5.947	0.000320
Species*Medium	6	8.1119	1.3520	12.259	0.000000
Error	31	3.4189	0.1103		
Total	44	67.0952			

(b)

Cell No.	LSD test; variable Growth rate (wood agar expt 1 data) Homogenous Groups, alpha = .05000 (Non-Exhaustive Search) Error: Between MS = .11029, df = 31.000									
	Species	Medium	Growth rate Mean	1	2	3	4	5	6	7
9	Ha	wood	2.301114	****						
11	Ha	wood + glucose	2.694209	****	****					
12	Ha	wood + soy protein	2.815590	****	****					
8	Ha	MEA + Phos + Ch	3.059065		****	****				
10	Ha	wood + cellulose	3.081292		****	****				
13	Ha	wood + KNO_3	3.186192		****	****				
14	Ha	Technical agar	3.572383			****	****			
7	Pg	Technical agar	3.882831				****	****		
4	Pg	wood + glucose	4.186575					****		
6	Pg	wood + KNO_3	5.152108						****	
2	Pg	wood	5.433735						****	****
1	Pg	MEA + Phos + Ch	5.444880						****	****
3	Pg	wood + cellulose	5.572590						****	****
5	Pg	wood + soy protein	5.752410							****

Table 5. Analysis of variance (a) and least significant difference test (b) of the effect of isolate, growth medium, temperature and two and three-way interactions on growth rates of *P. gigantea* and *H. annosum* on MEA and wood agar, at 25, 20 and 15 ºC.

(a)

Effect	Univariate Results for Each DV (Spreadsheet1) Sigma-restricted parameterization Effective hypothesis decomposition Degr. of Freedom	growth rate SS	growth rate MS	growth rate F	growth rate p
Intercept	1	4402.726	4402.726	15474.29	0.000000
isolate	10	132.968	13.297	46.73	0.000000
medium	1	61.189	61.189	215.06	0.000000
temp °C	2	197.431	98.716	346.96	0.000000
isolate*medium	10	45.196	4.520	15.89	0.000000
isolate*temp °C	20	20.552	1.028	3.61	0.000004
medium*temp °C	2	2.070	1.035	3.64	0.028991
isolate*medium*temp °C	20	15.336	0.767	2.70	0.000405
Error	132	37.556	0.285		
Total	197	512.299			

(b)

Cell No.	LSD test; variable growth rate (Spreadsheet1) Homogenous Groups, alpha = .05000 (Non-Exhaustive Search) Error: Between MS = .28452, df = 132.00 growth rate Mean	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
44	1.61666	****																					
43	2.00000	****	****																				
45	2.10000	****	****	****																			
58	2.16666	****	****	****																			
28	2.66666		****	****	****																		
47	2.70000		****	****	****	****																	
59	2.83333		****	****	****	****	****																
10	2.91666			****	****	****	****																
4	3.23333				****	****	****	****															
53	3.25000				****	****	****	****															
40	3.33333				****	****	****	****	****														
22	3.35000				****	****	****	****	****	****													
52	3.38333				****	****	****	****	****	****													
55	3.38333				****	****	****	****	****	****													
11	3.46666				****	****	****	****	****	****													
16	3.51666				****	****	****	****	****	****	****												
60	3.55000					****	****	****	****	****	****	****											
29	3.55000					****	****	****	****	****	****	****											
46	3.56666						****	****	****	****	****	****											
49	3.58333						****	****	****	****	****	****											
34	3.83333							****	****	****	****	****	****										
35	3.83333							****	****	****	****	****	****										
17	4.00000							****	****	****	****	****	****	****									
7	4.03333							****	****	****	****	****	****	****									
23	4.13333								****	****	****	****	****	****	****								
8	4.16666								****	****	****	****	****	****	****								
56	4.20000									****	****	****	****	****	****								
5	4.35000										****	****	****	****	****	****							
13	4.38333											****	****	****	****	****	****						
25	4.45000												****	****	****	****	****						
41	4.45000												****	****	****	****	****						
64	4.51666												****	****	****	****	****	****					
48	4.55000												****	****	****	****	****	****					
54	4.58333												****	****	****	****	****	****	****				
65	4.80000													****	****	****	****	****	****	****			
61	4.81666													****	****	****	****	****	****	****			
26	4.91666														****	****	****	****	****	****			
12	4.91666														****	****	****	****	****	****			
37	4.93333														****	****	****	****	****	****			
31	4.93333														****	****	****	****	****	****			

Table 6. Analysis of variance (a) and least significant difference test (b) of the effect of isolate, temperature, water activity and two and three-way interactions on growth rates of *P. gigantea* and *H. annosum* paired in interaction studies grown on MEA, at 25, and 15 ºC, at 0.995 and 0.973 a_w.

(a)

Effect	Univariate Results for Each DV (Spreadsheet2) Sigma-restricted parameterization Effective hypothesis decomposition Degr. of Freedom	Growth rate SS	Growth rate MS	Growth rate F	Growth rate p
Intercept	1	1287.507	1287.507	6403.872	0.000000
Isolate	5	44.507	8.901	44.274	0.000000
Temp	1	167.104	167.104	831.150	0.000000
aw	1	183.815	183.815	914.270	0.000000
Isolate*Temp	5	2.132	0.426	2.121	0.064931
Isolate*aw	5	103.582	20.716	103.040	0.000000
Temp*aw	1	5.852	5.852	29.107	0.000000
Isolate*Temp*aw	5	37.470	7.494	37.274	0.000000
Error	180	36.189	0.201		
Total	203	574.893			

(b)

Cell No.	LSD test; variable Growth rate (Spreadsheet2) Homogenous Groups, alpha = .05000 (Non-Exhaustive Search) Error: Between MS = .20105, df = 180.00 Isolate	Temp	aw	Growth rate Mean	1	2	3	4	5	6	7	8	9	10	11	12
1	PG 21	15	973	0.572259	****											
9	B20/5	15	973	0.615216	****											
13	G11	15	973	0.783696	****	****										
5	Rotstop	15	973	0.798278	****	****										
3	PG 21	25	973	1.075658		****	****									
17	LAEL 19	15	973	1.085424		****	****									
21	FERN 8	15	973	1.127975		****	****									
18	LAEL 19	15	995	1.327485			****	****								
14	G11	15	995	1.412281			****	****								
11	B20/5	25	973	1.703008				****								
7	Rotstop	25	973	1.733918				****								
2	PG 21	15	995	2.233918					****							
10	B20/5	15	995	2.590643					****	****						
15	G11	25	973	2.661184					****	****						
20	LAEL 19	25	995	2.686813						****						
16	G11	25	995	2.743590						****						
19	LAEL 19	25	973	2.954887						****	****					
22	FERN 8	15	995	3.286550							****	****				
6	Rotstop	15	995	3.526316								****	****			
23	FERN 8	25	973	3.716374									****	****		
24	FERN 8	25	995	4.076923										****		
4	PG 21	25	995	5.625000											****	
12	B20/5	25	995	5.875000											****	****
8	Rotstop	25	995	6.312500												****

Table 7. Analysis of variance of the effect of inoculum age, incubation time and two-way interaction (a) and least significant difference test of the effect of inoculum age (b) and incubation time (c) on the number of *P. gigantea* spores produced in submerged fermentation in malt extract broth at 25 ºC.

(a)

Effect	Univariate Results for Each DV (Spreadsheet1) Sigma-restricted parameterization Effective hypothesis decomposition Degr. of Freedom	Spore production (log_{10} ml^{-1}) SS	Spore production (log_{10} ml^{-1}) MS	Spore production (log_{10} ml^{-1}) F	Spore production (log_{10} ml^{-1}) p
Intercept	1	1000.081	1000.081	12441.55	0.000000
Inoculum age (days)	5	2.516	0.503	6.26	0.000236
Incubation (days)	5	2.187	0.437	5.44	0.000683
Error	39	3.135	0.080		
Total	49	10.471			

(b)

Cell No.	LSD test; variable Spore production (log_{10} ml^{-1}) (Spreadsheet1) Homogenous Groups, alpha = .05000 Error: Between MS = .08038, df = 39.000 Inoculum age (days)	Spore production (log_{10} ml^{-1}) Mean	1	2	3
4	13	5.647945			****
6	20	6.293183	****		
2	8	6.384620	****	****	
1	5	6.397082	****	****	
3	11	6.401546	****	****	
5	15	6.646489		****	

(c)

Cell No.	LSD test; variable Spore production (log_{10} ml^{-1}) (Spreadsheet1) Homogenous Groups, alpha = .05000 Error: Between MS = .08038, df = 39.000 Incubation (days)	Spore production (log_{10} ml^{-1}) Mean	1	2	3
1	4	5.217738		****	
2	5	5.906304			****
3	7	6.317334	****		
4	8	6.366049	****		
5	10	6.495765	****		
6	13	6.611136	****		

Table 8. Analysis of variance (a) and least significant difference test (b) of the effect of media, incubation time and two-way interaction on the number of *P. gigantea* spores produced in submerged fermentation in nutrient modified malt extract broth at 25 ºC.

(a)

Effect	Univariate Results for Each DV (Spreadsheet1) Sigma-restricted parameterization Effective hypothesis decomposition Degr. of Freedom	Spore count (log_{10} ml^{-1}) SS	Spore count (log_{10} ml^{-1}) MS	Spore count (log_{10} ml^{-1}) F	Spore count (log_{10} ml^{-1}) p
Intercept	0				
Media	2	2.45278	1.226389	6.882058	0.075703
Incubation (days)	3	1.35964	0.453212	2.543265	0.231756
Media*Incubation (days)	11	6.04027	0.549116	3.081441	0.192322
Error	3	0.53460	0.178201		
Total	21	11.08173			

(b)

Cell No.	LSD test; variable Spore count (log_{10} ml^{-1}) (Spreadsheet1) Homogenous Groups, alpha = .05000 (Non-Exhaustive Search) Error: Between MS = .17820, df = 3.0000 Media	Incubation (days)	Spore count (log_{10} ml^{-1}) Mean	1	2	3
1	ME + Phos + pept	7	5.096910	****		
2	ME + Phos + pept	8	6.096910	****	****	
15	ME + pept	11	6.138303	****	****	****
19	ME	10	6.948168	****	****	****
7	ME + Phos	8	6.954243	****	****	****
6	ME + Phos	7	6.966142	****	****	****
5	ME + Phos + pept	11	7.223434		****	****
12	ME + pept	8	7.402495		****	****
13	ME + pept	9	7.427324		****	****
20	ME	11	7.486076		****	****
10	ME + Phos	11	7.518514		****	****
14	ME + pept	10	7.579784		****	****
3	ME + Phos + pept	9	7.638489		****	****
4	ME + Phos + pept	10	7.640978		****	****
8	ME + Phos	9	7.733398		****	****
17	ME	8	7.770727			****
18	ME	9	7.945099		****	****
16	ME	7	8.026329			****
9	ME + Phos	10	8.035930			****

Table 9. Analysis of variance (a) and least significant difference test (b) of the effect of media on the number of *P. gigantea* spores produced in submerged fermentation in nutrient modified malt extract broth at 25 ºC.

(a)

Effect	Univariate Results for Each DV (Spreadsheet1) Sigma-restricted parameterization Effective hypothesis decomposition Degr. of Freedom	Spore production ($\log_{10}$ ml^{-1}) SS	Spore production ($\log_{10}$ ml^{-1}) MS	Spore production ($\log_{10}$ ml^{-1}) F	Spore production ($\log_{10}$ ml^{-1}) p
Intercept	1	1832.866	1832.866	10103.37	0.000000
Treatment	6	6.863	1.144	6.31	0.000068
Error	46	8.345	0.181		
Total	52	15.208			

(b)

Cell No.	LSD test; variable Spore production ($\log_{10}$ ml^{-1}) (Spreadsheet1) Homogenous Groups, alpha = .05000 Error: Between MS = .18141, df = 46.000 Treatment	Spore production ($\log_{10}$ ml^{-1}) Mean	1	2	3
5	E	5.529606	****		
7	G	5.537770	****		
6	F	5.742807	****		
4	D	5.808785	****		
2	B	5.912712	****	****	
3	C	6.285829		****	****
1	A	6.569164			****

Table 10. Analysis of variance and least significant difference test of the effect of fermentation media of *P. gigantea* spores produced in submerged fermentation in nutrient modified malt extract broth at 25 ºC, on the visual appearance score on wood agar (a) and (b), MEA at 0.994 (c) and (d), 0.980, (e) and (f), 0.954 (g) and (h), 0.940 a_w.(i) and (j).

(a)

Effect	Univariate Results for Each DV (Restricted media C N quality data) Sigma-restricted parameterization Effective hypothesis decomposition Degr. of Freedom	wdA 0.994 SS	wdA 0.994 MS	wdA 0.994 F	wdA 0.994 p
Intercept	1	338.8339	338.8339	573.9601	0.000000
Treatment	6	5.7244	0.9541	1.6161	0.171041
Error	36	21.2524	0.5903		
Total	42	26.9767			

(b)

LSD test; variable wdA 0.994 (Restricted media C N quality data)
Homogenous Groups, alpha = .05000
Error: Between MS = .59034, df = 36.000

Cell No.	Treatment	wdA 0.994 Mean	1
5	E	2.666667	****
6	F	2.714286	****
4	D	2.714286	****
7	G	2.714286	****
2	B	2.800000	****
3	C	3.500000	****
1	A	3.500000	****

(c)

Univariate Results for Each DV (Restricted media C N quality data)
Sigma-restricted parameterization
Effective hypothesis decomposition

Effect	Degr. of Freedom	MEA 0.994 SS	MEA 0.994 MS	MEA 0.994 F	MEA 0.994 p
Intercept	1	331.5610	331.5610	1770.198	0.000000
Treatment	6	2.7452	0.4575	2.443	0.059789
Error	21	3.9333	0.1873		
Total	27	6.6786			

(d)

LSD test; variable MEA 0.994 (Restricted media C N quality data)
Homogenous Groups, alpha = .05000
Error: Between MS = .18730, df = 21.000

Cell No.	Treatment	MEA 0.994 Mean	1	2	3
4	D	3.000000		****	
3	C	3.333333	****	****	
5	E	3.333333	****	****	
6	F	3.500000	****	****	****
7	G	3.800000	****		****
1	A	3.800000	****		****
2	B	4.000000			****

(e)

Univariate Results for Each DV (Restricted media C N quality data)
Sigma-restricted parameterization
Effective hypothesis decomposition

Effect	Degr. of Freedom	MEA 0.980 SS	MEA 0.980 MS	MEA 0.980 F	MEA 0.980 p
Intercept	1	260.1690	260.1690	987.9836	0.000000
Treatment	6	0.8074	0.1346	0.5110	0.792894
Error	20	5.2667	0.2633		
Total	26	6.0741			

(f)

LSD test; variable MEA 0.980 (Restricted media C N quality data)
Homogenous Groups, alpha = .05000
Error: Between MS = .26333, df = 20.000

Cell No.	Treatment	MEA 0.980 Mean	1
4	D	3.000000	****
3	C	3.000000	****
6	F	3.000000	****
2	B	3.200000	****
7	G	3.200000	****
5	E	3.333333	****
1	A	3.500000	****

(g)

Univariate Results for Each DV (Restricted media C N quality data)
Sigma-restricted parameterization
Effective hypothesis decomposition

Effect	Degr. of Freedom	MEA 0.954 SS	MEA 0.954 MS	MEA 0.954 F	MEA 0.954 p
Intercept	1	115.2672	115.2672	703.7899	0.000000
Treatment	6	2.9952	0.4992	3.0480	0.017470
Error	33	5.4048	0.1638		
Total	39	8.4000			

(h)

LSD test; variable MEA 0.954 (Restricted media C N quality data)
Homogenous Groups, alpha = .05000
Error: Between MS = .16378, df = 33.000

Cell No.	Treatment	MEA 0.954 Mean	1	2	3
5	E	1.333333	****		
4	D	1.428571	****		
6	F	1.666667	****	****	
1	A	1.857143	****	****	****
7	G	2.000000		****	****
2	B	2.000000		****	****
3	C	2.166667			****

(i)

Univariate Results for Each DV (Restricted media C N quality data)
Sigma-restricted parameterization
Effective hypothesis decomposition

Effect	Degr. of Freedom	MEA 0.940 SS	MEA 0.940 MS	MEA 0.940 F	MEA 0.940 p
Intercept	1	34.16362	34.16362	687.1219	0.000000
Treatment	6	0.30952	0.05159	1.0376	0.418503
Error	34	1.69048	0.04972		
Total	40	2.00000			

(j)

Cell No.	Treatment	MEA 0.940 Mean	1	2
	LSD test; variable MEA 0.940 (Restricted media C N quality data) Homogenous Groups, alpha = .05000 Error: Between MS = .04972, df = 34.000			
6	F	0.857143	****	
7	G	1.000000	****	****
4	D	1.000000	****	****
5	E	1.000000	****	****
2	B	1.000000	****	****
1	A	1.000000	****	****
3	C	1.166667		****

Table 11. Analysis of variance and least significant difference test of the effect of fermentation media on *P. gigantea* spores produced in submerged fermentation in nutrient modified malt extract broth at 25 ºC, on growth rate on wood agar (a) and (b), MEA at 0.994 (c) and (d), 0.980, (e) and (f), 0.954 (g) and (h), 0.940 a_w. (i) and (j).

(a)

Effect	Degr. of Freedom	WdA 0.994 SS	WdA 0.994 MS	WdA 0.994 F	WdA 0.994 p
	Univariate Results for Each DV (Restricted media C N quality data) Sigma-restricted parameterization Effective hypothesis decomposition				
Intercept	1	1165.523	1165.523	472.2614	0.000000
Treatment	6	21.264	3.544	1.4360	0.223300
Error	43	106.122	2.468		
Total	49	127.386			

(b)

Cell No.	Treatment	WdA 0.994 Mean	1	2
	LSD test; variable WdA 0.994 (Restricted media C N quality data) Homogenous Groups, alpha = .05000 Error: Between MS = 2.4680, df = 43.000			
3	C	3.885000	****	
4	D	4.122500	****	
7	G	4.669048	****	****
6	F	4.777143	****	****
2	B	5.278750	****	****
1	A	5.288333	****	****
5	E	6.017222		****

(c)

Effect	Degr. of Freedom	MEA 0.994 SS	MEA 0.994 MS	MEA 0.994 F	MEA 0.994 p
	Univariate Results for Each DV (Restricted media C N quality data) Sigma-restricted parameterization Effective hypothesis decomposition				
Intercept	1	981.8202	981.8202	327.8322	0.000000
Treatment	6	11.7937	1.9656	0.6563	0.684891
Error	43	128.7801	2.9949		
Total	49	140.5738			

(d)

Cell No.	Treatment	MEA 0.994 Mean	1
	LSD test; variable MEA 0.994 (Restricted media C N quality data) Homogenous Groups, alpha = .05000 Error: Between MS = 2.9949, df = 43.000		
4	D	3.857500	****
7	G	4.048095	****
3	C	4.063333	****
2	B	4.442500	****
1	A	4.477917	****
6	F	5.012381	****
5	E	5.338889	****

(e)

Effect	Degr. of Freedom	MEA 0.980 SS	MEA 0.980 MS	MEA 0.980 F	MEA 0.980 p
	Univariate Results for Each DV (Restricted media C N quality data) Sigma-restricted parameterization Effective hypothesis decomposition				
Intercept	1	443.6159	443.6159	694.7350	0.000000
Treatment	6	4.8967	0.8161	1.2781	0.293344
Error	34	21.7103	0.6385		
Total	40	26.6070			

(f)

Cell No.	Treatment	MEA 0.980 Mean	1	2
	LSD test; variable MEA 0.980 (Restricted media C N quality data) Homogenous Groups, alpha = .05000 Error: Between MS = .63854, df = 34.000			
4	D	2.991429	****	
5	E	3.009524	****	****
6	F	3.211667	****	****
7	G	3.472653	****	****
3	C	3.500000	****	****
2	B	3.838154	****	****
1	A	3.947143		****

(g)

Effect	Degr. of Freedom	MEA 0.954 SS	MEA 0.954 MS	MEA 0.954 F	MEA 0.954 p
	Univariate Results for Each DV (Restricted media C N quality data) Sigma-restricted parameterization Effective hypothesis decomposition				
Intercept	1	0.954688	0.954688	32.08788	0.000002
Treatment	6	0.030199	0.005033	0.16917	0.983505
Error	38	1.130587	0.029752		
Total	44	1.160786			

(h)

LSD test; variable MEA 0.954 (Restricted media C N quality data)
Homogenous Groups, alpha = .05000
Error: Between MS = .02975, df = 38.000

Cell No.	Treatment	MEA 0.954 Mean	1
5	E	0.111905	****
4	D	0.117143	****
6	F	0.135000	****
3	C	0.136667	****
1	A	0.166122	****
7	G	0.171429	****
2	B	0.184286	****

(i)

Univariate Results for Each DV (Restricted media C N quality data)
Sigma-restricted parameterization
Effective hypothesis decomposition

Effect	Degr. of Freedom	MEA 0.940 SS	MEA 0.940 MS	MEA 0.940 F	MEA 0.940 p
Intercept	1	0.164176	0.164176	11.69325	0.001647
Treatment	6	0.142008	0.023668	1.68573	0.154608
Error	34	0.477368	0.014040		
Total	40	0.619376			

(j)

LSD test; variable MEA 0.940 (Restricted media C N quality data)
Homogenous Groups, alpha = .05000
Error: Between MS = .01404, df = 34.000

Cell No.	Treatment	MEA 0.940 Mean	1	2
2	B	0.012000	****	
6	F	0.017143	****	
3	C	0.035000	****	
1	A	0.038036	****	
4	D	0.041667	****	
7	G	0.043061	****	
5	E	0.300000		****

Table 12. Analysis of variance and least significant difference test of the effect of restricted water availability in fermentation media on PG21 spore production. ME broth at 0.996 a_w modified with PEG 8000 to 0.991, 0.987 and 0.978 a_w. Spores harvested at 7 and 11 –14 days of fermentation at 25 ºC. 7-day (a) and (b), 10-day (c) and (d), 11-day (e) and (f), 12-day (g) and (h), 13-day (i) and (j), 14-day (k) and (l).

(a)

	Univariate Results for Each DV (restricted aw data) Sigma-restricted parameterization Effective hypothesis decomposition				
Effect	Degr. of Freedom	7 SS	7 MS	7 F	7 p
Intercept	1	401.3432	401.3432	2550.866	0.000000
media	3	3.2956	1.0985	6.982	0.005675
Error	12	1.8880	0.1573		
Total	15	5.1837			

(b)

	LSD test; variable 7 (restricted aw data) Homogenous Groups, alpha = .05000 Error: Between MS = .15734, df = 12.000				
Cell No.	media	7 Mean	1	2	3
4	D	4.342478			****
1	A	5.002100	****		
3	C	5.066540	****	****	
2	B	5.622434		****	

(c)

	Univariate Results for Each DV (restricted aw data) Sigma-restricted parameterization Effective hypothesis decomposition				
Effect	Degr. of Freedom	10 SS	10 MS	10 F	10 p
Intercept	1	568.1327	568.1327	5585.993	0.000000
media	3	2.3157	0.7719	7.589	0.002235
Error	16	1.6273	0.1017		
Total	19	3.9430			

(d)

	LSD test; variable 10 (restricted aw data) Homogenous Groups, alpha = .05000 Error: Between MS = .10171, df = 16.000			
Cell No.	media	10 Mean	1	2
4	D	4.777190		****
3	C	5.379165	****	
1	A	5.461563	****	
2	B	5.701233	****	

(e)

Effect	Univariate Results for Each DV (restricted aw data) Sigma-restricted parameterization Effective hypothesis decomposition Degr. of Freedom	11 SS	11 MS	11 F	11 p
Intercept	1	413.6059	413.6059	5742.700	0.000000
media	3	2.9468	0.9823	13.638	0.000503
Error	11	0.7923	0.0720		
Total	14	3.7390			

(f)

Cell No.	LSD test; variable 11 (restricted aw data) Homogenous Groups, alpha = .05000 Error: Between MS = .07202, df = 11.000 media	11 Mean	1	2
4	D	4.577980		****
1	A	5.298183	****	
3	C	5.607105	****	
2	B	5.684468	****	

(g)

Effect	Univariate Results for Each DV (restricted aw data) Sigma-restricted parameterization Effective hypothesis decomposition Degr. of Freedom	12 SS	12 MS	12 F	12 p
Intercept	1	451.8865	451.8865	2927.135	0.000000
media	3	3.5654	1.1885	7.698	0.003939
Error	12	1.8525	0.1544		
Total	15	5.4179			

(h)

Cell No.	LSD test; variable 12 (restricted aw data) Homogenous Groups, alpha = .05000 Error: Between MS = .15438, df = 12.000 media	12 Mean	1	2
4	D	4.577980		****
1	A	5.240244	****	
3	C	5.633129	****	
2	B	5.806270	****	

(i)

Effect	Univariate Results for Each DV (restricted aw data) Sigma-restricted parameterization Effective hypothesis decomposition Degr. of Freedom	13 SS	13 MS	13 F	13 p
Intercept	1	476.4903	476.4903	13648.30	0.000000
media	3	2.4207	0.8069	23.11	0.000028
Error	12	0.4189	0.0349		
Total	15	2.8396			

(j)

Cell No.	media	13 Mean	1	2	3
		LSD test; variable 13 (restricted aw data) Homogenous Groups, alpha = .05000 Error: Between MS = .03491, df = 12.000			
4	D	4.811243			****
3	C	5.520122	****		
1	A	5.664772	****	****	
2	B	5.832522		****	

(k)

Effect	Degr. of Freedom	14 SS	14 MS	14 F	14 p
		Univariate Results for Each DV (restricted aw data) Sigma-restricted parameterization Effective hypothesis decomposition			
Intercept	1	381.5069	381.5069	2915.591	0.000000
media	3	2.4045	0.8015	6.125	0.012359
Error	10	1.3085	0.1309		
Total	13	3.7130			

(l)

Cell No.	media	14 Mean	1	2
		LSD test; variable 14 (restricted aw data) Homogenous Groups, alpha = .05000 Error: Between MS = .13085, df = 10.000		
4	D	4.636501		****
3	C	5.239224	****	****
1	A	5.528810	****	
2	B	5.692655	****	

Table 13. Analysis of variance and least significant difference test of the effects of solutes used to restrict water availability in fermentation media on PG21 spore production and viability. ME broth treatments: A control 0.996 a_w, B PEG 8000 0.991, C PEG 8000 0.987, D glycerol 0.990, E glycerol 0.988, F sorbitol 0.984, G sorbitol 0.984 a_w. Spores harvested at 5, 10 and 15 days of fermentation at 25 ºC. Spore production analysis of variance (a), least significant difference test (b); viability as CFU on 0.998 a_w analysis of variance (c), least significant difference test (d), on 0.988 a_w analysis of variance (e), least significant difference test (f).

(a)

Effect	Degr. of Freedom	Spore counts log 10 SS	Spore counts log 10 MS	Spore counts log 10 F	Spore counts log 10 p
		Univariate Results for Each DV (restricted aw matric vs solute spore data) Sigma-restricted parameterization Effective hypothesis decomposition			
Intercept	0				
Treatment	5	4.48696	0.897392	5.506001	0.000810
Incubation time	1	0.02587	0.025874	0.158749	0.692802
Treatment*Incubation time	11	1.86429	0.169481	1.039858	0.435211
Error	34	5.54147	0.162984		
Total	53	26.44959			

(b)

Cell No.	Treatment	Incubation time	Spore counts log 10 Mean	1	2	3	4	5	6
	LSD test; variable Spore counts log 10 (restricted aw matric vs solute spore data) Homogenous Groups, alpha = .05000 (Non-Exhaustive Search) Error: Between MS = .16298, df = 34.000								
16	F	5	4.397940	****	****				
13	E	5	4.397940	****	****				
10	D	5	4.397940	****	****				
19	G	5	4.498283	****					
4	B	5	5.096910	****	****	****			
1	A	5	5.341041		****	****	****		
20	G	10	5.356294			****	****		
21	G	15	5.414346			****	****	****	
3	A	15	5.877718				****	****	****
8	C	10	5.990657				****	****	****
17	F	10	6.047313					****	****
9	C	15	6.056417					****	****
18	F	15	6.087181						****
12	D	15	6.216222						****
15	E	15	6.218239						****
2	A	10	6.234341						****
14	E	10	6.254203						****
11	D	10	6.328708						****
6	B	15	6.498428						****
5	B	10	6.504517						****

(c)

Effect	Univariate Results for Each DV (restricted aw matric vs solute CFU data) Sigma-restricted parameterization Effective hypothesis decomposition				
	Degr. of Freedom	).998 a $_w$ SS	).998 a $_w$ MS	).998 a $_w$ F	).998 a $_w$ p
Intercept	1	5502.574	5502.574	35109.27	0.00
Treatment	6	21.424	3.571	22.78	0.00
Incubation d	2	120.575	60.287	384.66	0.00
Treatment*Incubation d	12	34.775	2.898	18.49	0.00
Error	252	39.495	0.157		
Total	272	253.978			

(d)

LSD test; variable 0.998 a_w (restricted aw matric vs solute CFU data)
Homogenous Groups, alpha = .05000 (Non-Exhaustive Search)
Error: Between MS = .15673, df = 252.00

Cell No.	Treatment	Incubation d	0.998 a_w Mean	1	2	3	4	5	6	7	8	9	10	11	12
19	G	5	3.045354	****											
16	F	5	3.657488		****										
13	E	5	3.687927		****										
7	C	5	3.871345		****	****									
10	D	5	4.054513			****									
21	G	15	4.704983				****								
4	B	5	4.913089				****	****							
1	A	5	5.038444					****	****						
8	C	10	5.069472					****	****						
3	A	15	5.200687					****	****	****	****				
6	B	15	5.264468						****	****					
20	G	10	5.478082							****	****	****			
12	D	15	5.661708								****	****			
2	A	10	5.722209									****	****	****	
18	F	15	5.741599									****	****		
14	E	10	5.813120									****	****	****	
15	E	15	5.847265									****	****	****	
9	C	15	5.956925										****	****	****
17	F	10	5.985996										****	****	****
11	D	10	6.032293											****	****
5	B	10	6.204054												****

(e)

Univariate Results for Each DV (restricted aw matric vs solute CFU data)
Sigma-restricted parameterization
Effective hypothesis decomposition

Effect	Degr. of Freedom	0.988 a_w SS	0.988 a_w MS	0.988 a_w F	0.988 a_w p
Intercept	0				
Treatment	4	5.9370	1.48425	9.6503	0.000000
Incubation d	1	75.8046	75.80462	492.8673	0.000000
Treatment*Incubation d	10	13.9450	1.39450	9.0668	0.000000
Error	183	28.1460	0.15380		
Total	201	164.0893			

(f)

Cell No.	Treatment	Incubation d	).988 a_w Mean	1	2	3	4	5	6	7	8	9	10	11
LSD test; variable 0.988 a_w (restricted aw matric vs solute CFU data) Homogenous Groups, alpha = .05000 (Non-Exhaustive Search) Error: Between MS = .15380, df = 183.00														
19	G	5	3.390724	****										
16	F	5	3.666163	****	****									
13	E	5	3.781125		****									
7	C	5	3.873365		****									
10	D	5	4.010651		****									
21	G	15	4.653623			****								
1	A	5	4.784242			****	****							
8	C	10	4.811882			****	****	****						
4	B	5	4.950548				****	****						
15	E	15	5.021722			****	****	****	****					
12	D	15	5.215836					****	****	****				
20	G	10	5.325556						****	****	****			
18	F	15	5.398271							****				
14	E	10	5.588571							****	****	****		
11	D	10	5.604584							****	****	****		
9	C	15	5.676042								****	****		
2	A	10	5.776170									****	****	
17	F	10	6.091252										****	****
5	B	10	6.208080											****

Table 14. Analysis of variance (a) and least significant difference test (b) of the effects of a_w solute treatment in fermentation media, harvest time, spore type (oidia or conidia), a_w of CFU agar, two, three and four-way interactions on production and viability of propagules, as measured as CFU, produced in fermentation supported at liquid/air interface in ReacSyn ™ fermentation vessels.

(a)

Effect	Univariate Results for Each DV (ReacSyn aw data) Sigma-restricted parameterization Effective hypothesis decomposition				
	Degr. of Freedom	CFU SS	CFU MS	CFU F	CFU p
Intercept	1	3315.967	3315.967	26575.60	0.000000
Harvest	1	0.399	0.399	3.20	0.074718
Treatment	3	19.973	6.658	53.36	0.000000
Spore type	1	8.823	8.823	70.72	0.000000
CFU aw	1	0.067	0.067	0.54	0.464868
Harvest*Treatment	3	4.248	1.416	11.35	0.000000
Harvest*Spore type	1	0.040	0.040	0.32	0.569628
Treatment*Spore type	3	54.728	18.243	146.21	0.000000
Harvest*CFU aw	1	0.001	0.001	0.01	0.934126
Treatment*CFU aw	3	0.836	0.279	2.23	0.084663
Spore type*CFU aw	1	0.147	0.147	1.17	0.279456
Harvest*Treatment*Spore type	3	19.662	6.554	52.53	0.000000
Harvest*Treatment*CFU aw	3	0.029	0.010	0.08	0.972489
Harvest*Spore type*CFU aw	1	0.051	0.051	0.40	0.525141
Treatment*Spore type*CFU aw	3	0.479	0.160	1.28	0.281482
Harvest*Treatment*Spore type*CFU aw	3	0.387	0.129	1.03	0.377628
Error	282	35.187	0.125		
Total	313	161.120			

(b)

Cell No.	Harvest	Treatment	Spore type	CFU aw	CFU Mean	1	2	3	4	5	6	7	8	9
	LSD test; variable CFU (ReacSyn aw data) Homogenous Groups, alpha = .05000 (Non-Exhaustive Search) Error: Between MS = .12477, df = 282.00													
12	Primary	D	aerial	low	1.765412	****								
11	Primary	D	aerial	high	1.959877	****								
6	Primary	B	submerged	low	2.335330		****							
5	Primary	B	submerged	high	2.816068			****						
22	Secondary	B	submerged	low	2.907274			****	****					
28	Secondary	D	aerial	low	2.936221			****	****					
17	Secondary	A	submerged	high	2.999644			****	****					
27	Secondary	D	aerial	high	3.003755			****	****					
13	Primary	E	submerged	high	3.028243			****	****					
18	Secondary	A	submerged	low	3.047862			****	****					
14	Primary	E	submerged	low	3.068128			****	****					
26	Secondary	D	submerged	low	3.096492			****	****					
21	Secondary	B	submerged	high	3.111525				****					
2	Primary	A	submerged	low	3.168403				****					
1	Primary	A	submerged	high	3.190204				****					
25	Secondary	D	submerged	high	3.221835				****					
19	Secondary	A	aerial	high	3.563780					****				
29	Secondary	E	submerged	high	3.693613					****	****			
20	Secondary	A	aerial	low	3.695799					****	****			
9	Primary	D	submerged	high	3.721932					****	****			
31	Secondary	E	aerial	high	3.761443					****	****	****	****	
10	Primary	D	submerged	low	3.766847					****	****	****		
32	Secondary	E	aerial	low	3.773092					****	****	****	****	
30	Secondary	E	submerged	low	3.794255					****	****	****	****	
24	Secondary	B	aerial	low	3.806739					****	****	****	****	
15	Primary	E	aerial	high	3.906672						****	****	****	****
23	Secondary	B	aerial	high	3.916123						****	****	****	****
7	Primary	B	aerial	high	4.012082						****	****	****	****
16	Primary	E	aerial	low	4.064783							****	****	****
3	Primary	A	aerial	high	4.074226							****	****	****
8	Primary	B	aerial	low	4.101984								****	****
4	Primary	A	aerial	low	4.169891									****

Table 15 Analysis of variance and least significant difference test of the effect of immobilisation of inoculum in alginate beads in malt extract broth at 25 ºC at 150 rpm., fermentation time and interaction on the production of PG21 propagules (a) and (b); viability as CFU on malt extract agar (c) and (d).

(a)

Effect	Degr. of Freedom	Spore production SS	Spore production MS	Spore production F	Spore production p
	Univariate Results for Each DV (Beads PG21 immobilised spore data) Sigma-restricted parameterization Effective hypothesis decomposition				
Intercept	1	1188.990	1188.990	58410.46	0.000000
Treatment	1	1.585	1.585	77.87	0.000000
Fermentation (d)	3	10.508	3.503	172.07	0.000000
Treatment*Fermentation (d)	3	1.238	0.413	20.28	0.000000
Error	27	0.550	0.020		
Total	34	12.569			

(b)

LSD test; variable Spore production (Beads PG21 immobilised spore data)
Homogenous Groups, alpha = .05000
Error: Between MS = .02036, df = 27.000

Cell No.	Treatment	Fermentation (d)	Spore production Mean	1	2	3	4
1	Liquid	3	4.787016			****	
5	Beads	3	5.980755				****
3	Liquid	7	6.569323		****		
2	Liquid	5	6.616384		****		
7	Beads	7	6.901772	****			
8	Beads	10	6.981453	****			
4	Liquid	10	7.010132	****			
6	Beads	5	7.012409	****			

(c)

Univariate Results for Each DV (beads PG21 immobilised CFU data)
Sigma-restricted parameterization
Effective hypothesis decomposition

Effect	Degr. of Freedom	CFU log10 SS	CFU log10 MS	CFU log10 F	CFU log10 p
Intercept	1	2732.566	2732.566	287714.9	0.000000
Treatment	1	0.266	0.266	28.0	0.000002
Fermentation (d)	3	10.299	3.433	361.5	0.000000
Treatment*Fermentation (d)	3	4.474	1.491	157.0	0.000000
Error	64	0.608	0.009		
Total	71	15.647			

(d)

LSD test; variable CFU log10 (beads PG21 immobilised CFU data)
Homogenous Groups, alpha = .05000
Error: Between MS = .00950, df = 64.000

Cell No.	Treatment	Fermentation (d)	CFU log10 Mean	1	2	3	4	5
5	Beads	5	5.033448				****	
1	Liquid	5	5.999685					****
4	Liquid	15	6.231089	****				
6	Beads	7	6.278413	****	****			
2	Liquid	7	6.284485	****	****			
3	Liquid	10	6.370138		****			
8	Beads	15	6.503198			****		
7	Beads	10	6.583902			****		

Table 16 Analysis of variance and least significant difference test of the effect of wood chip moisture content on the production of PG21 oida in solid substrate fermentation at 25ºC (a) and (b) and viability measured as CFU on MEA (c) and (d).

(a)

Effect	Univariate Results for Each DV (Spreadsheet1) Sigma-restricted parameterization Effective hypothesis decomposition				
	Degr. of Freedom	Oidia log SS	Oidia log MS	Oidia log F	Oidia log p
Intercept	1	523.8598	523.8598	551.0416	0.000000
Treatment	11	454.1777	41.2889	43.4313	0.000000
Error	24	22.8161	0.9507		
Total	35	476.9938			

(b)

Cell No.	LSD test; variable Oidia log (Spreadsheet1) Homogenous Groups, alpha = .05000 Error: Between MS = .95067, df = 24.000				
	Treatment	Oidia log Mean	1	2	3
1	A	0.000000		****	
2	B	0.000000		****	
7	G	0.000000		****	
4	D	0.000000		****	
6	F	0.000000		****	
3	C	1.884404			****
5	E	6.444251	****		
11	K	7.147363	****		
8	H	7.313564	****		
10	J	7.333670	****		
12	L	7.634437	****		
9	I	8.018278	****		

(c)

Effect	Univariate Results for Each DV (PG woodchip 2nd pilot CFU data) Sigma-restricted parameterization Effective hypothesis decomposition				
	Degr. of Freedom	CFU log SS	CFU log MS	CFU log F	CFU log p
Intercept	1	162.5596	162.5596	3147.317	0.00
Treatment	11	250.9747	22.8159	441.738	0.00
Error	16	0.8264	0.0517		
Total	27	251.8011			

(d)

Cell No.	Treatment	CFU log Mean	1	2	3
	LSD test; variable CFU log (PG woodchip 2nd pilot CFU data) Homogenous Groups, alpha = .05000 Error: Between MS = .05165, df = 16.000				
1	A	0.000000	****		
2	B	0.000000	****		
3	C	0.000000	****		
4	D	0.000000	****		
5	E	0.000000	****		
6	F	0.000000	****		
7	G	0.000000	****		
9	I	5.500000			****
8	H	5.699592			****
10	J	6.289892		****	
11	K	6.340621		****	
12	L	6.520696		****	

Table 17 Analysis of variance (a) and (b) least significant difference test of the effect of sawdust moisture content and incubation time on the production of PG21 oida in solid substrate fermentation at 25ºC.

(a)

Effect	Degr. of Freedom	Spores log SS	Spores log MS	Spores log F	Spores log p
	Univariate Results for Each DV (PG sawdust temporal data) Sigma-restricted parameterization Effective hypothesis decomposition				
Intercept	1	1991.037	1991.037	23496.13	0.000000
MC	9	7.325	0.814	9.60	0.000000
Incubation	1	0.007	0.007	0.08	0.779870
MC*Incubation	9	0.992	0.110	1.30	0.272767
Error	34	2.881	0.085		
Total	53	11.172			

(b)

Cell No.	MC	Spores log Mean	1	2	3
	LSD test; variable Spores log (PG sawdust temporal data) Homogenous Groups, alpha = .05000 Error: Between MS = .08474, df = 34.000				
2	17	5.979790	****		
5	26	6.011092	****		
3	20	6.055228	****		
6	29	6.087055	****		
4	23	6.135709	****	****	
7	38	6.186430	****	****	
1	15	6.309891	****	****	
8	44	6.454041		****	
9	50	6.907399			****
10	55	7.064827			****

Table 18 Analysis of variance and least significant difference test of the effect of sawdust MC and incubation time on the production (a) and (b) and viability (c) and (d) of PG21 oida in solid substrate fermentation at 25ºC for 3-12 weeks.

(a)

Effect	Univariate Results for Each DV (Spreadsheet7) Sigma-restricted parameterization Effective hypothesis decomposition Degr. of Freedom	Oidia log SS	Oidia log MS	Oidia log F	Oidia log p
Intercept	1	2959.576	2959.576	210766.3	0.000000
MC	4	0.716	0.179	12.8	0.000004
Incubation	2	17.280	8.640	615.3	0.000000
MC*Incubation	8	0.232	0.029	2.1	0.072204
Error	30	0.421	0.014		
Total	44	18.649			

(b)

Cell No.	LSD test; variable Oidia log (Spreadsheet7) Homogenous Groups, alpha = .05000 (Non-Exhaustive Search) Error: Between MS = .01404, df = 30.000 MC	Incubation	Oidia log Mean	1	2	3	4	5	6	7
4	67	3	7.467708	****						
2	50	8	7.493230	****						
10	80	3	7.540645	****						
1	50	3	7.542606	****						
13	83	3	7.596961	****	****					
7	75	3	7.660196	****	****					
5	67	8	7.741668		****					
14	83	8	7.790347		****	****				
11	80	8	7.963701			****				
8	75	8	7.969515			****				
3	50	12	8.743149				****			
6	67	12	8.838708				****	****		
15	83	12	8.976551					****	****	
12	80	12	9.135448						****	****
9	75	12	9.186107							****

(c)

Effect	Univariate Results for Each DV (aw vs temporal CFU data) Sigma-restricted parameterization Effective hypothesis decomposition				
	Degr. of Freedom	CFU log SS	CFU log MS	CFU log F	CFU log p
Intercept	1	27552.59	27552.59	367743.5	0.000000
MC	4	17.55	4.39	58.6	0.000000
Incubation	2	2.16	1.08	14.4	0.000001
Agar	1	0.17	0.17	2.3	0.132979
MC*Incubation	8	13.00	1.62	21.7	0.000000
MC*Agar	4	0.33	0.08	1.1	0.352816
Incubation*Agar	2	0.08	0.04	0.6	0.575955
MC*Incubation*Agar	8	0.13	0.02	0.2	0.986331
Error	477	35.74	0.07		
Total	506	69.13			

(d)

Cell No.	LSD test; variable CFU log (aw vs temporal CFU data) Homogenous Groups, alpha = .05000 (Non-Exhaustive Search) Error: Between MS = .07402, df = 492.00										
	MC	Incubation	CFU log Mean	1	2	3	4	5	6	7	8
6	67	12	6.914380	****							
2	50	8	7.060696		****						
5	67	8	7.154697		****	****					
15	83	12	7.179625		****	****					
3	50	12	7.190652			****					
14	83	8	7.386519				****				
1	50	3	7.408145				****	****			
7	75	3	7.465895				****	****	****		
12	80	12	7.498362				****	****	****		
4	67	3	7.510983				****	****	****	****	
10	80	3	7.521238					****	****	****	
13	83	3	7.537466					****	****	****	
11	80	8	7.567548						****	****	
8	75	8	7.625310							****	
9	75	12	7.949123								****

Table 19 Analysis of variance and least significant difference test of the effect of sawdust a_w and addition of CaCl on the production (a) and (b) and viability (c) and (d) of PG21 oida in solid substrate fermentation at 25ºC for 3 weeks.

(a)

Effect	Univariate Results for Each DV (ssf scale up and aw and cacl spore data) Sigma-restricted parameterization Effective hypothesis decomposition Degr. of Freedom	Oidia log SS	Oidia log MS	Oidia log F	Oidia log p
Intercept	1	1657.575	1657.575	227122.6	0.000000
aw	2	0.022	0.011	1.5	0.241499
cacl	2	0.133	0.067	9.1	0.001203
aw*cacl	4	0.040	0.010	1.4	0.272908
Error	23	0.168	0.007		
Total	31	0.378			

(b)

Cell No.	LSD test; variable Oidia log (ssf scale up and aw and cacl spore data) Homogenous Groups, alpha = .05000 Error: Between MS = .00730, df = 23.000 aw	cacl	Oidia log Mean	1	2	3	4
5	981	20	7.616576				****
9	991	40	7.694818	****			****
3	974	40	7.698952	****			****
6	981	40	7.727366	****	****		****
8	991	20	7.766776	****	****	****	
2	974	20	7.793548	****	****	****	
4	981	0	7.828123	****	****	****	
7	991	0	7.840794		****	****	
1	974	0	7.894600			****	

(c)

Effect	Univariate Results for Each DV (ssf scale up and aw and cacl cfu data) Sigma-restricted parameterization Effective hypothesis decomposition Degr. of Freedom	CFU SS	CFU MS	CFU F	CFU p
Intercept	1	11700.43	11700.43	242253.2	0.000000
aw	2	28.09	14.05	290.8	0.000000
cacl	2	0.29	0.15	3.0	0.049143
Test agar	1	0.18	0.18	3.8	0.051439
aw*cacl	4	0.53	0.13	2.7	0.028915
aw*Test agar	2	0.04	0.02	0.4	0.639398
cacl*Test agar	2	0.10	0.05	1.1	0.339857
aw*cacl*Test agar	4	0.46	0.11	2.4	0.051891
Error	325	15.70	0.05		
Total	342	47.36			

(d)

Cell No.	aw	cacl	Test agar	CFU Mean	1	2	3	4
	LSD test; variable CFU (ssf scale up and aw and cacl cfu data) Homogenous Groups, alpha = .05000 (Non-Exhaustive Search) Error: Between MS = .04830, df = 325.00							
6	974	40	M	6.071768	****			
3	974	20	H	6.162474	****			
5	974	40	H	6.165969	****			
2	974	0	M	6.189209	****			
4	974	20	M	6.259497	****	****		
1	974	0	H	6.427516		****		
10	981	20	M	6.909507			****	
14	991	0	M	6.932065			****	
18	991	40	M	6.978828			****	****
12	981	40	M	6.981334			****	****
15	991	20	H	6.994783			****	****
9	981	20	H	7.009202			****	****
16	991	20	M	7.015234			****	****
11	981	40	H	7.019590			****	****
7	981	0	H	7.021757			****	****
13	991	0	H	7.045698				****
17	991	40	H	7.075608				****
8	981	0	M	7.103096				****

Table 20 Analysis of variance and least significant difference test of the effect of sawdust a_w on the production (a) and viability (b) and (c) of PG21 oida in solid substrate fermentation at 25ºC for 3 weeks.

(a)

Effect	Degr. of Freedom	Oidia log SS	Oidia log MS	Oidia log F	Oidia log p
	Univariate Results for Each DV (ssf scale up aw modification sp data) Sigma-restricted parameterization Effective hypothesis decomposition				
Intercept	1	990.0890	990.0890	64710.55	0.000000
substrate aw	2	0.0231	0.0116	0.76	0.486994
Error	15	0.2295	0.0153		
Total	17	0.2526			

(b)

Effect	Degr. of Freedom	CFU log SS	CFU log MS	CFU log F	CFU log p
	Univariate Results for Each DV (ssf scale up aw modification cfu data) Sigma-restricted parameterization Effective hypothesis decomposition				
Intercept	1	9629.204	9629.204	154022.4	0.000000
substrate aw	2	52.408	26.204	419.1	0.000000
test agar aw	2	12.468	6.234	99.7	0.000000
substrate aw*test agar aw	4	2.158	0.539	8.6	0.000002
Error	239	14.942	0.063		
Total	247	76.929			

(c)

Cell No.	LSD test; variable CFU log (ssf scale up aw modification cfu data) Homogenous Groups, alpha = .05000 Error: Between MS = .06252, df = 239.00 substrate aw	test agar aw	CFU log Mean	1	2	3	4	5	6	7	8
1	974	974	5.527752		****						
2	974	988	5.807637			****					
3	974	998	5.994179				****				
4	981	974	6.242263					****			
7	991	974	6.603869						****		
8	991	988	6.736459							****	
5	981	988	6.860039								****
6	981	998	7.051042	****							
9	991	998	7.098202	****							

Table 21 Analysis of variance of the effect of sawdust mass on the viability of PG21 oida in solid substrate fermentation at 25ºC for 3 weeks.

Effect	Univariate Results for Each DV (ssf scale comparison data) Sigma-restricted parameterization Effective hypothesis decomposition Degr. of Freedom	CFU log SS	CFU log MS	CFU log F	CFU log p
Intercept	0				
ss mass g	1	24.78355	24.78355	679.0727	0.000000
test agar aw	1	0.38619	0.38619	10.5816	0.001436
ss mass g*test agar aw	4	0.21004	0.05251	1.4388	0.224445
Error	138	5.03647	0.03650		
Total	147	54.33713			

Table 22 Analysis of variance (a) and least significant difference test (b) of the effect of water availability on endogenous reserves of PG21 spores produced in solid substrate fermentation on Pinus sylvestris sawdust at 25 ºC for 3 weeks. Collected data from treatment groups at same a_w, but differing additions of $CaCl_2$.

(a)

Effect	Univariate Results for Each DV (ssf scale up and cacl endogenous data) Sigma-restricted parameterization Effective hypothesis decomposition Mannitol SS	Mannitol MS	Mannitol F	Mannitol p
Intercept	20.14146	20.14146	32.93857	0.000003
aw	5.32691	2.66345	4.35571	0.022178
Error	17.73308	0.61149		
Total	23.05998			

(b)

Cell No.	aw	Mannitol Mean	1	2
	LSD test; variable Mannitol (ssf scale up and cacl endogenous data) Homogenous Groups, alpha = .05000 Error: Between MS = .61149, df = 29.000			
2	981	0.239574	****	
3	991	0.880818	****	****
1	974	1.349099		****

Table 23 Analysis of variance (a) and least significant difference test (b) of the effect of water availability on endogenous reserves of PG21 spores produced in solid substrate fermentation on Pinus sylvestris sawdust at 25 ºC for 3 weeks.

(a)

Effect	Mannitol SS	Mannitol MS	Mannitol F	Mannitol p
	Univariate Results for Each DV (ssf scale up aw modification endogenous data) Sigma-restricted parameterization Effective hypothesis decomposition			
Intercept	1159.021	1159.021	4636.649	0.000000
substrate aw	1.960	0.980	3.921	0.042677
Error	3.750	0.250		
Total	5.710			

(b)

Cell No.	substrate aw	Mannitol Mean	1	2
	LSD test; variable Mannitol (ssf scale up aw modification endogenous data) Homogenous Groups, alpha = .05000 Error: Between MS = .24997, df = 15.000			
2	981	7.596709	****	
3	991	8.076294	****	****
1	974	8.400022		****

Appendix II

Presentations

II.I Oral presentations

September 2003, Forest Pathology Conference, Thetford, U.K.
Ecophysiology and production of the biocontrol agent *Phlebiopsis gigantea*.

Heterobasidion annosum, a tree pathogen, is ubiquitous in the environment and causes financial losses for the forestry industry. It has been found that the saprophyte, Phlebiopsis gigantea, can reduce losses from H. annosum, by out-competing for woody resources. The natural population of spores of P. gigantea can be augmented by the application of a spore suspension, to the stumps, at the time of tree-felling.

Preliminary studies have been carried out to asses liquid media for the production of inoculum. In N controlled ME broth blastospore production was low in rich nutrient broth, but more efficient in the absence of peptone. In a pilot study to produce oidia on wood chips, mycelial growth is visible in higher water activity treatments. The experiment is not ready for harvesting. Environmental studies have been carried out to assess the fitness of different isolates of these organisms in the environment. Growth rates were calculated by measuring the radii of growing colonies under a variety of conditions. Variables used included temperature, water activity and growth medium. P. gigantea and H. annosum isolates were inoculated onto MEA plus Phostrogen® modified with glycerol within the range of 0.940 to 0.995 aw. Plates were incubated at 25oC and 15oC, Initial results show that generally the H. annosum isolates grew faster under drier/cooler conditions and the P. gigantea isolates grew faster under wetter/warmer conditions. Interaction studies have been carried out under differing environmental regimes. These indicate that the antagonist is not able to suppress the pathogen under all conditions.

June 2004, 5th Postgraduate Research Conference, Cranfield University, Silsoe, U.K.
Ecophysiology and production of the biocontrol agent *Phlebiopsis gigantea*.

Heterobasidion annosum, a tree pathogen, is ubiquitous in the environment and causes financial losses for the forestry industry. It has been found that the saprophyte, Phlebiopsis gigantea, can reduce losses from H. annosum, by out-competing for woody resources. The natural population of spores of P. gigantea can be augmented by the application of a spore suspension, to the stumps, at the time of tree-felling.

Environmental studies have been carried out to assess the fitness of different isolates of these organisms in the environment. Growth rates were calculated by measuring the radii of growing colonies under a variety of conditions. Variables used included temperature, water activity, growth medium and competition with other species. A variety of *P. gigantea* and *H. annosum* isolates were inoculated onto MEA plus Phostrogen® modified with glycerol within the range of 0.940 to 0.995 a_w. Plates were incubated at 25 ºC and 15 ºC, growth rates were calculated. Initial results show that generally the *H. annosum* isolates grew faster under drier/cooler conditions and the *P. gigantea* isolates grew faster under wetter/warmer conditions.

Interaction studies have been carried out under differing environmental regimes. These indicate that the antagonist is not able to suppress the pathogen under all conditions.

Submerged culture and solid substrates have been studied as methods for production of *P. gigantea* spores.

June 2005, 6th Postgraduate Research Conference, Cranfield University, Silsoe, U.K.
Production of the biocontrol agent *Phlebiopsis gigantea*.

Throughout the temperate zone the forestry industry suffers a financial loss due to a wood rotting disease, root and butt rot that reduces both quality and quantity of timber produced. The application of a spore suspension of the saprophytic fungus, *Phlebiopsis gigantea*, at the time of thinning, reduces the ability of the pathogenic fungus, *Heterobasidion annosum*, to colonise the freshly cut stumps.

Environmental studies were carried out to assess the fitness of different isolates of the pathogen and antagonist. These showed that competitiveness was affected by environmental factors, particularly water availability. Studies have been carried out, using liquid and solid substrate fermentations, to examine the possibility of improving production methods of viable spores of the antagonist. Liquid culture studies produced variable results, with a maximum production of $Log_{10}7$ submerged conidia ml^{-1} media with different imposed ecophysiological stresses. However, temporal studies on solid substrate, *Pinus sylvestris* sawdust, produced $>Log_{10}7$ oidia g^{-1}, with viability from 90% - 100% in the best moisture content treatments. Analysis of the endogenous reserves is in progress to identify specific quality characteristics.

September 2006, British Mycological Society, Annual Scientific Meeting: Stress in Yeasts and Filamentous Fungi, Birmingham, U.K.
Effect of environmental factors on interactions between the pathogen *Heterobasidion annosum* and antagonist *Phlebiopsis gigantea*.

Heterobasidion annosum, the causative agent of the disease “root and butt rot” in conifers, causes severe financial losses to the forestry industry throughout the temperate zone. The normal route of infection is as basidiospores that land on the freshly cut stump surface during thinning operations, the colonisation of the stump providing sufficient inoculum for the fungal hyphae to invade healthy roots of adjoining trees. The most successful disease control measures involve depriving the pathogen the opportunity to become established in the food source of the fresh cut stump. Frequently killing the surface of the stump with a chemical treatment does this, but there is also some success with a biocontrol method. When the antagonist *Phlebiopsis gigantea* is inoculated onto the stump surface it rapidly invades the tissue thus preventing the pathogen access to the resource.

Environmental studies have been carried out to assess the fitness of different isolates of the antagonist relative to the pathogen. Competitiveness was affected by environmental factors; water availability, temperature, nutrition, and substrate. These indicate that the antagonist is not able to suppress the pathogen under all conditions. Generally, in wet warm conditions the antagonist is dominant, but in drier, cooler conditions the pathogen is dominant. Consideration needs to be given to possible field conditions at the time of application to maximise the establishment of the antagonist.

II.II Poster presentations

September 2003, Basidio 2003: Conference on the Biology of Basidiomycete Fungi, Horticulture Research International, Warwick, U.K.
Relative impact of environmental regimes on the biocontrol agent *Phlebiopsis* gigantea and the pathogen *Heterobasidion annosum.*

September 2004, British Mycological Society, Annual Scientific Meeting: Fungi in the environment, Nottingham, U.K.
Impact of environmental factors on growth and interactions between *Phlebiopsis* gigantea and *Heterobasidion annosum.*

April 2005, Basidio 2005: Conference on the Biology of Basidiomycete Fungi, Horticulture Research International, Warwick, U.K.
Development of production methods for the biocontrol agent *Phlebiopsis gigantea*.

September 2005, British Mycological Society, Annual Scientific Meeting: Exploitation of Fungi, Manchester, U.K.
Production of the biocontrol agent *Phlebiopsis gigantea* in controlled environmental and nutritional media.