STUDIES ON SEEDLING MORTALITY ASSOCIATED WITH *EUCALYPTUS REGNANS* FOREST REGENERATION IN SOUTHERN TASMANIA

by
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This thesis contains no material which has been accepted for the award of any other degree or diploma in any tertiary institution and that to the best of my knowledge and belief, the thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

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ABSTRACT

Burning of logging slash resulted in apparent sterilisation of the soil surface to a depth of about 2cm and also increased soil surface pH, which in localised areas reached sufficiently high values to be toxic to seedlings. *In vitro* studies showed that high pH conditions caused seedling mortality in *E. regnans* although the lethal effect of a transient elevated pH was reduced at temperatures sufficiently low to delay germination. The pH above which detrimental effects were expected was in the range 8.4 to 9.0.

Suppression of seedling growth was observed in field soil with glasshouse studies showing that this suppression was partly overcome by prior slash-burning or by addition of N and P fertilisers.

Availability of soil moisture appeared to be the main factor limiting seedling establishment in the field. Slash-burning was associated with increased seedling emergence but also with increased mortality rates compared to an unburnt control.

Although fungi were found to be associated with the majority of dying seedlings from the field, their involvement in seedling pathology was unclear. Most common among these isolates was a species of *Phoma*, representative isolates of which caused discolouration and stunting of *E. regnans* seedlings *in vitro*, but were not otherwise markedly pathogenic. Species of *Alternaria* and *Cladosporium* were also commonly found on seedlings and probably present as saprophytes. Pythiaceous fungi were isolated from seedlings but pathogenicity of these was not examined. *Rhizoctonia solani* (pectic zymogram groups ZG5, ZG7 and ZG10) were isolated from seedlings that had died in field soil under glasshouse conditions. Representatives of *R. solani* groups ZG5 and ZG7 were pathogenic to *E. regnans in vitro* however *Rhizoctonia* was not commonly isolated from the field.

Three bacterial rhizosphere isolates (two *Pseudomonas fluorescens* and *Bacillus circulans*) and a fungus (*Epicoccum purpurascens*) from *E. regnans* were evaluated as fungal antagonists. Three of these were shown to reduce mortality (due to *R. solani* ZG7) of seedlings *in vitro* following inoculation in the vicinity of seeds, with *E. purpurascens*

providing 100% protection after 30 days (compared with 22% survival of controls). Surprisingly, *B. circulans* and *E. purpurascens* also increased seed germination rates. Of the antagonists, only *E. purpurascens* improved seedling establishment in a glasshouse trial, both in the presence and absence of *R. solani* ZG7. However neither treatment with *E. purpurascens* nor the fungicide Raxil (applied to seed in combination with Mancozeb) resulted in improvement in seedling establishment in a small field trial.

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TABLE OF CONTENTS

1 INTRODUCTION

The Australian Newsprint Mills (ANM) is responsible for managing a 160,000 hectare forest concession, producing pulp for paper production as well as supplying most of the state's veneer production and over 25% of sawlogs used within Tasmania. Forest resources of the company are managed by ANM Forest Management.

Impetus for this project came as a result of concern over high seedling mortality in some ANM regeneration areas in the year following sowing. In one such area known as Jungle-17 which was sown in 1991, seedling mortality had been almost total, requiring subsequent cultivation of the soil surface and hand planting of nursery-grown stock. The high incidence of seedling mortality over the affected areas indicated that a fungal pathogen may have been responsible.

Although often regarded as a cause of mortality there is little known about fungi responsible for seedling mortality in eucalypt forest areas in Tasmania. The principal aim of this project was to examine the causes and control of mortality of eucalypt seedlings with emphasis on *Eucalyptus regnans*. This work consisted of several parts, these being:

- Investigation of the pattern of seedling establishment and mortality in an affected area following slash-burning
- Further investigations into the effects on seedling establishment following slash-burning
- Identification and evaluation of pathogenicity of fungi found associated with seedlings at the time of mortality
- Evaluation of the effect of some fungicides on seedling mortality
- Isolation and evaluation of microbial antagonists to potential fungal pathogens

Part of the results of this thesis has been published:

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2 LITERATURE REVIEW

2.1 *Eucalyptus regnans* - natural distribution and silvicultural practices for forest regeneration from field sown seed

Eucalyptus regnans is endemic to parts of Victoria and Tasmania (Hall *et al.*, 1975) being one of the most important commercial hardwood tree species in these states. It is valued for pulping and for timber (which together with that of *E. delegatensis* and *E obliqua*, is marketed as "Tasmanian oak"). In Victoria *E. regnans* is mainly found on the mountains to the east of Melbourne, with a small population in the Otways to the south-west while in Tasmania it occurs in the north-east, south-east and in the valleys of the Derwent and Huon rivers. In Victoria this tree grows within an altitude range of 150 to 900 metres and in Tasmania it can be found between near sea level and about 600 metres.

While climate is the main factor controlling the distribution of *E. regnans* (Cunningham 1960), it prefers fertile loamy soils. Forests of *E. regnans* are confined to areas with cool summers and cool to cold winters, with annual rainfall ranging from 750 to over 1650 mm. Light to moderate snowfalls and frosts can occur within these areas throughout the year (Hall *et .al.*, 1975).

Under natural conditions *E. regnans* is usually found in pure even-aged stands (Cunningham, 1960; Gilbert, 1958; Hall *et al.*, 1975). It is a fire-sensitive species with few trees surviving fire (Gilbert, 1958). Natural regeneration follows the occasional catastrophic wildfires which kill all trees. Following such fires there is a prolific fall of seed from standing trees over a period of several months ensuring regeneration and vigorous competition in developing regrowth.

2.1.1 Early studies on *E. regnans* forest regeneration

A need to understand the regeneration of *E. regnans* forest to enable good management practices was recognised in the 1950s prompting research in Tasmania and Victoria. Between 1955 and 1958 Gilbert (1958) conducted a study into forest ecology of the Florentine Valley in Tasmania which included an investigation of the factors influencing eucalypt seedling establishment, with particular emphasis on

E. regnans. At about the same time Cunningham (1960) conducted a detailed study of factors affecting the regeneration of *E. regnans* in the Ada River area of Victoria, including investigation of the fate of seed and young seedlings. Cremer (1962) continued the work of Gilbert in the Florentine Valley. Collectively these researchers also explored silvicultural techniques and their findings formed the basis for subsequent silvicultural practices used in the management of *E. regnans*. Among the significant findings of Gilbert (1958) and Cunningham (1960) was demonstration of the need for burning in regeneration of this species, leading to the adoption of regeneration burning as a standard practice in this type of forest (Shea *et al.*, 1981).

2.1.2 Regeneration procedures

Although based on the natural regeneration process, the established procedures for regeneration from seed differ in a number of important ways. These are:

a) Forest is harvested in distinct coupes (ranging in size between about 20 and 100 hectares) while surrounding forest may remain undisturbed.

b) Vegetation is completely cleared from the site.

c) A smaller amount of seed is applied and normally only once.

2.1.3 Seedbed preparation

The process of seedbed preparation begins with the harvesting of existing forest on the site. Harvesting normally occurs by clear felling which results in disturbance of the soil surface and accumulation of logging debris as 'slash cover'. After harvesting operations in each coupe are completed the slash layer is burnt (normally in the early autumn following harvesting) to remove logging residues and to provide a suitable seedbed. Each of these activities can have effects on eventual seedling emergence and mortality rates.

2.1.4 Sowing of seed

In Tasmania sites that are resown are normally aerially sown with seed collected from a similar region and ideally from the same coupe. Sowing procedures are specified by the Tasmania Forestry Commission (Lockett, 1991). Sowing rates are based on previous experience of field conditions and are normally around 60 000 viable seeds per hectare (ranging between about 40 000 and 90 000). This figure is somewhat lower than that suggested by Cunningham (1960) who based his recommendation of 150 000 viable seed per acre (370 000 per hectare) on an assumption that insecticide treatment would be applied. Neumann (1992) suggested sowing rates after logging of about 200 000 viable seeds per hectare (equivalent to the normal annual seed fall) to ensure successful regeneration in the presence of insect predators. He also recommended insecticide treatment when sowing at lower rates to ensure adequate regeneration.

2.2 Factors affecting mortality

2.2.1 Use of pesticide treatments for insect and fungal control

Recognising a need to protect seed from seed harvesting insects, Gilbert (1958), Cunningham (1960) and Neumann (1992) have recommended treatment of seed or seedbeds with insecticide. It has been reported that over 80% of *E. regnans* seed sown on burnt sites is harvested or destroyed by insects (Cunningham, 1960; Cremer, 1966). Soil treatment with organochlorine insecticides (dieldrin or DDT) was demonstrated (Gilbert, 1958; Cunningham, 1960) to substantially improve seedling establishment. However this approach for insect control was not recommended due to possible adverse effects on non-target organisms and the high cost of broadscale application. Seed pelletisation incorporating DDT and the fungicide thiram in the pellet was adopted for field sowing following demonstration that this could improve seedling survival (Cremer, 1966). It has now been shown (Neumann and Kassaby, 1986; Neumann *et al.*, 1978) that fungicides commonly applied in pelletisation (including thiram) are however phytotoxic at the levels needed to control fungal pathogens. Although DDT has been used extensively for many years its use has been discontinued due to its toxicity and persistence in the environment.

Pelletisation of eucalypt seed is no longer carried out in Tasmania and fungicides or insecticides are not otherwise applied (E. Lockett, pers. comm.).

2.2.2 Pattern of germination and effect of sowing time

With broadcast sowing a limited amount of seed is sown and at one time only. There is always a risk of failure due to unfavourable weather conditions. Hence timing of sowing is important. Cunningham (1960) considered autumn sowing to be more reliable than spring sowing due to the risk of unfavourable climatic conditions following spring sowing. A seed bed of mineral soil such as that produced by burning was very suitable for autumn germination but dried out rapidly making spring sowing risky. In contrast, Cremer (1962) suggested that the ideal time for germination would be "when growth commences after winter" and proposed that in the Florentine Valley July/August would probably be the best sowing time followed by June and September.

Tasmanian Forestry Commission guidelines (Lockett, 1991) recommend sowing at a time of year favourable for germination and as soon as possible after seedbed preparation. In practice sowing is normally carried out in the autumn following logging.

Germination of *E. regnans* normally occurs soon after sowing if conditions are suitable. In the studies by Gilbert (1958) 50% germination occurred within 60 days and between 70% and 97% germination occurred within three months of sowing. Each time sowing was followed by favourable weather. Cremer (1962) also found that the majority of germination occurred within three months of sowing, noting that winter or hot dry conditions resulted in delayed germination.

Reserves of viable ungerminated *E. regnans* seed are unlikely to persist in soil in the field for more than one year according to Cunningham (1960) who conducted a study into the fate of seed on the ground. He concluded that over 80% of seed not collected by insects germinates in the field at least to the stage of radicle emergence, the remainder being probably killed by fungi or by other causes.

2.2.3 Patterns of seedling mortality

Most observed mortality of *E. regnans* seedlings occurs at the cotyledon stage with progressively improved survival with increasing seedling size (Gilbert ,1958; Cunningham, 1960).

Climate has an important effect on mortality rates, with a marked increase in seedling death rate during the winter and spring being noted by Gilbert (1958) and Cremer (1962). Cunningham(1960), also found winter seedling losses were most severe, accounting for over 90% of seedlings germinating in autumn and a further 70% of surviving seedlings which had germinated in the previous spring. During summer 50% of new seedlings present at the end of spring were killed.

2.2.4 Seed dormancy

Seed dormancy in eucalypts is confined to those species that normally grow in the cooler alpine and sub-alpine areas of south eastern Australia (Turnbull and Doran, 1987). Little is known about the mechanisms of dormancy in eucalypt seed (Turnbull and Doran, 1987). Grose (1957) noted that dormancy resulted in slow or delayed germination of some *E. regnans* seed with the rate of germination being increased by stratification. Dormancy in *E. regnans* can be removed by holding imbibed seed at 3°C to 5°C for three weeks or longer (Boland *et al.*, 1980). Such stratification can occur naturally on the ground over the winter period or can be induced artificially. Cunningham (1960) noted that dormancy was induced in much of the *E. regnans* seed shed over the summer months. He proposed that the function of this dormancy was to ensure that seeds do not germinate during cool moist periods in summer. He observed that dormancy was induced in a proportion of imbibed seeds at temperatures above 90°F (32°C). At 110°F (43.3°C) 50 - 60% of seed became dormant and over 10% were killed. At 130°F (54.4°C) all imbibed seeds were killed in six hours, with survival at this temperature progressively increasing at lower moisture contents. Dormancy was not induced by temperatures between 90 and 130°F (32°C and 54.4°C) at moisture contents below 30%. This dormancy could be largely removed by a short period of stratification at 40 to 50°F (4.4 to 10.0°C). Temperatures sufficient to strengthen dormancy (>32°C) may be experienced during seed extraction and handling procedures or after sowing.

2.2.5 Insect predation of seed

In mature unburnt *E. regnans* forest, insects are believed to remove a significant proportion of seed that falls to the forest floor. Ashton (1979) observed that in a forest at Wallaby Creek in Victoria at least 60% of the seed that fell to the forest floor was harvested by ants. He suggested that successful germination after wild fires was due to interference with ant foraging activity, followed by saturation of foraging capacity with a massive oversupply of seed falling from standing trees. Grose (1960) reported that in Victoria the lygaeid bug *Dieutches notatus* destroys up to 60% of the seed of *Eucalyptus delegatensis*. In Tasmania the lygaeid bug *Euander lacertosus* has been found collecting seed of *E. regnans* (Cremer, 1965). Cunningham (1960) concluded that under natural conditions over 80% of seed was harvested by insects.

Forest burning results in an immediate reduction in arthropod species diversity and activity. A wildfire in a Victorian *E. regnans* forest in 1983 resulted in a 93% reduction in arthropod activity and 62% drop in diversity (Neumann, 1991). However ecological changes associated with burning can favour some species. Foraging ant populations were found to be substantially increased for several months in the same forest noted above (Neumann, 1992) in response to abundant seed fall from standing trees. Predator satiation occurred and there was sufficient seed remaining to ensure forest regeneration.

2.2.6 The effect of soil disturbance

The effects of soil disturbance, slash accumulation and fire intensity on regeneration of *E. regnans* from seed is the subject of a recent study by King *et al.* (1993). Seedbed condition was found to have a significant influence on rates of germination. Soil compaction was the most significant of these factors on seedling establishment. Seedling density was reduced 68% and seedling growth reduced 66% on severely compacted soil compared to uncompacted soil. Soil profile disturbance resulted in increased germination rates but reduced seedling growth. Williamson (1990) studied the effects of mechanised forest harvesting operations on soil and found similar findings on the influence of soil compaction on growth and competitive development.

2.2.7 Effect of slash cover and forest litter

In the undisturbed forest, accumulation of litter results in the development of a distinct layer of organic matter above the mineral soil. Logging debris provides an additional measure of organic matter which can act as a barrier to seedling establishment (Gilbert, 1958; Harris, 1989; King *et al.*, 1993). Although the effect of slash accumulation was found by King *et al.* (1993) to be variable, heavy slash cover generally resulted in poor germination and growth. In their study the litter layer was the main barrier to seedling establishment. Removal of this layer by mechanical disturbance or fire was required for adequate seedling establishment. Germination and growth were increased with increased fire intensity and maximum seedling establishment was achieved on areas that had been subjected to a high intensity burn. However fire was not essential for successful regeneration where there had been general topsoil disturbance. Gilbert (1958) found that mineral soil was best for *E. regnans* germination, followed by burnt slash then unburnt slash. Seedling mortality was generally lower on mineral soil than on burnt or unburnt slash.

Some of the inhibition due to the litter layer may be the result of allelopathic effects. *Eucalyptus* leaf-litter and bark-litter may be a source of allelopathic substances, interfering with seed germination and growth of plants (May and Ash, 1990). This effect was found (May and Ash, 1990) to be reduced by decomposition of the litter or by increased rainfall. Ashton and Willis (1982) found that allelopathic factors inhibited the establishment of *E. regnans* seedlings in mature forests in the absence of fire.

2.2.8 Effect of slash burning on seedbed conditions

2.2.8.1 Effects of fire on chemical and physical properties of soil

Fire can have a substantial modifying effect on soil properties which in turn can have a significant effect on subsequent growth and survival of seedlings and activity of soil microbiota. The effects of fire on the chemical and physical properties of soil have been reviewed by Raison (1979), Humphreys and Craig (1981) and Chandler *et al.* (1983). There have also been a number of studies on the solution chemistry of soil following fire in eucalypt forest (Ellis *et al.*, 1982; Ellis and Graley,

1983; Grove *et al.*, 1986; Khanna and Raison, 1986; Tomkins *et al.*, 1991; Weston and Attiwill, 1990). Immediate effects on the soil include heating, the mineralisation of organic matter and deposition of ash. Release of intense heat is associated with the combustion of organic matter with about 5% of this heat energy being transferred to the soil (Packham, 1970). Fire can cause significant increase in temperatures at the soil surface resulting in sterilisation of the surface soil and if intense it can cause structural changes to soil minerals (Humphreys and Craig, 1981). Soil temperatures reached during fires at a range of depths are reviewed by Cromer (1967) and Humphreys and Craig (1981). Depth and intensity of soil heating as well as duration of this heating was in proportion to the amount of fuel burnt. Except under very heavy fuel loads the main effect of heat penetration was restricted to the top 2 to 5 cm of soil. Combustion of organic matter in mineral soil does not occur except where the fire has been very intense.

Burning of slash results in the release of most of the C, H, O, N, and organic P and S to the atmosphere (Raison, 1979). Non-volatile residues, consisting largely of carbonates of alkali and alkaline earth metals are deposited as ash. Increase in surface soil pH after burning is mainly attributable to hydrolysis of basic cations on subsequent wetting of the ash producing an alkaline residue. The magnitude of pH increase (which may vary from less than one pH unit to three or more,) is a function of the amount of ash deposited, buffering capacity of the soil and the amount of precipitation (Raison, 1979; Chandler *et al.*, 1983). Plant nutrients are generally observed to become more available following fire although the extent and duration of this increased availability depends on the behaviour of the individual elements in the soil. Movement of ions through the soil depends on their mobility and the pattern and extent of rainfall following burning. The more mobile ions (particularly K^+) are readily leached to lower depths in the soil while calcium, being less mobile, may persist in the surface soil layer (~0-5 cm) for a period of a year or more following burning (e.g. Ellis and Graley, 1983; Khanna and Raison, 1986).

Although combustion of organic matter results in loss of the majority of the nitrogen to the atmosphere it is generally observed that the level of available nitrogen in soil is increased for a period following burning (eg. Chandler *et al.*, 1983). Confirming the observations of other

researchers (in other temperate and tropical forest types) Weston and Attiwill (1990) found that the principal cause of reduction in the amount of inorganic N in soil after fire in an *E. regnans* forest was microbial immobilisation rather than leaching.

Levels of available phosphorus in the soil surface are increased as a result of deposition in ash and the effect of heat on soil minerals (Chandler *et al.*, 1983; Humphreys and Craig, 1981).

2.2.8.2 The "ash-bed effect"

Exposure of forest soils to high intensity fires can promote marked stimulation of plant growth (Pryor, 1963). Numerous reasons have been suggested for this response, known in Australia as the ash-bed effect. Pryor (1963) found that this could be duplicated by dry heating to 150°C or by the addition of N and P fertilisers. He proposed that many factors were involved in the response including a likely microbiological component. The stimulatory effect of heated soil was gradually lost with time in warm moist glasshouse conditions but could be regained by reheating. The rate of loss of growth stimulus could be increased by inoculation with a small amount of unheated soil. Microbial immobilisation of available nutrients was seen as the likely cause of this change. Cromer (1967) similarly concluded that the effect was due to heating and likely to be the combined result of soil sterilisation and enhanced nutrient availability.

Ashton and Willis (1982) theorised that microbial activity associated with the litter layer was a major inhibitor of regenerating *E. regnans* and also suggested that the ashbed effect was at least partly due to sterilisation of the soil and colonisation of the rhizosphere with more favourable microbiota.

2.2.8.3 Effect of fire on soil microbiology

Renbuss *et al.* (1973) studied the microbiology of an ash-bed formed by burning a log pile on the soil surface. Temperatures of greater than 100°C were maintained for more than 6 hours down to a depth of 20 cm. Assays immediately following the fire showed that the soil had been apparently sterilised down to 25 cm. The soil was rapidly recolonised by bacteria following the fire, reaching levels greater than for unheated soil. Recolonisation by actinomycetes and fungi proceeded

more slowly. The early bacterial and fungal recolonisers were found to include many types not detected in the untreated soil. This distinctive ash-bed microbiota was observed to persist for about a year before reverting to that more normally found. As this time period correlated with the time during which the soil remained stimulatory to the growth of eucalypt seedlings a causal relationship was postulated.

Theodorou and Bowen (1982) studied the changes in soil microbiology following a fire of moderate intensity in dry sclerophyll forest. They observed a reduction in bacterial and fungal numbers to a depth of 2 cm following burning compared to an unburnt control. Fluorescent pseudomonads were the most severely affected, with reduction in their numbers occurring to a greater depth in the soil than other bacteria and fungi. They were however the first to recover in numbers. Two months after burning (and after rain) counts of all microbial groups under investigation with the exception of actinomycetes had risen to above the corresponding levels for the unburnt control. After 20 months there was no significant difference in microbial numbers between the burnt and unburnt areas.

2.2.8.3.1 Pyrophilous fungi

The incidence of pyrophilous fungi on forest soil following fire has been discussed by Warcup (1981, 1990). Fructifications of these (which may include many colourful macrofungi) are much more commonly observed on burnt soil and some of these fungi have been shown to form mycorrhizae with eucalypt seedlings. Germination of some fungal spores in soil including of ascospores of *Anthracobia melaloma*, a known pyrophilous species have been shown by Warcup and Baker (1963) to be stimulated by heating. Soil pH appears to be an important factor determining the occurrence of pyrophilous fungi (Warcup, 1981) with many species being stimulated by, or tolerant of, alkaline conditions.

2.2.9 Effects of vegetation clearing on soil temperatures

Soil surface temperatures can be substantially modified by clearing of vegetation and litter (which otherwise provides shading plus insulation from solar radiation and re-radiation from the soil surface). Blackening of the soil surface as a result of fire also promotes solar

heating (Raison, 1979). Raison *et al.* (1986) observed that mean summer day time soil surface temperatures were substantially higher (elevated by up to 8°C) and daily minima were unchanged or slightly lower in recently burnt *E. pauciflora* forest compared to unburnt forest. This difference was largely ascribed to removal of the understorey vegetation and also due to removal of leaf litter.

Nunez and Bowman (1986) found that the level of nocturnal cooling at ground level was directly related to stand density of *E. delegatensis* forest. Minimum soil surface temperatures were substantially lower in a clearfelled area than in an equivalent unlogged region and in contrast to the clearfelled area the surface temperatures in the unlogged region remained above freezing in winter. Model estimates showed a 5.7°C difference (between these regions) in minimum temperature during cloudless conditions and a 3.0°C difference during cloudy conditions.

2.2.10 Soil moisture

The limiting effect of moisture deficits on survival of *E. regnans* seedlings in the field has been noted by early researchers (Cunningham, 1960; Cremer, 1962). Cremer (1962) considered that a significant proportion of seedling deaths were due to desiccation and noted that only very young seedlings were affected. Cunningham (1960) attributed most of the summer losses to desiccation and observed that death due to desiccation was mainly confined to very young seedlings. This occurred as a result of drying of the surface 1 to 3 cm of soil and affected seedlings with roots which did not penetrate below this depth. Drying was most severe on mineral soil and in exposed situations. Dry soil was also more likely to reach high surface temperatures. He proposed that summer losses could be reduced by provision of some shade.

Effects of soil moisture on germination and survival of eucalypt seedlings has been reviewed by Stoneman (1994), who considered moisture deficits as an important cause of seedling mortality. Battaglia and Reid (1993) have found that small-scale variation in the soil surface topography (over tens of centimetres) had a sufficient effect on microclimate to significantly affect germination and survival of

seedlings of *E. delegatensis*. The influence of soil moisture was seen as the main cause of this microsite variation.

2.2.11 Frost

Frost can cause damage to seedlings either directly or via frost heave. The extent of frost damage also depends on the stage of seedling development and the extent of "hardening off" that has taken place. Cremer and Mucha (1985) found that dry seed was more resistant to frost damage than imbibed seed and imbibed seed more resistant than germinating seed. There is little available information on frost tolerance of very young (cotyledon stage) *E. regnans* seedlings, with most of the available data being derived from study of more advanced seedlings. Ashton (1956, 1958) studied the effect of frost on *E. regnans* seedlings and observed that frost resistance could be induced by hardening the seedlings by prior exposure to cool temperatures for several weeks. With artificial frost conditions un-hardened seedlings were killed at temperatures below 25-27°F (-3.9 to -2.8°C) while well hardened seedlings were killed at temperatures below 20-22°F (-6.7 to -5.6°C). The lethal frost temperature in the field was observed to be even lower. Ashton (1958) noted that cotyledon and two leafed seedlings were very resistant to frost damage. Layton and Parsons (1972) found that 10 day old seedlings were very resistant to frost, with this resistance being uniform regardless of provenance.

Frost tolerance of well established *E. regnans* seedlings was found (Griffin *et al.*, 1982; Rook *et al.*, 1980) to vary between provenances with most tolerant provenances being from frost prone highland areas of Victoria and Tasmania. Hardening had a significant effect on frost tolerance. Rook *et al.*, (1980) found that the most frost tolerant provenance could withstand -5.5°C in autumn (May), -7.0°C in winter (mid-July) and -4.5°C in spring (early October). Menzies *et al.*, (1981) assessed the seasonal frost tolerance of seedlings of three eucalypt species (*E. saligna, E. regnans* and *E. fastigata*) acclimatised at Rotorua in New Zealand. Based on foliage damage, frost tolerance of *E. regnans* ranged from -3.5°C in summer to -9°C in winter.

Neither Gilbert (1958) nor Cunningham (1960) considered frost to be a significant cause of *E. regnans* seedling mortality in their studies although Cunningham (1960) speculated that infection by fungi

could follow frost damage. The minimum temperature recorded by Cunningham (1960) at 5cm above the ground was 23.5°F (-4.7°C) and there were few frosts below 28°F (-2.2°C). Although frost damage was not a major cause of death, heavy losses occurred in small localised areas. Frost heave (as opposed to frost damage) can cause heavy losses of seedlings in some areas. Frost heave exposes seedling roots making seedlings more susceptible to desiccation or other damage. Although shelter provided protection from frost the associated reduction in light intensity increased susceptibility to fungal attack.

Frost may have some role in increasing the susceptibility of seedlings to pathogen attack. Cunningham (1960) considered it possible that winter infection by fungi followed frost damage although frost damage to cotyledon stage or two leaf stage seedlings was not observed.

2.2.12 Browsing (by marsupials, rabbits and/or insects)

Browsing of *E. regnans* was discussed in detail by Gilbert (1958) and by Cremer (1962). Browsing of very young seedlings was not observed by Gilbert (1958) to occur, presumably because of their small size and sparse distribution. He noted that browsing did not occur until seedlings had reached a "browsable" size at about six months following germination. Defoliation by browsing animals did nevertheless have a serious effect on growth of established seedlings. Browsing of established seedlings is often a serious problem in forest regeneration. Insect grazing of seedlings was recognised by Gilbert (1958) but regarded as rarely a cause of seedling deaths. Browsing was not considered to be a major cause of seedling mortality overall in the area under investigation by Cunningham (1960).

2.2.13 Involvement of fungi in *E. regnans* seedling mortality

Early studies on regeneration of *E. regnans* (Cremer, 1962; Cunningham, 1960; and Gilbert, 1958) have included some reference to fungal pathogens but do not appear to adequately quantify their significance, or identify the fungal species involved. Fungi were considered by Cunningham (1960) and Gilbert (1958) to be a major contributor to seedling deaths in areas that they studied, with most seedling mortality being observed at the cotyledon stage and within

weeks of emergence. The majority of winter seedling losses were attributed by Cunningham (1960) to infection by soil borne fungi.

Among associated factors investigated by these researchers were the effects of frost and of light intensity. Cunningham (1960) found that fungal attack was increased in areas where light intensity was low and air movement restricted such as amongst slash cover. Ashton (1956) also found that infection of *E. regnans* seedlings by fungi was affected by light intensity and increased at low light intensity. At 1.5% of full sunlight very heavy losses occurred and only 2% of seedlings survived 6 months.

2.3 Involvement of fungal pathogens in eucalypt seedling pathology

2.3.1 Fungal pathogens

The fungi causing damping-off type symptoms are the main pathogens of very young seedlings. Since seedlings are most prone to damping-off prior to the development of secondary tissues (with lignification), damping-off is normally restricted to seedlings at or prior to the cotyledon stage. At a later stage small seedlings may also be killed by pathogens causing blights or wilts. Pre-emergence damping-off of ungerminated seed or seedlings prior to emergence may in practice be difficult to quantify but could commonly be of greater significance than the more readily visible post-emergence damping-off. Fungi most commonly associated with damping-off of crops world-wide include species of *Pythium, Rhizoctonia* and *Fusarium.* Most reports of damping-off refer to seedlings grown in nurseries where such disease can cause significant seedling losses and measures can be taken to control the fungi responsible. In forest situations, seedling death due to damping-off normally goes unnoticed.

2.3.2 Involvement of fungal pathogens in early mortality of eucalypt seedlings

There has been little published research on the role of fungal pathogens in early seedling mortality in eucalypt forests. The only investigation specifically concentrating on this aspect was that by Mwanza and Kellas (1987) who identified five species of *Pythium*, two species of *Fusarium* and *Cylindrocarpon destructans* (all isolated from

a Victorian forest soil) as being capable of causing post-emergent damping-off of *E. delegatensis* and *E. radiata* seedlings. These fungi also caused pre-emergence damping-off in the laboratory. It was hypothesised that much of the eucalypt germination failure in the field may be due to pre-emergence damping-off. Damping-off was widespread in the field although the proportion of germinated seedlings killed by damping-off was less than that for other causes (Mwanza, 1986).

2.3.3 Reports of fungi causing damping-off of eucalypts outside Australia

There are few published records identifying the microorganisms associated with damping-off diseases of eucalypt seedlings in Australia (detailed later in this review) and much of what has been recorded refers to pathogen incidence in seedling nurseries. Accounts from overseas of fungi causing seedling diseases in eucalypt nurseries are a little more numerous (Table 2.3.3.1 lists reports of fungi causing damping-off of eucalypt seedlings). Records of fungi observed to be pathogenic to eucalypt seedlings outside of Australia do not necessarily mean that these pathogens are present or even potentially pathogenic in Australia. They can nevertheless give an indication of the types of pathogens that may be expected.

Table 2.3.3.1 Reports of fungi causing damping-off of eucalypt seedlings outside Australia.

Pathogen	*Eucalyptus* species	Country	Reference
Cylindrocladium scoparium	*E.* spp.	Argentina	Jauch, (1943)
	E. spp.	Brazil	Batista, (1951)
	E. spp.	Brazil	Blum *et al.*, (1992)
C. clavatum	*E.* spp.	Brazil	Blum *et al.*, (1992)
C. quinqueseptatum	*E. grandis*	India	Sharma & Mohanan, (1992)
Fusarium solani	*E.* spp.	Egypt	Michail *et al.*, (1986)
F. oxysporum	*E.* spp.	Iraq	Taha *et al.*, (1987)
	E. spp	USA	Hepting, (1971)
Macrophomina phaseolina	*E.* spp.	Iraq	Taha *et al.*, (1987)
Pythium myriotilum	*E.* spp.	India	Sharma *et al.*, (1984)
P. deliense	*E.* spp.	India	Sharma *et al.*, (1984)
Pythium spp.	*E. grandis*	India	Sharma & Mohanan, (1992)
Rhizoctonia solani	*E.* spp.	Iraq	Taha *et al.*, (1987)
	E.tereticornis	India	Sharma *et al.*, (1984)
	E. grandis	India	Sharma *et al.*, (1984)

2.3.4 Damping-off pathogens affecting eucalypt species in Australia

2.3.4.1 Pythiaceae (*Pythium* and *Phytophthora*)

Commonly associated with root diseases the soil borne fungi, *Phytophthora* and *Pythium* species have a cosmopolitan distribution. These genera are morphologically similar, with the difference between them being possibly of taxonomic significance only (Podger, 1979). *Pythium* species mainly infect juvenile tissue and as such pathogenicity is normally restricted to young seedlings although root tips and feeder roots of older plants are also infected causing reduced vigour but seldom causing death (Hendrix and Campbell, 1973). They are the most common cause of pre- and post-emergence damping-off diseases of plants. Members of the genus *Phytophthora* are highly specialised plant inhabiting fungi with species often associated with root rots but more commonly attacking above-ground plant parts. Because they have a

mobile zoospore stage, infection and spread of these fungi are influenced by soil water activity.

In their synopsis of pests and diseases of nurseries and commercial eucalypt forests in Australia, Neumann and Marks (1976) considered *Pythium* species to be important pathogens causing damping-off of eucalypt species in forest nurseries. They listed *P. irregulare* as one of the species responsible. Podger (1979) reported "about 25" species of *Pythium* and *Phytophthora* that have been recorded from forest nurseries, plantations and forests in Australia. Species of *Pythium* or *Phytophthora* are not normally easy to isolate directly from soil and determination of their presence in any particular soil may require the application of a baiting technique such as with lupin seedlings (Chee and Newhook, 1965) or *E. sieberi* cotyledons (Marks and Kassaby, 1974). Reports of fungi isolated using baiting techniques should perhaps be treated with caution as the mere presence of a fungal species does not necessarily indicate pathogenicity. A range of *Pythium* and *Phytophthora* species were isolated by Pratt and Heather (1973) from a large number of samples of forest soils collected throughout Australia. These fungi were isolated by lupin baiting and thus described as being potentially pathogenic. From *E. regnans* and *E. obliqua* forest in south-eastern Tasmania they obtained *Pythium (?)acanthophoron, P. (?)deliense, P. ultimum* var. *sporangiferum, P. splendens, Phytophthora drechsleri* (syn. *Ph. cryptogea*) and *Ph. cinnamomi.* They suggested that disease attributed to *Ph. cinnamomi* might be caused by other *Phytophthora* and *Pythium* species acting together or alone with or without *Ph. cinnamomi.*

Marks and Kassaby (1974) evaluated the pathogenicity of *Pythium debaryanum, P. irregulare, P. mamillatum, P. ultimum P. vexans, Ph. drechsleri, Ph. cinnamomi* and three unidentified *Pythium* species from eucalypt forest soil on juvenile (two leaf stage) and intermediate seedlings of *E. sieberi* and juvenile seedlings of other eucalypt species. Of the *Pythium* species, only *P. debaryanum, P. irregulare* and *P. ultimum* could kill seedlings at the two-leaf stage and neither of the *Pythium* isolates nor *Ph. drechsleri* could kill seedlings at the intermediate stage of growth. *Ph. cinnamomi* showed very distinct pathogenicity, killing seedlings quickly.

Mwanza and Kellas (1987) identified five species of *Pythium* (*P. mamillatum, P. ultimum, P. paroecandrum, P. irregulare* and *P. perplexum*) as being capable of causing post-emergent damping-off of *E. delegatensis* and *E. radiata* seedlings. These fungi also caused pre-emergence damping-off in the laboratory.

Ph. cinnamomi is a pathogen reported to cause serious plant disease throughout the world (Zentmyer, 1980) and its implication as the cause of jarrah dieback in Western Australia (Podger, 1972) promoted considerable concern that this pathogen could be a threat to forests in other parts of Australia. Consequently this fungus has been the subject of extensive research in this country (much of which has been reported in Old, 1979). The biology of *Phytophthora* in general is also reviewed in Erwin *et al.*, 1983.

Although widespread in Tasmania, *Ph. cinnamomi* is not known in the coldest and driest regions being mainly restricted to coastal areas (Podger *et al.*, 1990b). It is not regarded as a serious threat to Tasmanian forests that are used for wood production. In Victoria *Ph. cinnamomi* is also mainly limited to coastal areas, rarely found in mountain forests and then only at roadsides (Marks and Kassaby, 1974). Bioclimatic analysis of the distribution of *Ph. cinnamomi* in Tasmania (Podger *et al.*, 1990a) has suggested that damage due to this fungus is unlikely where mean annual temperature does not exceed 7.5°C or where annual mean rainfall is below 600mm. Pathogenicity of *Ph. cinnamomi* is affected by soil type and damage due to this pathogen has not been recorded in Tasmania in forest growing on krasnozem soils on basalt or heavy textured soils on ultramafics (Podger *et al.*, 1990a).

2.3.4.2 *Rhizoctonia*

Although one of the most commonly reported pathogens causing damping-off of crop plants world-wide, there are few records of eucalypt disease caused by *Rhizoctonia* species. There appear to be no reports of *Rhizoctonia* species pathogenicity to eucalypts in forest areas within Australia, although a*Rhizoctonia* species has been reported among fungi associated with damping-off of *E. botryoides* in a South Australian nursery (Vaartaja, 1967).

2.3.4.3 *Cylindrocarpon*

Although commonly found in the rhizosphere, *Cylindrocarpon* species are rarely regarded as pathogenic (Domsch *et al.*, 1980). Pathogenicity when it occurs appears to be associated with toxin production.

Florence and Cocker (1962) found that growth of blackbutt (*E. pilularis*) seedlings was severely inhibited in mature blackbutt forest soil. Heating of soil for two days at 35°C or 70°C was found to result in vigorous seedling growth. As sterilisation of the soil by irradiation resulted in healthy growth of seedlings, they concluded that the stunting observed in forest soil was caused by microbial antagonism. Evans *et al.* (1967) isolated *Cylindrocarpon destructans* (syn. *radicola*) (= *Nectria radicola*) from roots of *E. pilularis* and demonstrated that a toxin produced by the fungus caused severe stunting and blackening of roots of seedlings at concentrations as low as 2ppm. These effects were characteristic of the symptoms induced when seedlings in aseptic culture were inoculated with *C. destructans* or when seedlings were grown in infested soil. The toxin, for which the name nectrolide had been proposed (Evans, 1964), was found to be identical to brefeldin A also produced by *Penicillium brefeldianum.* It was suggested that this compound may be involved in a number of plant diseases where the cause was otherwise unclear.

Ashton and Willis (1982) found *C. destructans* to be common in the rhizosphere of *E. regnans* in the Wallaby Creek area of Victoria. Culture filtrates caused deformation and blackening of roots of *E. regnans* seedlings similar to that reported by Evans *et al.* (1964) for *E. pilularis.* Ashton and Willis (1982) theorised that microbial activity associated with the litter layer was a major antagonistic factor in the regeneration of *E. regnans* and also suggested that the ashbed effect was at least partly due to sterilisation of the soil and colonisation of the rhizosphere with more favourable microbiota.

Jehne (1982) noted the effect of soil sterilisation on a previously undescribed *Cylindrocarpon* species associated with root decay and crown dieback of *E. obliqua* in southern Tasmania. Levels of *Cylindrocarpon* inoculum in the surface layers of soil were low at one year after burning and subsequently increased with age of the

regenerating forest. Mwanza and Kellas (1987) found *C. destructans* among pathogens causing damping-off of *E. obliqua* and *E. radiata* in a Victorian forest.

2.3.4.4 *Fusarium*

Like those of the related genus *Cylindrocarpon,* species of *Fusarium* are common rhizosphere inhabitants and although normally saprophytic some species (most notably *F. oxysporum*) can be serious pathogens of a wide range of plant species. There are few reports of pathogenicity to eucalypts within Australia. Mwanza and Kellas (1987) identified two species of *Fusarium (F. avenaceum and F. longipes)* isolated from a Victorian forest soil as being capable of causing post-emergent damping-off of *E. delegatensis* and *E. radiata* seedlings. These fungi also caused pre-emergence damping-off in the laboratory. *Fusarium oxysporum* was found to be among species associated with damping off of *E. blakelyi* in a South Australian nursery (Vaartaja, 1967).

2.3.4.5 *Botrytis cinerea*

This fungus has been reported to cause wilt (Neumann and Marks, 1976) and damping-off (Vaartaja, 1967) of eucalypt seedlings in Australian nurseries and has been associated with blight of eucalypt seedlings in nurseries and plantations at various places around the world (eg. Abrahão, 1948; Pennycook, 1989). Although *B. cinerea* is known to cause damping-off of other crops, reports of disease on eucalypts do not normally refer to damping off, nevertheless damping-off is considered by the present author to be within the range of possible disease symptoms.

2.3.4.6 *Alternaria*

Ashton (1956) reported that in heavily shaded areas, weak seedlings of *E. regnans* were commonly infected with species of *Alternaria* (syn. *Macrosporium*) This was also the most common fungal genus infecting seedlings in the greenhouse. *Alternaria* (and in particular *A. alternata*) is very commonly found on a wide variety of plants and other substrates (Domsch *et al.*, 1980). It is a common (although usually weak) plant pathogen and can be seed-borne.

2.3.4.7 *Ceuthospora innumera* and *Piggotia substellata*

These fungi were found by Ashton and Macauley (1972) to cause a serious leaf spot disease on *E. regnans* in central Victoria and were responsible for killing young seedlings with serious impairment of seedling survival in the first year of growth. These fungi were also colonisers of leaf litter and much of the seedling infection appeared to occur by rain splash of spores in soil onto leaves and stems. Incidence of this disease was greater in the cooler and wetter months of the year.

2.4 Pathogenic bacteria

There appear to be no records of eucalypt seedling mortality caused by bacteria. Nevertheless it is possible that bacteria could play a role in seedling mortality through facilitation of frost injury by ice nucleation (Lindow *et al.* 1978) or by direct pathogenicity, but this aspect has received little attention.

2.5 Mycorrhizal associations of *E. regnans*

Eucalyptus regnans is normally abundantly mycorrhizal (Ashton, 1976) with a wide range of forms being present. In mature forest, mycorrhizal fungi are mainly basidiomycetes. Mycorrhizal development of *E. regnans* was improved by high light intensity and inhibited by high levels of nitrogenous fertilisers, but did not appear to be affected by high levels of phosphate.

Malajczuk *et al.* (1982) reviewed the fungi known to form ectomycorrhizas with eucalypts. Of these, 16 were ectomycorrhizal on *E. regnans,* including 15 basidiomycetes previously reported by Ashton (1976) and one ascomycete (*Cenococcum geophilum*) previously reported by Chilvers (1968) and Chilvers and Pryor (1965). Two unidentified ascomycetes were also included in a list of fungi reported to be mycorrhizal with eucalypts in New Zealand by Chu-Chou and Grace (1982).

Warcup (1991) found that species of ascomycetes were the main early mycorrhizal colonisers of eucalypt seedlings following fire in regeneration coupes in Tasmania. Although 58 ascomycete isolates were shown to form mycorrhizae with *E. obliqua,* the identity of most of these remained undetermined. Ascocarps were formed by two species.

Seedlings were generally highly mycorrhizal and where *Acacia* and *Pomaderris* seedlings were also present these had ectomycorrhizal fungi in common with the eucalypts. These results in combination with similar results from South Australia (Warcup, 1990), show that ascomycetes are important mycorrhizal colonisers following fire of seedlings of eucalypts and other plants.

Warcup (1981) reported that *E. regnans* seedlings growing in forest soils that had been steam treated at 60-71°C for 30 minutes were commonly ectomycorrhizal with ascomycetes, while in untreated soil basidiomycete mycorrhizae were more common. Greatly increased growth was reported for seedlings in the steamed soil compared to those on untreated soil.

3. MATERIALS AND METHODS

3.1 General

3.1.1 Field site description

The field sites were located near Mt Thunderbolt, approximately 80km north-west of Hobart, Tasmania (Tasmaps sheet 4629 -"Ouse") at altitude 550-650m, latitude 42°29' south, longitude 146°35' (Figure 3.1.1.1). Soil in the area was a krasnozem overlying dolerite. Prior to logging the vegetation of the area consisted of tall open forest dominated by *E. regnans* with a wet sclerophyll understorey consisting predominantly of *Bedfordia salicina* D.C. and *Pomaderris apetala* Labill.

In 1992 studies were conducted on a coupe that had been subjected to a hot burn in April 1992 (Jungle-19) and an adjacent coupe (Jungle-18) that had been logged but left unburnt (Figure 3.1.1.1). One trial site (Site C, Figure 3.1.1.2) was set up on the unburnt coupe and two on the burnt coupe (Sites A & B, Figures 3.1.1.3 to 3.1.1.5). Fencing (to exclude animals) was provided around 1992 trial sites by ANM Forest Management personnel, who also collected temperature and rainfall data for the area. In 1993 studies were conducted on an adjacent coupe (Jungle-24) which had been subjected to a hot burn in April 1993. Two trial sites (D & E, Figures 3.1.1.6 and 3.1.1.7) were set up on Jungle-24. All coupes had been logged in the year prior to study.

3.1.2 Glasshouse conditions for pot experiments

Glasshouse temperatures were generally maintained between 10 and 25°C and soil moisture in pots kept at field capacity by daily watering. No fertiliser was added unless otherwise noted.

Figure 3.1.1.1 Map of Jungle area showing locations of experimental sites and transects.

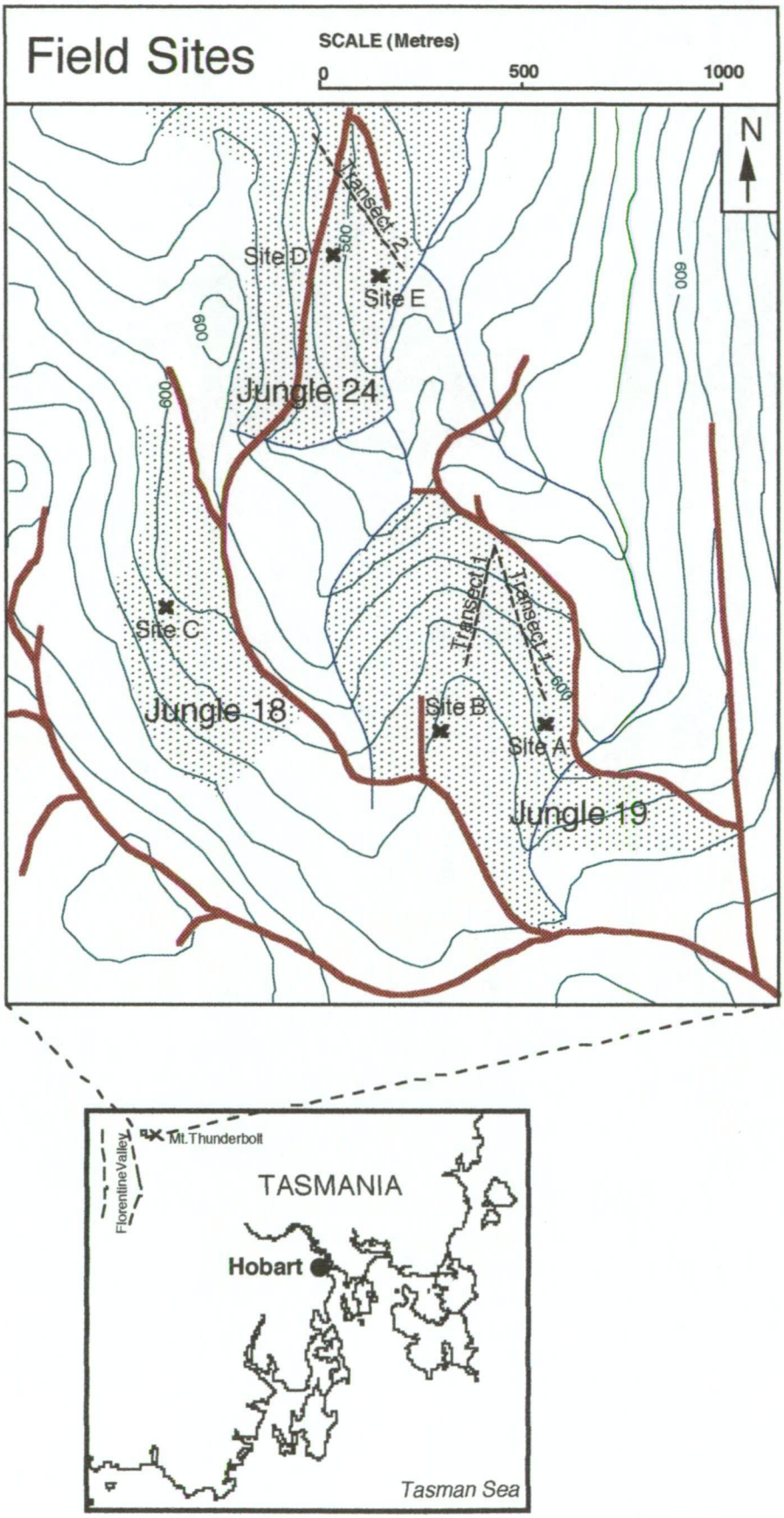


Figure 3.1.1.2 Experimental site C on unburnt coupe Jungle-18 (within fenced area).

Figure 3.1.1.3 Experimental site A on burnt coupe Jungle-19 (within fenced area).

Figures 3.1.1.4 and 3.1.1.5 Experimental site B on burnt coupe Jungle-19 (within fenced area).

Figure 3.1.1.6 Experimental site D on burnt coupe Jungle-24 (in foreground).

Figure 3.1.1.7 Experimental site E on burnt coupe Jungle-24 (in foreground).

3.1.3 Plant potting mix

Potting mix was used in some glasshouse experiments and consisted of a 1:1 mixture of coarse sand and peat amended with 1.5 kgm^{-3} of dolomite, 1.2 kgm^{-3} of limil (calcium hydroxide) and 1.0 kgm^{-3} of 'Osmocote' fertiliser (NPK= 15.5:5.2:12.5). Potting mix was steam sterilised (80°C for 45 minutes) at the time of preparation.

3.1.4 Tests of seed viability

Seed viability is known to vary between seed lots and with the age of the seed sample. Germination tests were carried out on seed in association with experiments where a measure of seed viability was required.

Five lots of 0.20 g of seed were surface sterilised by wetting for 30 seconds with ethanol and soaking for a further two minutes in a solution of 2% sodium hypochlorite. Seed was then rinsed three times with sterile water before placing onto 1.5% tap water agar in petri plates. Plates were placed in plastic bags and incubated at room temperature under indirect natural daylight. Counts were made of the number of seedlings at intervals until no further germination was apparent. The final germination total thus obtained was treated as the number of viable seeds per gram of seed.

3.1.5 Culture and storage of fungi and bacteria

Routine culturing of fungi was on BBL potato dextrose agar (PDA). For long term storage of fungi the method of Muir (1988) was used. Four small pieces (3 mm^3) of actively growing PDA culture were placed into 4 ml of sterile 0.85% saline in Bijoux bottles. Bottles were sealed and stored at room temperature.

Medium term storage of fungi was also achieved by placing 5 mm^2 blocks of PDA culture onto tap water agar (TWA, Appendix 1.1.4) in petri plates. Plates were sealed and stored at 10°C.

The medium used for routine culturing of bacteria was trypicase soy agar (TSA, Appendix 1.1.1). Slants of TSA were used for medium term storage of bacteria for up to four months at 4°C.

3.1.6 Preparation of millet cultures of fungal isolates

Conical flasks (500 ml) containing 100 g white millet plus 80 ml water were sealed with cotton stoppers and aluminium foil then, after soaking overnight, autoclaved for 20 minutes at 121°C. On cooling these were aseptically inoculated then kept at 22°C and shaken at least twice weekly.

3.1.7 Soil pH

The pH of air-dried soil samples was measured by glass electrode using a 1:5 (wt/vol) soil-water (distilled) suspension. On addition of water the soil suspensions were shaken vigorously for 15 seconds and then stirred for at least 30 seconds immediately prior to and during pH measurement. The electrode was fully immersed in the suspension but not touching any sediment.

3.2 Studies on the effect of burning on soil conditions and on the germination and survival of *E. regnans*

3.2.1 Location and sampling of field sites

In April 1992 the coupe Jungle-19 had been subjected to a hot fire and an adjacent coupe (Jungle-18) had been logged but left unburnt. Two representative experimental sites were set up on the burnt coupe (300 m apart) and another 800 m away on the unburnt coupe (Figure 3.1.1.1). Each site comprised five replicate 10 m^2 plots.

Soil was sampled at eight days after burning (27/4/92) and again at 133 days (31/8/92). Sampling was at 0-2, 2-5 and 5-10 cm, with each sample comprising pooled duplicate sub-samples randomly collected from each sample plot. Any surface ash was included in the 0-2 cm sample. Adhering soil particles were brushed from the sampling trowel between samples.

Samples were subjected to a number of studies including pH measurement of all samples and estimates of fungal and bacterial numbers for those taken at eight days. Recombined samples from each plot were also used for glasshouse seedling trials (Methods 3.2.3).

3.2.2 Estimation of fungal and bacterial numbers in soil at 8 days after burning

A measure of the bacterial and fungal populations in the soil samples were obtained by dilution plating:- One gram of each sample was suspended in 100 ml of 0.85% saline and agitated in a "Stomacher" for two minutes. Serial dilutions (0.1 ml)were spread onto Rose Bengal Agar (RBA, Appendix 1.1.6) and TSA (amended with 80 000 and100 000 units per litre respectively of Penicillin G and Nystatin after autoclaving and cooling to 60°C) with five replicates per sample. Plates were incubated at 25°C in the dark for one week after which counts of bacterial colonies (on TSA) and of fungal colonies (on RBA) were made.

Moisture determinations were carried out on separate soil sub-samples and microbial numbers calculated on a soil dry weight basis.

3.2.3 Seedling establishment in the glasshouse on field soil collected at 8 and 133 days

Soil collected from field sites at eight days after burning was recombined in 12.5 cm diameter pots in the depth order in which it had been collected. Equal weights of *E. regnans* seed, (0.50 g) were sown for each of the five replicate plots from each sample site. Seedling emergence and mortality was determined at two day intervals until no further change was apparent and then monitored at weekly intervals. Seedlings that had died within 40 days of sowing were collected for isolation of associated fungi (Methods 3.5).

Soil samples collected at 133 days were sieved (7 mm mesh) to remove coarse material before placing soil into 12.5 cm pots as described above except that duplicate pots per sample were used. Four pots of potting mix were included as a control. Seed of *E. regnans* was sown at 0.25 g per pot. Counts were made at intervals of the number of seedlings per pot. At three months, seedling heights were measured. Selected pots were later used in an experiment to determine the effect of added N and P (Methods 3.2.7).

3.2.4 Germination and mortality of *E. regnans* in burnt and unburnt areas in the field

Patterns of germination and seedling mortality on burnt and unburnt sites were investigated in an experimental trial during 1992. Five representative 1 m^2 plots spaced at least 10 m apart were set up at each of sites A, B (burnt) and the unburnt site C (Figure 3.1.1.1). Each plot was sown with 0.25 g of *E. regnans* seed on 27th April 1992. Insufficient germination was expected with this amount of seed for reliable comparisons to be made between treatments. Therefore plots were sown with an additional gram of seed on 13th May 1992, giving a total of 1.25 g per plot (244 viable seeds per plot based on germination test).

Germination and seedling mortality counts were made at intervals of three to four weeks until 14th January '93. Germination was assessed as the emergence of identifiable seedlings. New germinants were marked by placing a peg 2 cm from the seedling (Figure 3.2.4.1) and mortality was determined by the absence of live seedlings near previously placed pegs (which were then removed). Observations were also made of field conditions which may have influenced germination and mortality.

Figure 3.2.4.1 Example of seedling plot (site A1, 1m^2 rectangle between white tags and larger wire peg) showing method for marking seedlings with (blue) pegs.

3.2.5 Additional soil pH investigations

3.2.5.1 Seedling establishment on ash

Bulk samples of ashbed soil (to a depth of 10 cm) and grey ash were collected from Jungle-19 at 66 days after burning. These were sieved (7 mm mesh) and mixed to uniform consistency. A pot trial was set up to compare seedling establishment on ash, soil and a 1:1 mixture (wt/wt) of ash and soil at five replicate pots per treatment.

3.2.5.2 Variation in soil pH across a coupe transect, before and soon after burning

A measure of the variability of soil surface pH over a coupe two weeks before and also two weeks after burning was obtained by sampling a transect of Jungle-24 (Figure 3.1.1.1) in April 1993. The transect covered areas of disturbed and undisturbed soil with moderate to heavy slash cover over about 30% and light slash cover over the remainder. The majority of the area had been burnt at the second sampling. Forty samples of approximately 100 g of the top 2 cm of soil were collected at 5 m intervals along the transect.

3.2.5.3 Variation in soil pH across a coupe transect at 133 days after burning

In addition to sampling trial sites on Jungle-19 at 133 days, surface soil samples were taken at this time across a transect (Figure 3.1.1.1) of a larger part of this coupe for pH measurement. This transect covered terrain which had undergone variable soil disturbance and intensity of burning. Forty-three samples of approximately 50 g of the top 2 cm of soil were collected at intervals of twenty paces (approx. 10 m) along the transect.

3.2.6 Studies on the effect of pH on germination and survival of *E. regnans* seedlings *in vitro*

3.2.6.1 Effect of pH in the range 8.0 to 10.4 on germination and survival of *E. regnans* seedlings *in vitro*

The effect of pH on seed germination and seedling survival was investigated using 0.1 M Tris(hydroxymethyl)aminomethane HCl buffers (Tris HCl, Appendix 1.2.1) for pH values of 7.8 to 9.0 or 0.1 M sodium carbonate-bicarbonate (Appendix 1.2.2) buffers for higher pH values. Plates of these buffers solidified with 1.5% agar were sown with 100 mg seed per plate (with five replicate plates per determination) and kept at 15°C under continuous light. Germination was recorded at intervals with total germination and number of live seedlings being determined after three weeks (no further germination was evident after this time).

3.2.6.2 Effect of temperature-induced dormancy on susceptibility of seeds to elevated pH *in vitro*

The effect of temperature and pH on germination was studied as described above except that number of replicates was increased to 10, with incubation at 4°C and 20°C at either pH 8.0 or pH 10.2. Numbers of germinated seeds were recorded after 21 days. Ungerminated seeds were then rinsed three times with sterile water and transferred to pH 7.2-buffered (Tris HCl) medium with incubation for a further 28 days at 20°C. The numbers of additional seedlings were then recorded.

3.2.7 Effect of phosphorus and nitrogen nutrition on seedling growth rates in field soil in the glasshouse

Three-month-old seedlings growing on field soil collected at 133 days after burning (Methods 3.2.3) were used to determine whether any improvement in growth could be obtained with the addition of nitrogen and phosphorus fertilisers.

Nine pots of seedlings (out of 10) from the unburnt area were sorted into three replicate groups of three, such that the variability in seedling size and number was the same in each group. Treatments applied were: control ; P added; N+P added. There were more pots of

seedlings on soil from the burnt area to choose from (20) allowing four treatments and four replicates (with pots otherwise sorted as above). Treatments applied to pots from the burnt area were: control; P added; N+P added; N added.

Nitrogen and phosphorus were applied using a method based on that of Ellis and Pennington (1992). Nitrogen as $(NH_4)_2SO_4$ was applied at 0 or 100 μgg^{-1} soil and phosphorus as H_3PO_4 at 0 or 20 μgg^{-1} soil. These nutrients were added in three stages at rates equivalent to 5, 20 and 75 percent of the total respectively with these additions separated by two weeks (the final 75% was applied in five equal amounts at two-day intervals).

Individual heights of all seedlings were measured to the top leaf node and mean seedling heights per pot were determined at the commencement of the experiment and again after 11weeks.

3.3 Sowing-time trial

Four sowing dates at six week intervals during 1992 (13th May, 24th June, 4th August and 14th September) were compared in a field trial using four replicate 1 m^2 plots per sowing date in a Latin Square arrangement. Each plot received 1.5 g of *E. regnans* seed (~291 viable seeds). Assessment was as in Methods 3.2.4 and was carried out at intervals of three weeks until 25th November. This trial was located in the fenced area at site B in Jungle-19 (Figures 3.1.1.4 ,3.1.1.5).

3.4 Assessment of fungi associated with a sample of *E. regnans* seed

Five hundred seeds were randomly selected from a 2 g seed sample and spaced at least 1 cm apart on TWA in petri plates. Plates were incubated for three weeks at 21°C in plastic bags under natural daylight conditions after which fungal genera associated with seeds were identified.

3.5 Studies on fungi associated with seedling mortality

3.5.1 Collection of seedlings for isolation and identification of associated fungi

Positions of seedlings in the field experiments to assess mortality rate (Methods 3.2.4) had been marked with pegs and hence dead and dying seedlings could be collected for examination. Healthy seedlings from field sites and dying seedlings from glasshouse trials on field soil were also collected. Seedlings collected in the field were placed separately onto moist tissue paper in a sealed container to be assessed for fungal associates within five hours of collection as described in Methods 3.5.2.

3.5.2 Assessment of fungi associated with seedlings

Whole seedlings were washed with sterile water to remove adhering soil, surface sterilised for two minutes in 1% sodium hypochlorite then rinsed in three changes of sterile water before placing on tap-water-agar (TWA). After two days at 25°C associated fungi were identified (where possible) on conidia and conidiophore structure or hyphal morphology. Presence of pycnidial fungi was determined at five days. Using a needle, emergent hyphal tips of representative fungi were dissected out over approximately two weeks and subcultured onto PDA. Cultures of all field isolates were stored in saline (Methods 3.1.5).

3.5.3 Identification of fungi

Identification of most fungi was based on morphological characteristics with the assistance of published keys and descriptions of Barnett and Hunter (1972), Domsch *et al.* (1980), Waterhouse (1967) and Sweetingham and MacNish (1991). Electrophoretic comparison of pectic enzymes was also used for elucidation of *Rhizoctonia* biotypes (Methods 3.5.4).

3.5.4 Electrophoretic comparison of pectic enzymes of *Rhizoctonia* isolates

Isolates were grown for seven days on 2 ml of pectin medium at 25°C in bijoux bottles (Appendix 1.1.7) after which culture fluid

samples were assayed using the electrophoretic method of Cruickshank and Wade (1980), with modifications by Cruickshank (1983). Electrophoresis proceeded at 5°C, and at a constant current determined by a starting voltage of 8 Vcm^{-1} across the gel. Gels were stained using the method of Cruickshank (1983).

3.5.5 Nuclear staining of *Rhizoctonia* isolates

The number of nuclei per cell of representative *Rhizoctonia* isolates was determined using the 0.5% safranin O in 3% KOH technique of Bandoni (1979).

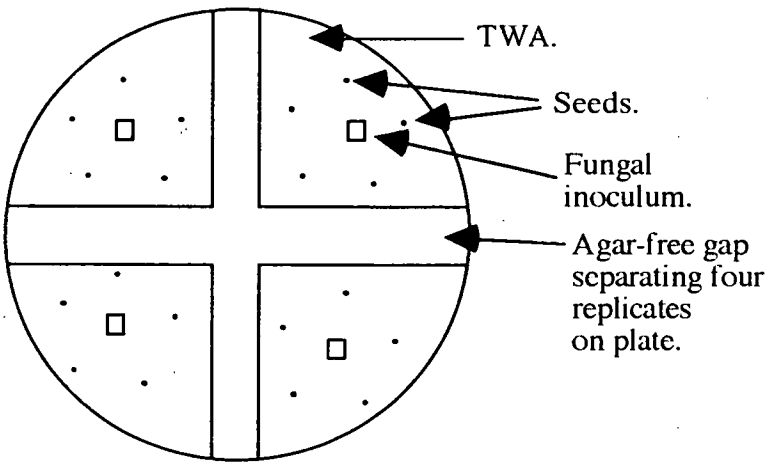
3.5.6 Induction of zoosporangia production by Pythiaceae

Induction of zoosporangia production was attempted using the method of Waterhouse (1967) with the exception that cultures were grown on PDA and stream water was substituted for pond water.

3.5.7 Test of pathogenicity of representative fungal isolates *in vitro*

Seed of *E. regnans* was surface sterilised by wetting for 30 seconds with ethanol, soaking for two minutes in 1% sodium hypochlorite then washing in three changes of sterile water. Seeds were placed at least 5 mm apart in four groups of five seeds on TWA plates (Figure 3.5.7.1) and a 3 mm^3 block of seven day PDA culture of the fungal isolate placed within each group of seeds at about 10 mm from each seed. This was replicated four times for each fungal isolate. Plates were sealed with parafilm then incubated at 10°C under continuous fluorescent light with weekly monitoring of germination and seedling mortality. A final assessment was made at 40 days and fungi were regarded as potentially pathogenic if one or more seedlings had been killed.

Figure 3.5.7.1 Plate arrangement for *in vitro* assay of fungal pathogenicity to *E. regnans* seedlings



3.5.8 Glasshouse testing of pathogenicity of selected isolates in field soil and investigation of the effect of steam-treatment on field soil

Mineral soil (top 10 cm) collected from Jungle-24 was sieved then mixed to uniform consistency before removing a sub-sample equivalent to the amount required for 1.5 experimental treatments. The remainder was steam sterilised at 80°C for 45 minutes. Volumes of 70 cm^3 of undried five-week millet culture (Methods 3.1.6) of each fungal isolate were mixed with 2.40 kg of steam sterilised soil (sufficient to fill six 9.5 cm pots per treatment). One isolate each of *Alternaria*, *Cylindrocarpon*, *Phoma* and two of *Rhizoctonia* were tested. A control received 70 cm^3 of autoclaved (dead) *Alternaria* millet culture. Other treatments were sterilised soil, unsterilised soil and sterilised soil to which 5% unsterilised soil had been added. Pots were placed in the glasshouse with daily watering and sown after one month with *E. regnans* seed at 0.25g per pot . Numbers of seedlings were recorded at weekly intervals with a final count being made at 30 days following sowing. No attempt was made to reisolate fungi from any seedlings that had died.

3.6 Studies on the use of fungicides for control of pathogens

3.6.1 Effects of Raxil and Sumisclex on fungi *in vitro*

The effects of a low concentration of these two fungicides on a selection of fungal isolates was evaluated *in vitro*. TWA media was prepared and amended with either Raxil or Sumisclex at 10 ppm active ingredient (tebuconazole or procymidone, respectively) after it had cooled to about 60°C following autoclaving and before pouring into plates. Using a 8 mm cork borer, plugs of both fungicide media were aseptically placed onto opposite sides of PDA plates. These plates were then centrally inoculated with cork borer plugs of a 5-day-old culture of the fungus to be tested. Plates were incubated at 25°C in the dark and widths of any fungal inhibition zone around fungicide plugs were recorded after fungal growth had reached the edge of each plate (13 to 28 days).

3.6.2 Fungicide phytotoxicity tests

Phytotoxicity of a range of concentrations of each of Sumisclex, Raxil and Mancozeb when applied as a seed dressing to *E. regnans* seed was evaluated in glasshouse trials. Concentrations were selected to cover the likely range to be suitable for seed treatment. Fungicides were mixed with 0.1 ml of 1.5% carboxy methyl cellulose solution per gram of seed, prior to application to the seed in 5ml glass beakers, stirring with a glass rod until seed was dry. Seed was coated one day prior to use. Sumisclex treated seed was sown onto potting mix with five replicates of 0.20 g seed per treatment. Raxil and Mancozeb treatments were sown onto unsterilised field soil at 0.20 g of seed per pot and 4 pots per treatment. Number of seedlings per pot were assessed at intervals and final counts were taken at 60 days for Sumisclex and 45 days for Raxil and Mancozeb.

3.7 Studies on biological control of pathogens

3.7.1 Isolation of bacterial antagonists

Healthy one-year-old *E. regnans* seedlings and associated soil were collected from field sites at Jungle-19. Roots were washed to remove soil before being surface sterilised for 3 minutes in 2% sodium

hypochlorite then rinsed with 5 changes of sterile water. Two grams of root material were placed in a stomacher bag with 100ml of sterile 0.85% saline and pummeled for 5 minutes. Serial dilutions were spread onto King's B agar (Appendix 1.13) or 0.3% TSA (Appendix 1.1.2) and after six days at 25°C plates were overlayed with a thin layer of PDA which was then streaked with a spore suspension of *Phoma* isolate B42-2. Bacterial colonies demonstrating inhibition to fungal growth were selected for further study.

3.7.2 Inhibition of fungal growth by bacterial isolates *in vitro*

Plugs of a 5-day PDA culture of *Rhizoctonia* isolate RZ08 were cut with a cork borer and centrally inoculated onto plates of PDA and TSA. Plates were kept at 25°C and after fungal growth had proceeded 5 mm beyond the plugs a loopful of each bacterial isolate was spot inoculated at 20 mm from the mycelial edge. On an additional 5 days incubation (or when fungal growth had reached the edge of the plate), widths of inhibition zones around bacterial colonies were recorded.

3.7.2.1 Influence of iron concentration on the level of antagonism

The method was as described in Methods 3.7.2 except that King's B agar (Appendix 1.1.3) amended with $FeCl_3$ at concentrations of 0, 0.25, 1.25, 2.5 or 5 μM was used.

3.7.3 Test of inhibition of fungal growth by *Epicoccum purpurascens* isolates *in vitro*

Five isolates of *E. purpurascens* were screened for antagonism to five potentially pathogenic fungi. Isolates and test fungi were inoculated at opposite edges of PDA plates and incubated for two weeks at 25°C. The isolate showing greatest inhibition was selected for further study.

3.7.4 Microbial antagonism to *Rhizoctonia solani* on *E. regnans* seedlings *in vitro*

The influence of three bacterial isolates and one *E. purpurascens* isolate on the germination of *E. regnans* seed and on

seedling survival in the presence of *R. solani* isolate RZ08 was tested *in vitro*. Antagonists, in association with seed, were individually tested with and without *R. solani*. Untreated and *R. solani*-only controls were also included.

Seeds of *E. regnans* were sorted to exclude those which were damaged or otherwise not likely to be viable and at least 100 randomly selected seeds from the resultant seed pool used per treatment. The seed was surface sterilised by wetting for 30 seconds with ethanol, soaking for two minutes in 2% sodium hypochlorite then washing in three changes of sterile water. Bacterial inoculation of seed was by soaking for about 30 minutes in suspensions of 10^7 cells ml^{-1} in sterile 0.85% saline. Seed was then spaced at least 1cm apart on water agar (0.3% agar to allow bacterial motility) at 5 seeds per plate.

Nutrient free inoculum of the *E. purpurascens* antagonist was prepared prior to seed inoculation by placing a 3 mm^3 block of PDA culture of the fungus onto water agar followed by incubation for one week at 25°C. Approximately 1 mm^3 of water agar containing hyphae was then placed on top of each seed (previously placed on test agar plates as described above).

For treatments with *R. solani*, plugs of five day PDA culture were cut from a plate and placed 10 mm from each seed using a 7 mm cork borer. Plates were kept at 20°C under continuous fluorescent light. Germination and mortality were monitored, with final assessment at 30 days. Chi-squared comparisons were made between antagonists and associated controls with probabilities calculated by Monte Carlo simulation.

3.7.5 Glasshouse testing of antagonists in field soil

A sample of the top 10 cm of mineral soil from a burnt forest site was sieved then mixed to uniform consistency before steam sterilising at 80°C for 45 minutes. Soil was placed into 12.5 cm diameter pots with six replicate pots per treatment. Treatments with *R. solani* were inoculated with undried 52-day-old millet culture which was mixed with the top 3 cm of soil at a rate equivalent to 5 cm^3 of culture per pot.

Seed of *E. regnans* was weighed out in lots of 0.400 ±0.005 g (~ 44 viable seeds) and microbial antagonists applied to seed before sowing. Growth from five-day-old bacterial cultures on TSA was scraped from plates and suspended in 18 ml sterile 0.85% saline with a drop of 0.1% (vol/vol) Tween 80 as wetting agent. Bacterial suspensions (1.0 ml with densities of the three isolates ranging between $4x10^8$ and $4x10^9$ cells ml^{-1}) were applied to seed lots and left to soak for four hours. When seed was sown the associated bacterial suspension was also poured evenly over the soil surface.

The *E. purpurascens* isolate was inoculated onto the seed by applying 0.5 ml of a suspension containing spores and sporodochia (approx. 10^3 spores ml^{-1}) in a 1.5% (wt/vol) carboxy methyl cellulose solution to each 0.400 g lot of seed. An amount of 1 cm^3 of air dried millet culture of this isolate was also added to the soil surface of inoculated pots.

Pots were spaced 15 cm apart to minimise cross contamination by soil splashing during watering. Seedling counts were made at weekly intervals for six weeks with seedling mortality being noted. Statistical analysis was by Student's t-test with paired comparisons to the controls.

3.7.6 Identification of bacterial antagonists

Bacterial antagonists were identified with the assistance of keys and descriptions included in *Bergey's Manual of Systematic Bacteriology*, Vol. 1 (Krieg and Holt, 1984), Vol. 2 (Sneath *et al.*, 1986) and *The Prokaryotes*, (2nd Ed.) Vol. 2 (Balows *et al.* 1992). The following tests were performed.

3.7.6.1 Gram stain and cell morphology

Cultures grown on TSA for 48 hours were stained by the Gram method and examined by light microscopy.

3.7.6.2 Motility

Bacterial motility was determined using 24 hour broth cultures and examined by phase contrast microscopy.

3.7.6.3 Oxidative /fermentative metabolism

Two tubes of Hugh and Leifson medium (Appendix 1.1.8) per isolate were stab inoculated. One of each pair was incubated for 24 hours aerobically and the other anaerobically (by covering with sterile paraffin). Colour change in the aerobic tube only indicated oxidative metabolism of glucose whilst a colour change in the anaerobic tube indicated glucose fermentation.

3.7.6.4 Oxidase

With a glass tube a small amount of culture from TSA medium was placed onto filter paper moistened with a few drops of a freshly prepared 1% aqueous NNNN'tetramethyl-*p*-phenylene diamine dihydrochloride solution. Presence of oxidase was indicated by development of purple colouration within 10 seconds.

3.7.6.5 Catalase

Bacterial biomass on glass slides were covered with a few drops of 3% H_2O_2. Immediate production of gas bubbles indicated presence of catalase.

3.7.6.6 Fluorescence (pyoverdin production)

Cultures grown on plates of King's B agar (Appendix 1.1.3) were examined at one, three and five days under 366nm ultraviolet light. A positive result was indicated by fluorescence of bacterial colonies and surrounding media.

3.7.6.7 Utilisation of citrate

Utilisation of citrate was determined by the development of turbidity in Koser's citrate medium (Appendix 1.1.9) after 24 hour incubation.

3.7.6.8 Voges-Proskauer test

Bijoux bottles of V-P broth (Appendix 1.1.10) were inoculated and incubated for seven days. Approximately 1 ml of 40% NaOH and a knife point (approx. 1 mg) of creatine was then added and the bottles

shaken. A positive result was recorded if pink colouration developed after standing for thirty minutes.

3.7.6.9 Acid production in V-P medium (methyl-red test)

Bijoux bottles of V-P broth (Appendix 1.1.10) were inoculated and incubated for seven days. On addition of few drops of methyl red solution (Appendix 1.2.3) a red colour indicated a positive result (pH ≤4.2) with yellow colouration being negative.

3.7.6.10 Indole production

Bijoux bottles of 1% Oxoid tryptone water were inoculated and incubated for seven days. A few drops of Kovacs reagent (Appendix 1.2.5) was then added and bottles briefly shaken. Development of a red colouration by the layer of reagent within ten minutes was an indication of the presence of indole.

3.7.6.11 Poly-ß- hydroxy butyrate (PHB) accumulation

Cultures were grown for seven days at 25°C in bijoux bottles of Palleroni and Doudoroff medium (Appendix 1.1.19, containing NH_4Cl at one-fifth normal concentration and amended with 0.5% *DL*-ß-hydroxy butyrate). Staining was by the modified Neisser's method of Cruickshank *et al.*, (1975)

3.7.6.12 Arginine dihydrolase production

Tubes of Thornley's semisolid medium (Appendix 1.1.11) with and without arginine were stab inoculated then sealed with sterile paraffin. Arginine hydrolase production was indicated by development of red colour after seven days incubation in the arginine amended medium.

3.7.6.13 Nitrate reduction

Bijoux bottles of nitrate reduction medium (Appendix 1.1.12) were inoculated and incubated for seven days. A knifepoint (approx. 1 mg) each of KI and soluble starch were placed together on a clean surface (petri dish) and dissolved in a drop of 0.1 N HCl. Colour change

to black on addition of a drop of culture suspension indicated the presence of nitrate and thus the absence of nitrate reduction.

3.7.6.14 Levan formation from sucrose

Plates of nutrient agar (Appendix 1.1.13) amended with 4% sucrose, were inoculated and incubated for seven days. Viscous colony consistency indicated levan production.

3.7.6.15 Hydrolysis of gelatin

Bijoux bottles of nutrient gelatin (Appendix 1.1.14) were stab inoculated and incubated for up to seven days at 20°C. A positive result was indicated by liquefaction of the medium.

3.7.6.16 Hydrolysis of casein

Plates of milk agar (Appendix 1.1.15) were inoculated and incubated for up to seven days. A positive result was indicated by development of a translucent zone around the growth.

3.7.6.17 Hydrolysis of starch

Plates of starch agar (Appendix 1.1.16) were inoculated and incubated for seven days. Starch hydrolysis was indicated by clear zones around the growth on flooding plates with Gram's iodine solution.

3.7.6.18 Tyrosine utilisation

Plates of tyrosine agar (Appendix 1.1.17) were inoculated and incubated for up to seven days. A positive result was indicated by clearing of the tyrosine crystals from around the growth.

3.7.6.19 Acid/gas from glucose

Bijoux bottles of glucose peptone broth (Appendix 1.1.18) with bromocresol-purple indicator (0.0006% w/v) plus inverted Durham tubes were inoculated and incubated for up to seven days. Colour change from purple to yellow indicated acid production while presence of gas in the Durham tube indicated its production.

3.7.6.20 Hydrolysis of Tween 80

Plates of TSA were inoculated and after colonies had formed (on incubation), drops of sterile 1% (vol/vol) aqueous Tween 80 were placed on the media between colonies. Lipolytic activity was indicated by the development of an opaque deposit within the media near colonies after incubation for a further 24 hours.

3.7.6.21 Growth at various temperatures

Plates of TSA were inoculated then sealed with parafilm before incubation at the selected temperature for up to seven days.

3.8 1993 Field Trial - Comparison of two sowing times with evaluation of fungicide, biocontrol and fertiliser treatments at the latter sowing time

A field trial was set up on Jungle-24 following slash-burning of the site in early April '93. Five treatments were randomly arranged in two blocks (Sites D & E) of four replicates. Seed of *E. regnans* was sown at 1.25 g per 1 m^2 plot (208 viable seeds per plot). Treatment details were as shown in table 3.8.1. Plots were assessed as in the 1992 field experiments (Methods 3.2.4) but normally at 4 week intervals.

Table 3.8.1 Treatment details for 1993 field trial

Code	Sowing Date	Other details
A	20/4/93	Untreated.
B	30/6/93	Untreated.
C	30/6/93	Seed dressed at 20g Raxil and 5g Mancozeb per kg.
D	30/6/93	Millet culture of *E. purpurascens* isolate B60 applied to soil surface at 35 cm^3 per plot.
E	30/6/93	Osmocote (NPK 14:6.1:11.6) applied to soil surface at 40 g per plot.

4. RESULTS

4.1 Estimates of fungal and bacterial numbers in soil at 8 days after burning

In the absence of slash-burning, neither mean fungal nor bacterial colony forming units (CFU) were observed to vary greatly with soil depth to 10 cm (Figure 4.1.1 B,D). However burning was associated with a significant reduction in fungal CFU for the top 2 cm of soil (Figure 4.1.1 A) and although a similar trend was noted for bacteria, high counts for a small number of samples (presumably less affected by

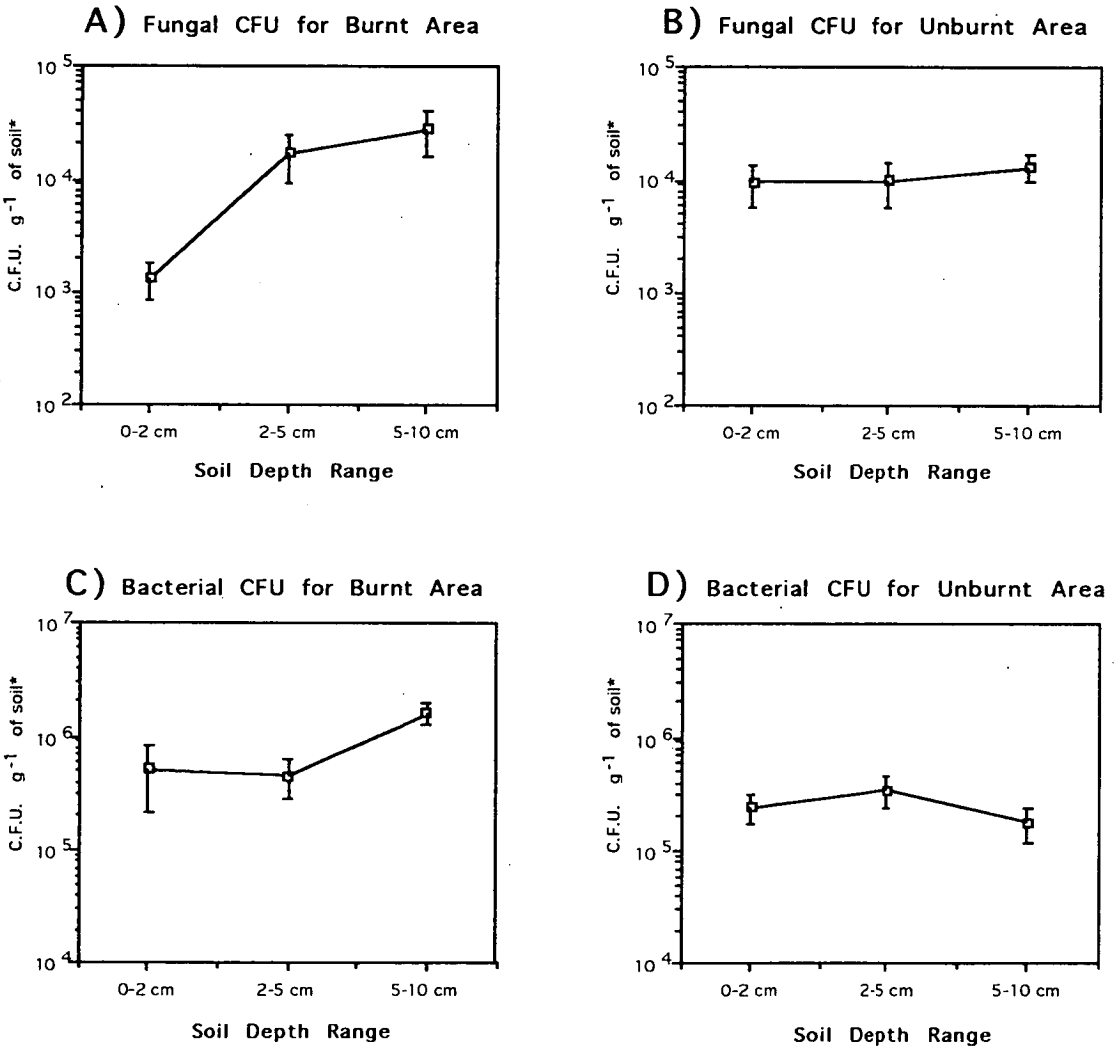


Figure 4.1.1 Estimates of fungal and bacterial CFU in soil at 8 days after slash-burning. Each point is the mean of 10 and 5 replicates for the burnt and unburnt areas respectively. Bars show ± standard errors.

* C.F.U. = colony forming units

the burn) resulted in a 0-2 cm mean which was approximately the same as for the 2-5cm depth range (Figure 4.1.1 C).

4.2 Observation of ascomycete growth on soil surface following fire

At approximately one month after burning of both Jungle-19 and Jungle-24 coupes brightly coloured (mostly orange) fungal growth was commonly found in moist areas on the burnt soil surface, being particularly prominent under the shelter of rocks and pieces of wood (Figure 4.2.1). Microscopic examination of two samples revealed the presence of asci and ascospores, confirming that the fungus was a discomycete. Within one month of its appearance this type of fungal growth could no longer be seen on the burnt areas.

Figure 4.2.1 Example of ascomycete growth on the soil surface at about one month after burning.

4.3 Soil pH at 8 and 133 days after burning

Mean pH of the top 2 cm of soil at eight days after the fire was 8.3. Surface pH in the burnt area was variable (ranging from 7.0 to 10.0) reflecting variability in the intensity of burning and ash deposition. Increasing acidity with increasing depth was noted for these samples in contrast to equivalent samples from the unburnt site, where mean pH of the top 2 cm was 5.2 (ranging from 4.9 to 5.3.) with no apparent change with depth (Figure 4.3.1(A), Appendix 4).

At 133 days after the fire the mean pH of the surface 2 cm of soil from the burnt sites had dropped to 7.2, with a corresponding decrease in variability (Figure 4.3.1(B)). This drop in pH was likely to be the result of leaching of mobile ions from the soil. Total rainfall on the site during the intervening period (according to data provided by ANM Forest Management) had been 425 mm.

Across a 400 m transect of Jungle-19 at 133 days, soil surface pH varied between 4.4 and 8.0 with a median of 7.2 (Figure 4.3.2). Values of less than 6.5 represented samples taken from unburnt or poorly burnt areas. The soil surface pH distribution was similar to that found within experimental sites on Jungle-19 on the same day and indicates that the sample sites were representative of the coupe as a whole.

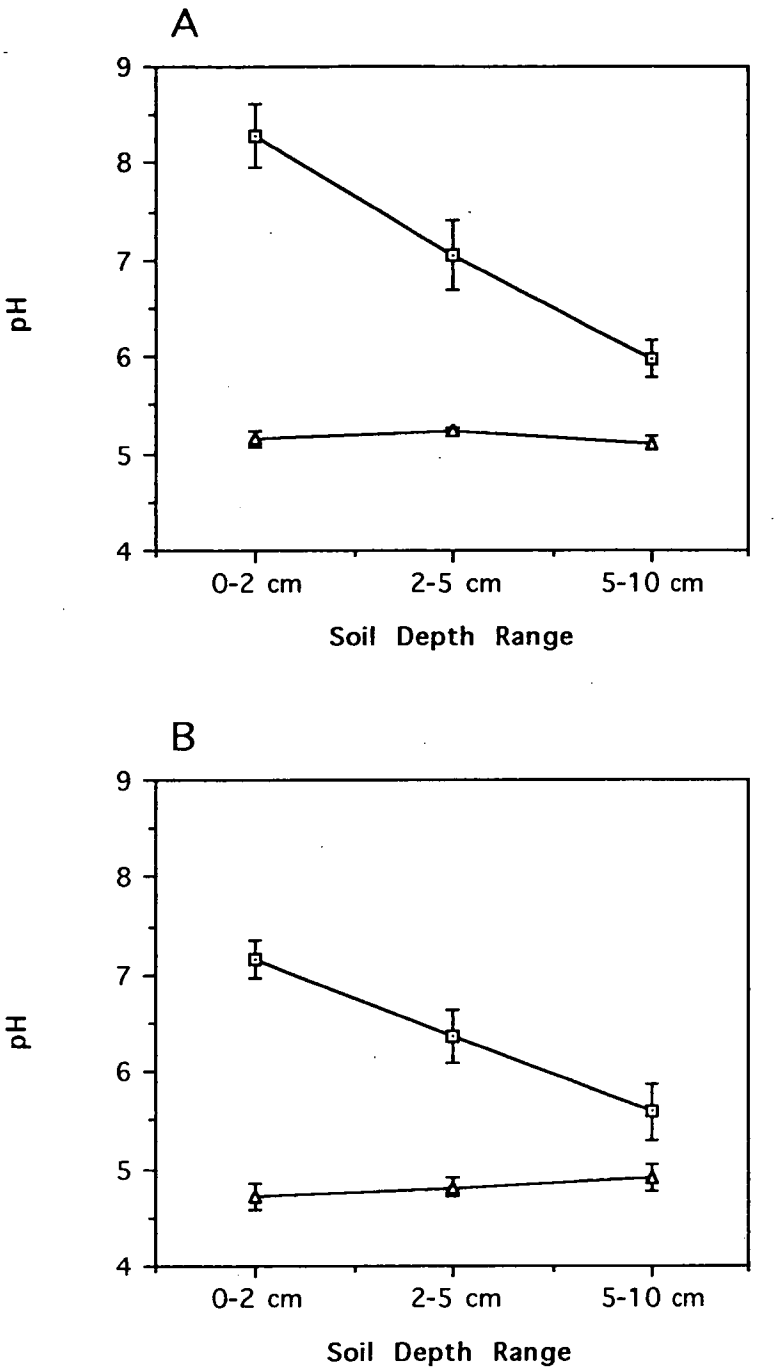


Figure 4.3.1 Variation in mean soil pH with depth at 8 days (A) and 133 days (B), after slash burning in burnt area (□) and unburnt control (△). Bars show ± standard errors.

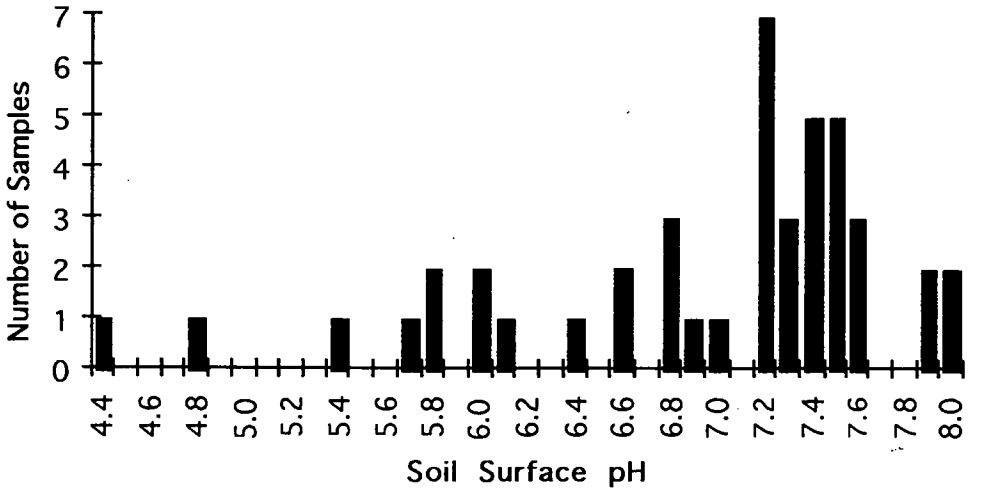


Figure 4.3.2 The pH of soil surface samples taken from a transect of Jungle-19 on 31/8/92 (133 days after burning). Number of samples = 44.

4.4 Influence of soil pH on seedling establishment in the glasshouse on field soil collected at 8 days

As noted in Results 4.3, there was wide variability in pH of surface soil samples collected from the burnt area at eight days after the fire. Germination rates of *E. regnans* were also variable when grown on samples of this soil in the glasshouse, being clearly affected by pH of the particular soil sample. Numbers of seedlings at 20 days in different field soil samples increased up to pH 8.5, with a decline in germination at higher pH levels (Figure 4.4.1(A)). Of the 21 seeds observed to germinate on the soil sample with a surface pH of 10.0 none survived beyond two weeks (Figure 4.4.2).

A similar trend was observed for survival of seedlings after a three month period on these soil samples although actual surface pH had declined by this time (Figure 4.4.1(B)).

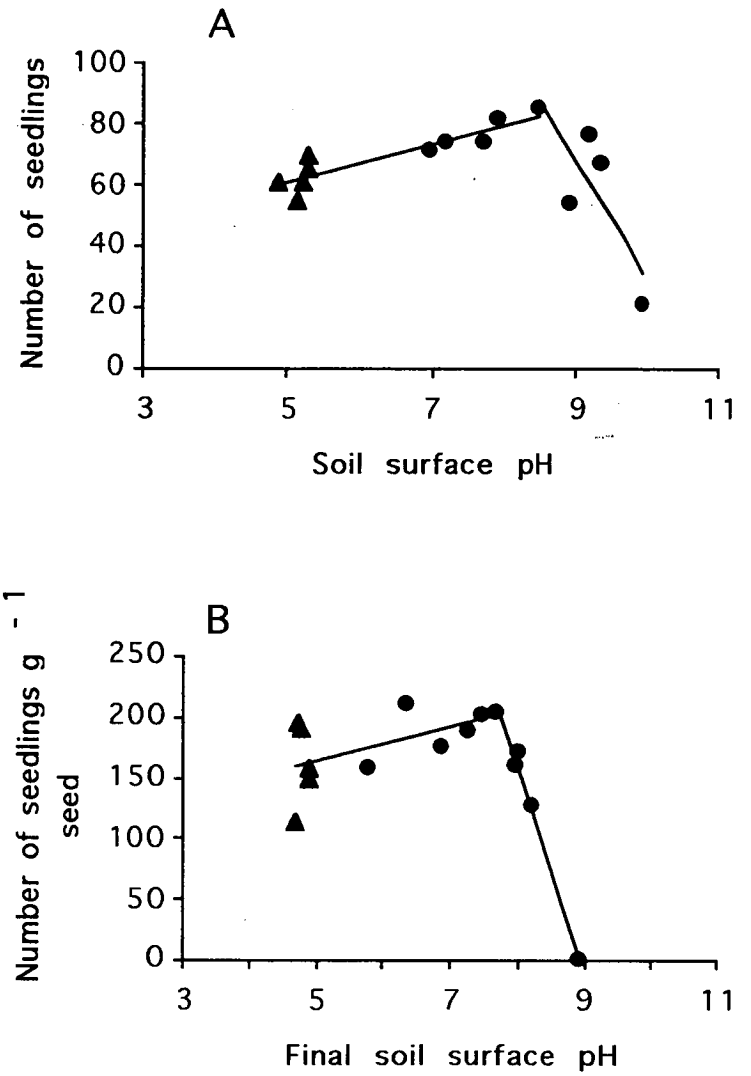


Figure 4.4.1 A) Germination of *E. regnans* at 20 days in field soil at varying pH values. B) Survival of seedlings after three months in field soil at varying pH values. Pots of field soil from burnt areas (●) and unburnt area (▲) were sown with 0.5 g of seed and maintained under glasshouse conditions. Two regression lines have been fitted to each of the data.

Figure 4.4.2 *E. regnans* seedling establishment at 20 days after sowing on field soil samples with surface pH of 8.5 (left) and 10.0 (right).

4.5 Seedling establishment on ash

A significant reduction in establishment of seedlings on ash or on a 1:1 soil-ash mixture was apparent when compared with seedling establishment on soil alone (Table 4.5.1). This reduction was correlated with increasing pH of the ash mixtures, although other chemical and physical properties of the ash may have had some effect.

Table 4.5.1 Seedling establishment on ash, soil and a 1:1 mixture of ash and soil.

Soil description	pH	Seedling establishment at 50 days ± s.e.
Soil	7.8	184 ±10.1 a
1:1 Soil-ash mixture	9.2	16 ± 5.1 b
Ash	10.1	4 ± 2.9 b

Five replicate pots each received 0.20g of seed at 195±24 viable seed per gram.
Values followed by the same letter do not differ significantly ($P<0.01$) according to Duncan's New Multiple-Range test.

4.6 Variation in soil pH across a coupe transect before and soon after burning

Soil surface pH on Jungle-24 prior to burning ranged between 3.1 and 5.6 (Figure 4.6.1 (A)). Two weeks after burning, surface pH ranged between 5.4 and 9.1 with ten percent of samples having a pH greater than 8.4 (Figure 4.6.1 (B)). The mean elevation in pH was 2.5 units.

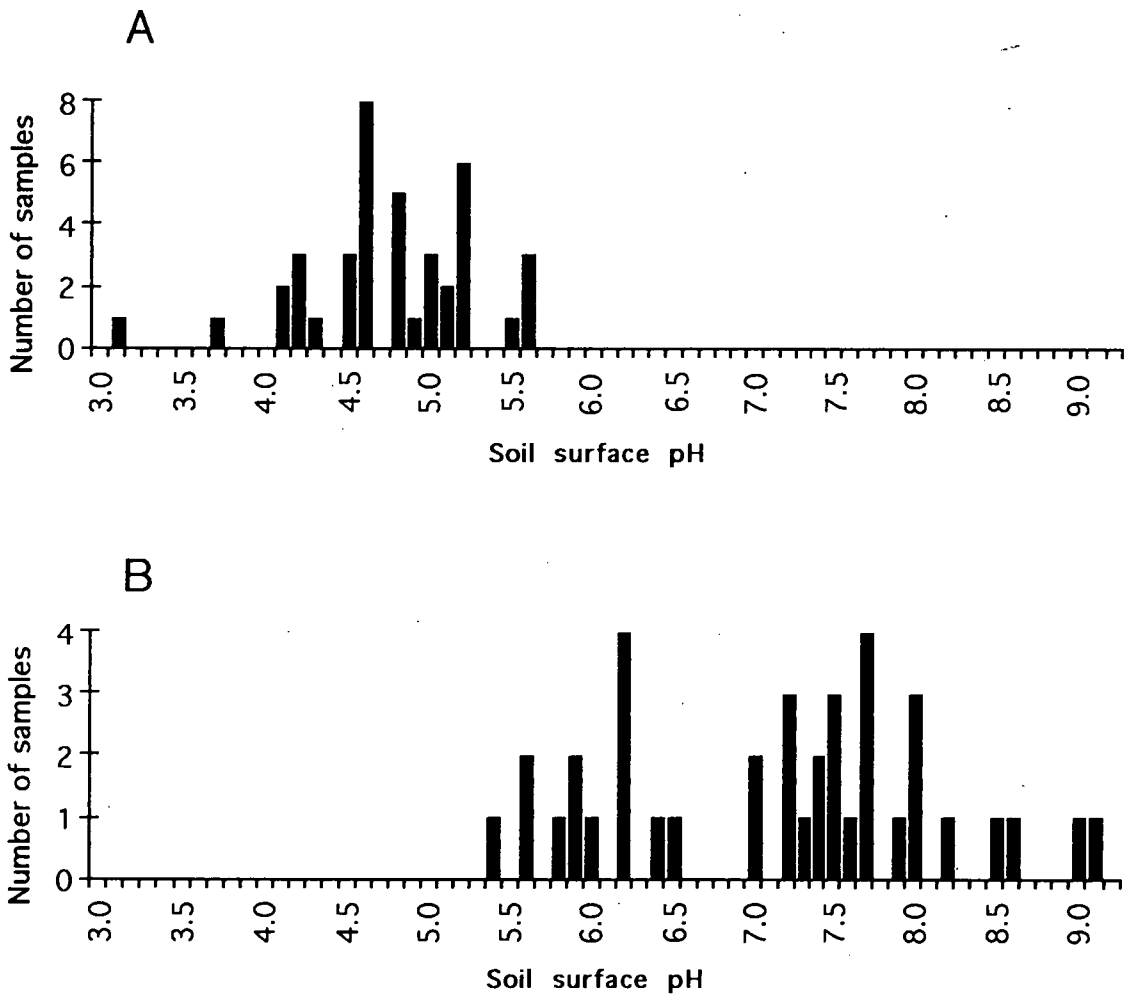


Figure 4.6.1 Soil surface pH distributions for samples taken along a transect of Jungle-24 at: A) two weeks before (40 samples collected) and; B) two weeks after (38 samples) slash-burning.

4.7 Effect of pH in the range 8.0 to 10.4 on germination and survival of *E. regnans* seedlings *in vitro*

A marked drop in germination of *E. regnans* seeds was observed *in vitro* with increasing pH (Figure 4.7.1(A)), which was also associated with a progressive decline in seedling survival (Figure 4.7.1(B)).

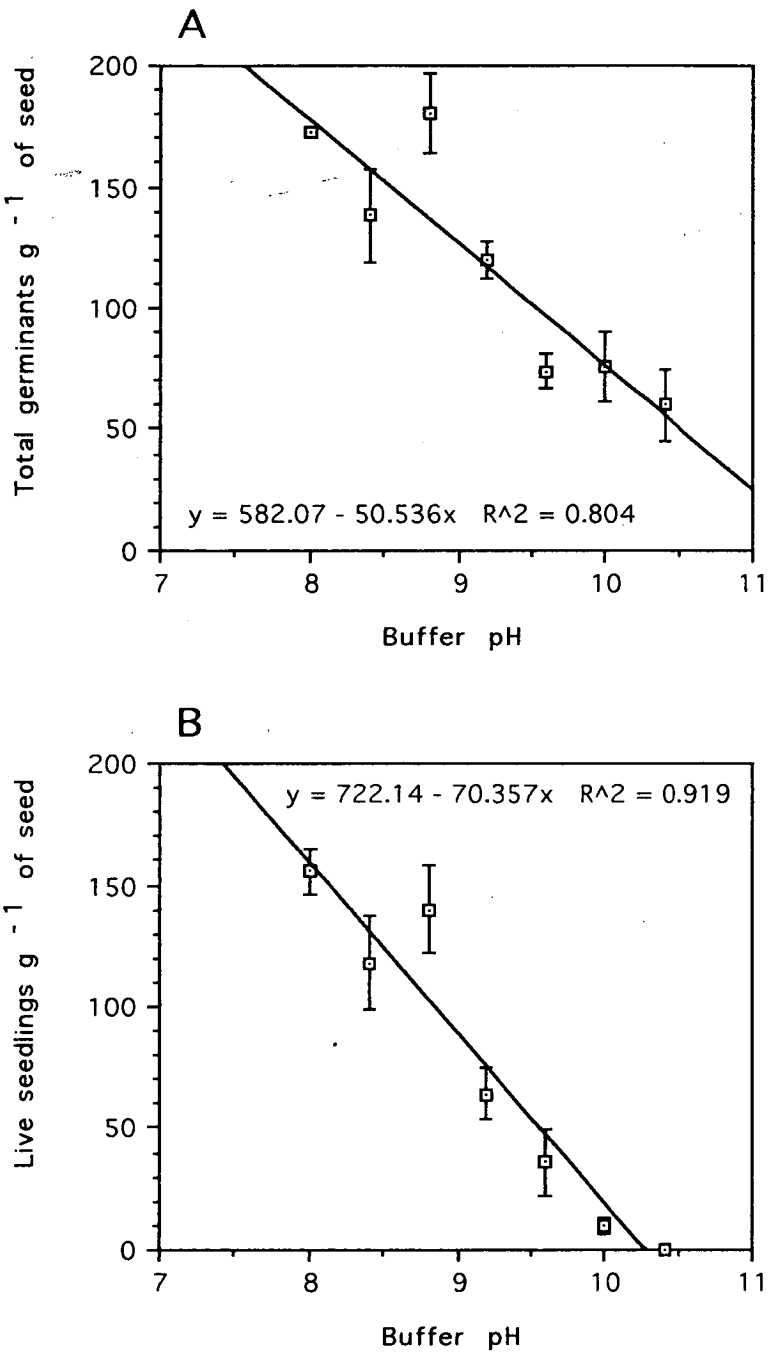


Figure 4.7.1 Effect of pH on seed germination (A) and seedling survival (B), *in vitro* . Buffers (0.1 M) were Tris (pH 8.0 - 9.0) or sodium carbonate-bicarbonate (pH 9.2 - 10.4). Incubation was at 15°C for 21 days. Each point is the mean of five replicate determinations. Bars show ± standard errors.

4.8 Effect of temperature-induced dormancy on susceptibility of seedlings to elevated pH *in vitro*

Low temperature was found to have a significant effect on the response of seed to elevated pH (Table 4.8.1). There was no germination at 4°C regardless of incubation pH. At 20°C germination occurred but was much reduced at pH 10.2 relative to pH 8.0 ($p<0.01$).

After transfer to pH 7.2 medium there was strong germination of seed that had been at 4°C. Germination of seed that had been at pH 10.2 and 4°C was found to be significantly less than that of seed that had been at pH 8.0 and 4°C, ($p<0.05$). There was some additional germination of seed that had previously been at 20°C. Total germination of seed that had initially been at pH 10.2 and 20°C, was significantly less ($p<0.01$) than for any other treatment, indicating that many of the seeds that were not observed to germinate at pH 10.2 may have commenced the germination process and subsequently died. Low temperature had a protective effect on the seed from high pH conditions, possibly by inhibiting germination.

Table 4.8.1 Effect of pH and temperature combinations on germination of *E. regnans* seed.

Initial incubation temperature and pH	4°C and pH 8.0	4°C and pH 10.2	20°C and pH 8.0	20°C and pH 10.2
Germination at 21 days *:	0	0	103 a	24 b
Additional germination after transferring to pH 7.2 at 20°C for 28days*:	199 a	169 b	36 c	42 c
Total germination*	199 a	169 b	139 c	66 d

* Number of seedlings.

Ten replicate plates each sown with 0.100 g of seed at 195±24 viable seed per gram.

Values in each row followed by the same letter do not differ significantly ($P<0.05$) according to Duncan's New Multiple-Range test. The nil values were not included in the statistical analysis.

4.9 Comparison of seedling growth rates in the glasshouse on field soil collected at 133 days after burning

Growth suppression was evident amongst seedlings grown in soil from field sites when compared with controls grown in potting mix (Figure 4.9.1). This was reflected in mean seedling heights which although approximately 50% greater on the burnt soil than on the unburnt soil, were only about half that reached on potting mix (Table 4.9.1). Mean heights on potting mix were greater than for all other treatments ($p<0.01$) while, on application of a log(x-18) transformation to the data, a difference ($p<0.05$) was found between means for sites C and B (18mm being the approximate mean cotyledon height).

Figure 4.9.1 *E. regnans* seedlings after 16 weeks growth in (from left to right): soil from unburnt site C; soil from burnt sites A & B; and potting mix.

Table 4.9.1 Comparison of seedling heights on burnt and unburnt field soil and potting mix.

Soil source	Mean seedling heights at three months, (mm).± standard error §.	Transformed data ±standard error#
Site A (burnt)	31.0 ± 4.5 a*	0.92 ±0.24 ab*
Site B (burnt)	35.1 ± 3.6 a*	1.18 ±0.12 b*
Site C (unburnt)	22.8 ± 0.09 a*	0.66 ±0.07 a*
Potting Mix	72.9 ±10.9 b**	1.71 ±0.09 c**

§ Sites A to C ,5 replicates; Potting mix, 4 replicates.

Log(x-18) transformation, (18 mm being the approximate mean cotyledon height above the soil surface).

Figures followed by the same letter are not significantly different according to Duncan's New Multiple-Range test at *P<0.05 or **P<0.01.

4.10 Effect of nitrogen and phosphorus addition on seedling growth rates on field soil in the glasshouse

Visual assessment at 11 weeks of seedlings growing on soil from the unburnt area revealed a distinct increase in seedling size for the N + P treatment as well as a lesser but noticeable improvement for those that had received P alone (Figure 4.10.1). This manifest difference was not strongly reflected in measurements of seedling heights (Table 4.10.1) although a significant difference ($P<0.05$) was found for the N + P treatment compared to that which received no nutrients.

On soil from the burnt sites neither application of N, P nor N+P was associated with a statistically significant increase in seedling height ($p<0.05$) indicating that availability of nitrogen or phosphorus was not greatly limiting seedling growth in burnt soil (Table 4.10.2). A slight increase in size with N+P or N alone was however noted on visual assessment of treatments (Figure 4.10.2). In retrospect, final seedling dry weights may have been used to further compare treatments and might have been a more reliable measure of seedling size.

Table 4.10.1 Growth of *E. regnans* seedlings in soil from the unburnt site after fertiliser treatments*

Soil source and Treatment	Initial mean seedling height, (mm)	Final mean height after 11 weeks, (mm)	% Height Increment, (100x difference/initial)
Unburnt	32.1 a#	43.7	33.7 a
Unburnt +P	34.4 a	51.3	58.8 ab
Unburnt +N+P	34.3 a	66.3	96.5 b

* Three replicate pots per treatment, prepared as outlined in Methods 3.2.7.

\# Figures in each column followed by the same letter are not significantly different ($p<0.05$) according to Duncan's New Multiple Range test.

Figure 4.10.1 Effect of fertiliser treatments on growth of *E. regnans* seedlings after 11 weeks in soil from the unburnt site. Treatments (from left to right): untreated control; +P; +N&P.

Table 4.10.2 Growth of *E. regnans* seedlings in soil from the burnt site after fertiliser treatments *

Soil source and treatment	Initial mean seedling height, (mm)	Final mean height after 11 weeks (mm)	% Height Increment, (100x difference/initial)
Burnt	62.8 a#	81.4	29.7 a
Burnt +P	58.5 a	76.2	32.8 a
Burnt +P+N	62.5 a	100.2	64.6 a
Burnt +N	60.6 a	94.5	58.7 a

* Four replicate pots per treatment, prepared as outlined in Methods 3.2.7

Figures in each column followed by the same letter are not significantly different ($p<0.05$) according to Duncan's New Multiple Range test.

Figure 4.10.2 Effect of fertiliser treatments on growth of *E. regnans* seedlings after 11 weeks in soil from burnt sites. Treatments (from left to right): untreated control; +P; +N&P; +N.

4.11 Assessment of fungi associated with *E. regnans* seed

Even when not surface sterilised *E. regnans* seed used in experimental procedures was generally free of fungal contamination. Most common contaminants, affecting a total of less than 5% of unsterilised seed were *Epicoccum purpurascens*, *Ulocladium* and *Alternaria* species while species of *Penicillium* and Mucoraceae were normally absent. However one atypical seed sample (not used in experimental work) with a high level of fungal contamination was examined in detail and Table 4.11.1 lists fungi present on 500 seeds from this sample. None of these was observed to be pathogenic to the seedlings.

Table 4.11.1 Fungi present on a sample of 500 *E. regnans* seeds

Fungal taxa	Percentage of seeds affected
Penicillium	39%
Alternaria	<1%
Ulocladium	11%
Mucoraceae	10%
Trichoderma	<1%

4.12 Field Studies

Findings of all field experiments are reported here. Rainfall and temperature data collected by ANM Forest Management personnel in 1992 at a site on Jungle-19 and in 1993 at Islet-12 (approximately 1km east from Jungle-24) are included in Appendix 2.

4.12.1 Effects of site burning on germination and seedling mortality

Although total seedling numbers varied between sites germination on all sites in this experiment followed the same pattern (Figure 4.12.1, Appendix 5). Following sowing in mid-May germination was sparse until reaching a distinct peak rate in August and then afterwards tapered off. An apparent delay of about two to three weeks in the germination peak was noted for site B when compared with sites A and C. Total observed germination was significantly lower ($p<0.05$) on the unburnt site (C) than on the burnt sites A and B (Table 4.12.1.1).

The main seedling mortality peak commenced in late August and reached a maximum rate during September and October, about a month after the germination peak (Figure 4.12.1, Appendix 5). Although germination on the burnt sites had been about six times greater than that on the unburnt site, a higher seedling mortality rate on the burnt sites ($p<0.05$, Table 4.12.1.1) resulted in no significant difference between them in the final number of live seedlings ($p<0.05$, Table 4.12.1.1). Likewise there was no significant difference in germination or mortality rates between the two burnt sites.

Table 4.12.1.1. Germination and mortality of *Eucalyptus regnans* seedlings on burnt and unburnt sites #

Site	% Germination §	% Live Seedlings §	Seedling Mortality, (% of germinants)
Site A (burnt)#	32.4% a* (393)	5.3% a (64)	87% a
Site B (burnt)	22.5% a (273)	5.2% a (63)	80% a
Site C (unburnt)	4.6% b (56)	2.3% a (28)	51% b

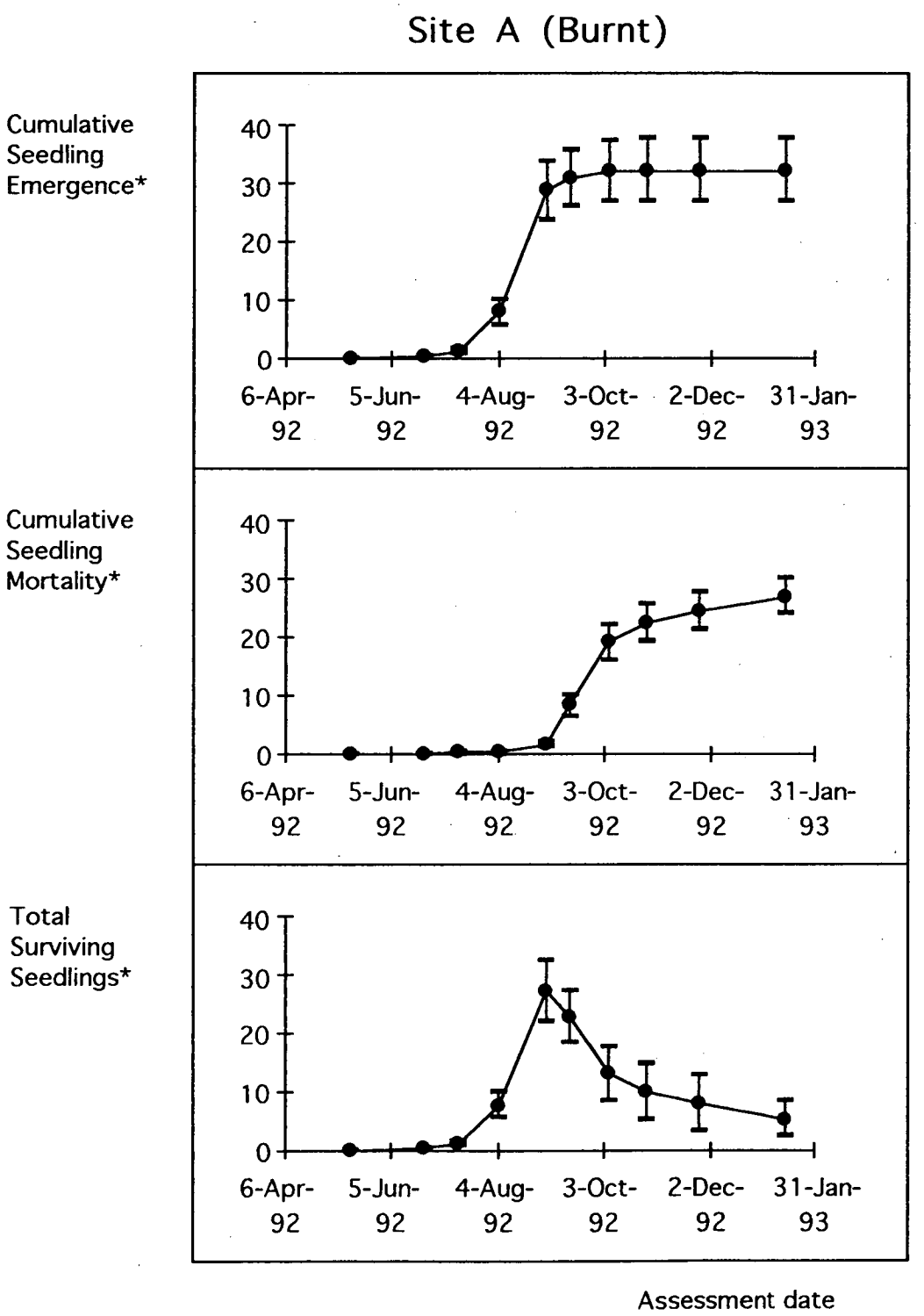
Sites A and B were located on Jungle-19 which was burnt on 19/4/92 and site C on Jungle-18 which was logged at the same time but was left unburnt. Figures shown refer to seedling totals until 14/1/93 (246 days after sowing).

§ Based on 244 viable seeds per plot. Seedling numbers are shown in brackets.

* Figures in each column followed by the same letter are not significantly different according to Duncan's New Multiple-Range (p=0.05).

Figure 4.12.1 Emergence, mortality and survival (over a 9 month period) of seedlings sown in sites A, B and C.

*Numbers of seedlings are expressed as a percentage of viable seed sown. Each point is the mean from 5 replicate $1m^2$ plots per site, each sown with 1.25 g of *E. regnans* seed (~244 viable seeds). Bars show ± standard errors.



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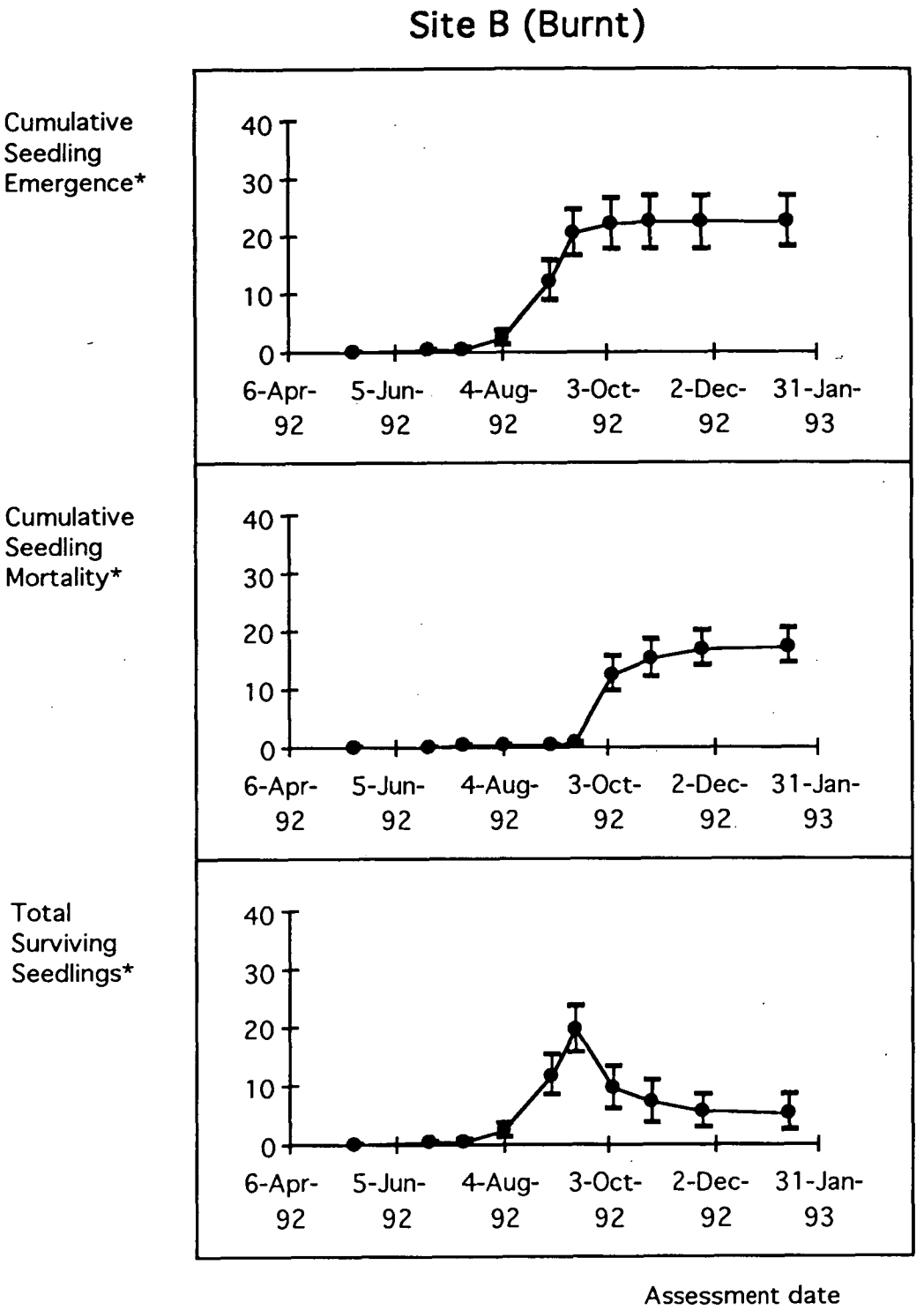

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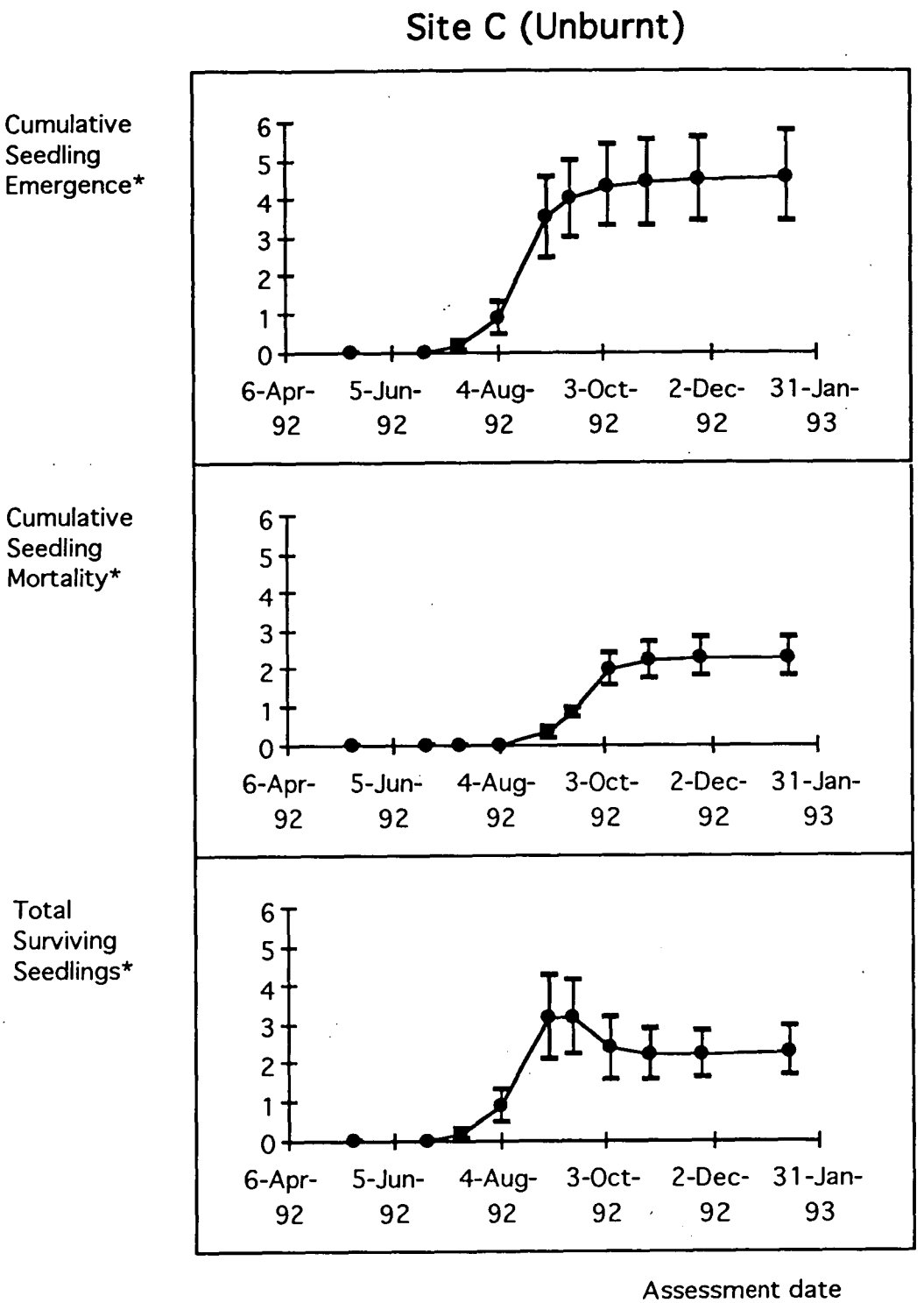


4.12.2 Sowing-time trial

This trial was run from sowing in May - September '92 until 25th November '92 after which excessive weed growth made accurate counting difficult. The greatest germination totals were for the first two sowing dates (Table 4.12.2.1; Appendix 6) which were not significantly different ($p<0.05$). However a marked reduction in germination resulted from later sowings in August and September with almost no seedlings arising from the September sowing. This period corresponded with the onset of warmer (higher maximum temperature) and generally drier weather (Appendix 2) while an abundant population of seed-feeding Lygaeid bugs (associated with the weed growth) was present on the site from October onward. These factors may have contributed to reduced germination of the later sown seeds. Also the time available after sowing of the last treatment until cessation of assessment may not have been sufficient for full germination to occur in the later sown treatments.

Table 4.12.2.1 Sowing-time Trial: Summary of results to 25th November '92*

Sowing Date	% Germination §	% Live Seedlings §	Seedling Mortality, (% of germinants)
13th May '92	10.6 a# (124)	3.4 a (39)	69
24th June '92	11.5 a (134)	4.5 a (53)	60
4th August '92	4.4 b (49)	2.5 ab (29)	41
14th Sept. '92	0.2 c (2)	0.2 b (2)	0

* This trial was located at site B on Jungle-19 with 4 replicate 1 m^2 plots per sowing date in a Latin Square arrangement.

§ Based on 291 viable seeds per plot. Seedling numbers are shown in brackets.

\# Figures in each column followed by the same letter are not significantly different according to Duncan's New Multiple Range test ($p<0.05$).

4.12.3 1993 Field Trial - Comparison of two sowing times and evaluation of fungicide, biocontrol and fertiliser treatments at the later sowing time

Rainfall was unusually low during the course of this experiment with extended dry periods in winter and spring (Appendix 2). This was seen as a contributing factor to reduced germination and increased mortality, particularly for later sown treatments and plots in block 1.

Greater differences were found between blocks than between treatments. Significantly lower ($p<0.01$) germination in block 1 (Table 4.12.3.1) appeared to be due to the increased tendency for surface drying on this more exposed site with germination being restricted to microsites where surface water was likely to collect.

The next largest differences were between sowing times, with germination following sowing in April (treatment A, Table 4.12.3.2 i), being significantly higher (at $p<0.05$) than for all June-sown treatments with the exception in block 2 of the *E. purpurascens* treatment (D). There were no other significant differences between the later sown treatments. In block 1 mortality was lower for the fungicide treatment (C) compared to treatment A (Table 4.12.3.2 ii) but not lower than other June-sown treatments.

The final seedling percentage (Table 4.12.3.2 iii) did not vary significantly between treatments and represented about 12% of the germinants on both blocks (Table 4.12.3.1), with numbers being higher ($p<0.01$) in block 2.

Table 4.12.3.1 Comparison of block means for 1993 field trial.[a]

	Block 1	Block 2	Significance
Germination percentage[b]	6.1 ± 1.7	22.5 ± 2.7	Different at $p<0.01$
Mortality (percent of germinants)	82.0 ± 5.5	89.5 ± 1.8	No difference
Final seedling percentage[b]	0.79 ± 0.24	2.50 ± 0.49	Different at $p<0.01$

[a] Figures shown refer to totals up until 20th October '93 and each is the mean of 5 replicate 1 m^2 plots ± standard error. Block 1 and Block 2 were located at sites D & E respectively on Jungle-24.

[b] Based on 208 viable seeds per plot.

Table 4.12.3.2 Final germination and seedling mortality data for 1993 field trial*

i) Germination percentage#

Treatment§	Block 1	Block 2	Combined Blocks
A	15.8 ± 6.8 a	37.2 ± 4.6 a	26.5 ± 5.5 a
B	3.4 ± 1.7 b	23.5 ± 1.6 bc	13.4 ± 3.9 b
C	3.7 ± 0.7 b	12.9 ± 2.9 c	8.3 ± 2.2 b
D	3.6 ± 0.6 b	26.8 ± 6.9 ab	15.2 ± 5.4 ab
E	4.0 ± 0.8 b	12.4 ± 3.2 c	8.2 ± 2.2 b

ii) Mortality (percent of germinants)

Treatment	Block 1	Block 2	Combined Blocks
A	95.5 ± 3.0 a	89.9 ± 3.0 a	92.7 ± 2.3 a
B	88.7 ± 7.9 ab	89.2 ± 3.3 a	89.0 ± 4.0 ab
C	56.8 ± 21.1 b	87.7 ± 3.8 a	72.3 ± 11.5 b
D	79.2 ± 9.5 ab	88.4 ± 5.7 a	83.8 ± 5.4 ab
E	89.9 ± 6.2 ab	92.5 ± 5.2 a	91.2 ± 3.8 ab

iii) Final seedling percentage#

Treatment	Block 1	Block 2	Combined Blocks
A	1.20 ±1.05 a	3.37 ±0.52 a	2.28 ±0.68 a
B	0.36 ±0.23 a	2.52 ±0.75 a	1.44 ±0.54 a
C	1.32 ±0.57 a	1.56 ±0.63 a	1.44 ±0.39 a
D	0.60 ±0.23 a	3.97 ±2.04 a	2.28 ±1.14 a
E	0.48 ±0.28 a	1.08 ±0.69 a	0.78 ±0.36 a

* Figures shown refer to totals up until 20th October '93 and each is the mean of 4 replicate 1 m^2 plots per block ± standard error (8 replicate for combined blocks). Figures in each column followed by the same letter are not significantly different according to Duncan's New Multiple-Range test at $p<0.05$. Block 1 and Block 2 were located at sites D & E respectively on Jungle-24.

Based on 208 viable seeds per plot.

§ Treatments:

A) Sown 20th April 1993, untreated.

B) Sown 30th June 1993, untreated.

C) Sown 30th June 1993 with seed dressed at 20g Raxil and 5g Mancozeb per kg.

D) Sown 30th June 1993 and millet culture of *E. purpurascens* isolate B60 applied to soil surface at 35 cm^3 per plot.

E) Sown 30th June 1993 and Osmocote (NPK 14:6.1:11.6) applied to soil surface at 40 g per plot.

4.13 Field observations

During the course of this study numerous observations were made of conditions and events in the field that may have had an influence on eucalypt germination and mortality. These observations varied in type and were often of a non-quantitative nature. Many of these observations are summarised here.

4.13.1 Weed growth

The most abundant weed species observed in burnt areas (Jungle-19 in 1992 and to a lesser extent on Jungle-24 in 1993) was *Rorippa dictyosperma, a* "fireweed" species (Figures 4.13.1 and 4.13.2). This weed was found only in areas where the soil surface had been burnt. Germination of *R dictyosperma* was observed to commence in June with most of the early plants confined to sheltered microsites. Growth of this weed was observed to be much more vigorous than growth of eucalypt seedlings. By mid-September plants had progressed to the rosette stage. In late November 1992, flowering had commenced and plants were measured to be about 40 cm wide with flowering stalks 50 cm high. A blanketing cover of this weed was reached over parts of Jungle-19. A white rust fungus (*Albugo*) was present on leaves and stems.

On the unburnt coupe, (Jungle-18) *R. dictyosperma* was absent and overall weed growth was sparse, being confined to forest undergrowth species. The most common species was *Bedfordia salicyna.*

undergrowth species. The most common species was *Bedfordia salicyna*.

Figure 4.13.1.1 Weed growth (*Rorippa dictyosperma*) on site A (plot A1), 25th November '92.

Figure 4.13.1.2 Weed growth on same site , 14th January '93.

Weed growth was not considered to be a significant cause of seedling mortality. In places where weeds were present on experimental plots eucalypt seedlings persisted amongst them and appeared to benefit from the shelter that the weeds provided.

4.13.2 Arthropod activity

Insects were not commonly seen on burnt coupes for several months following burning. Small numbers of Lygaeid bugs were observed in the vicinity of site A on early October '92 progressing to plague proportions across Jungle 19 in the vicinity of plants of *R. dictyosperma* from late October onward. Larvae of *Plutella xylostella* (cabbage moth) could also be found on this plant. On 27th October some evidence of insect grazing of seedlings was observed on one plot at site A. Ants were not observed to be present on burnt coupes.

Insects were not observed to be common at Site C on the unburnt coupe, however the frequent observation of spiders on this site from October '92 onward indicated that insects were probably present. A small number of ants were observed on this site.

In general, insect grazing was not observed to be significant cause of mortality of established seedlings. Some insect grazing of cotyledon stage seedlings was observed on Jungle-24 in 1993 but based on counts where a cause of death could be ascertained this appeared to account-for only a small proportion of seedling deaths (probably less than 2%). Seed feeding insects such as Lygaeids bugs may have caused a reduction in seed numbers (particularly late sown seed) prior to germination.

4.13.3 Soil moisture

There was considerable variation in germination rates between experimental plots. This variation appeared to mainly reflect differences in moisture availability and exposure to soil drying and was also seen across microsites within plots. On drier plots, seedlings were mainly restricted to microsites where surface water was likely to collect. This was particularly noticeable in the 1993 field trial where one block of

experimental plots (site D) was in an exposed, well drained location and the other block (site E) was in an area where the most of the soil surface remained moist due to seepage. Although no quantitative measurements were made of soil moisture levels the consistent association of lack of seedlings with drier areas would strongly indicate that the availability of moisture was a major limiting factor to seedling establishment.

4.13.4 Frost and frost-heave

The significance of frost as a cause of seedling mortality was not clear. Frost *per se* did not appear to be a major cause of seedling mortality as indicated by the persistence of healthy seedlings amongst those that had died.

Frost-heave (Figure 4.13.4.1) was observed to occur in some locations, particularly in moist sheltered sites with friable burnt mineral soil. Frost heave appeared to dislodge seedlings from the soil leaving them prone to subsequent desiccation.

4.13.5 Fungal pathogens

The presence or absence of fungal pathogens could not be easily inferred by field observations alone. Many seedlings looked like they may have been killed by fungi but because of their small size and the general absence of identifiable fungal structures a conclusive assessment could not be made without attempted isolation of the fungi responsible.

4.13.6 Time of seedling mortality

Peak seedling mortality was observed to occur at about one month after the peak in germination regardless of time of sowing.

Figure 4.13.4.1 Frost heave was observed to occur in some locations.

4.14 Comparison of field and glasshouse germination and mortality rates

Both field germination and field mortality rates were different to corresponding rates on the same soils in the glasshouse (Table 4.14.1). Observed field germination generally accounted for less than 30% of the viable seed sown and the majority of germinants had died within six months. In contrast, on soils from the same sites sown in the glasshouse the majority of seed was found to germinate and seedling mortality was consistently low (normally less than 3%).

Table 4.14.1 Comparison of germination and seedling mortality rates in the 1992 field experiment with those in the glasshouse on soil collected from the same sites at 8 and 133 days after burning.

	Field site/soil source		
	Site A (burnt)	Site B (burnt)	Site C (unburnt)
Seed sown in the field			
Mean germination percentage[a]	32.4	22.5	4.6
Mortality (percent of germinants)	87	80	51
Seed sown in glasshouse on soil collected at 8 days after burning			
Mean germination percentage[a]	77.8 (97.2 [b])	98.3	82.4
Mortality (percent of germinants)	6.6 (1.3 [b])	0.6	2.0
Seed sown in glasshouse on soil collected at 133 days after burning			
Mean germination percentage[a]	55.9	55.3	65.5
Mortality (percent of germinants)	2.2	3.7	2.8

[a]Based on 194 viable seed per gram.

[b]Excluding soil from site A2 in which all seedlings died due to high surface pH.

4.15 Evaluation of fungi associated with seedling mortality

Fungi were observed to be associated with over 95% of dead and dying seedlings collected from burnt field sites during August to October 1992 and July to August 1993. The types of fungi that were found are illustrated for a sample of 63 dead and dying seedlings collected on 5th October '92 . Readily recognisable fungal taxa associated with these seedlings are listed in Table 4.15.1. Other fungal species were present but could not be reliably enumerated due to an absence of features useful for identification. The presence of these were inferred by evaluation of isolates taken from seedlings. Isolates from two seedlings were*Trichoderma* sp(p). which only sporulated in culture. Most of the remaining isolates did not form spores in culture but could be readily sorted into two types based on colony characteristics.

these groups appeared to be *Rhizoctonia*-like but were not further identified. A small number of uncommon fungal types were not identified.

A greater number of fungal types were found to be present on individual dead seedlings than on individual dying seedlings. This trend was also seen when comparing dying seedlings and healthy seedlings. A comparison of 16 apparently healthy and 24 dying seedlings collected from Jungle-24 on 30/7/93 and 25/8/93 revealed that the healthy seedlings were generally free of fungi (Table 4.15.2). Again the most common fungi present on the dying seedlings were the same as indicated in Table 4.15.1 although percentage of seedlings affected by individual fungal taxa (with the possible exception of Pythiaceae) were lower .

Of the fungi isolated from seedlings a species of *Phoma* was consistently the most common one isolated[1] (Figure 4.15.2.1).

The identity of pythiaceous fungi was not resolved as attempts at stimulating zoosporangia and zoospore formation were not successful. Some isolates were however observed to produce branched antheridia and would therefore be identified as *Pythium*.

Fungi isolated from seedlings that had died on field soil in the glasshouse (in order of reduced frequency of isolation) were species of *Rhizoctonia*, Pythiaceae, *Cylindrocarpon*, *Alternaria* and *Epicoccum*. Neither *Phoma* nor *Cladosporium* were recorded amongst 35 isolates from seedlings that had died in the glasshouse. None of 13 seedling isolates of *Cylindrocarpon* (8 from the glasshouse) was obtained from burnt sites or soil taken from burnt areas. Figure 4.15.2.2 shows some of the fungi commonly associated with seedling mortality.

[1] One *Phoma* isolate (B42-2) has been lodged with the International Mycological Institute and has been identified by Dr E. Punithalingam as resembling *P. sydowii* and *P. sorghina*. A preserved specimen has been placed in the IMI dried reference collection (IMI number 361246).

Table 4.15.1. Fungi found growing on dead and dying seedlings collected on 5/10/'92 from burnt sites on Jungle-19.

Fungal taxa	Number of seedlings affected.#	Percentage of total number of seedlings examined.#
Phoma	47	74.6%
Cladosporium	40	63.5%
Alternaria	30	47.6%
Epicoccum	9	14.3%
Ulocladium	2	3.2%
Mucoraceae	2	3.2%
Botrytis	1	1.6%
Pythiaceae	13	20.6%

A total of 63 seedlings were examined.

Table 4.15.2 Fungi found growing on healthy and dying seedlings collected on 30/7/93 and 25/8/93 from Jungle-24.

Fungal taxa	Number of dying seedlings affected.#	Number of healthy seedlings affected.#
Phoma	10 (42%)	0 (0%)
Cladosporium	8 (33%)	0 (0%)
Alternaria	4 (17%)	0 (0%)
Epicoccum	0 (0%)	2 (12.5%)
Pythiaceae	6 (25%)	0 (0%)
Rhizoctonia	1 (4%)	0 (0%)
Unknown	5 (21%)	0 (0%)
No fungi present	0 (0%)	14 (87.5%)

A total of 24 dying and 16 healthy seedlings were examined. Percentages of total number of seedlings in each group are given in brackets.

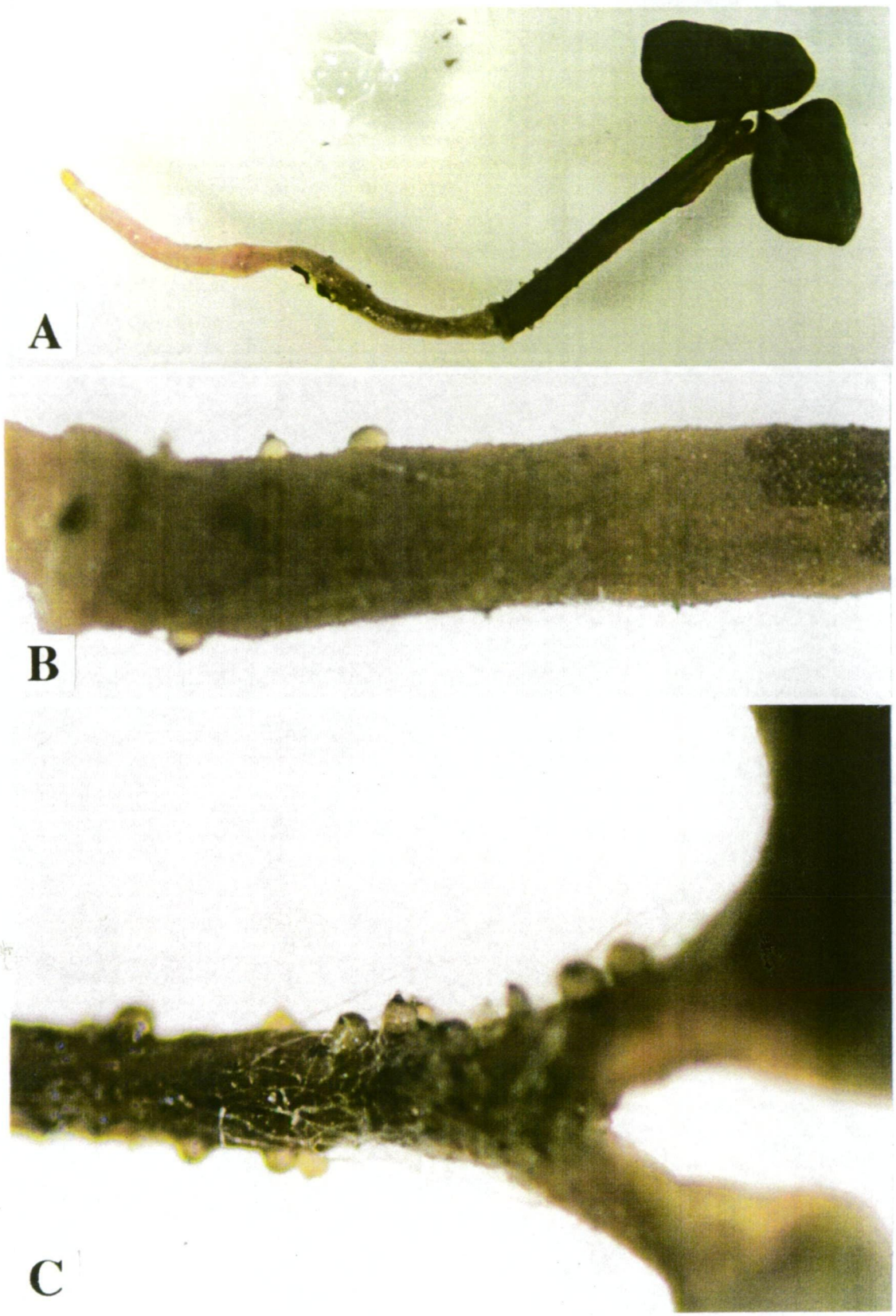


Figure 4.15.2.1 **A**) Dying *E. regnans* seedling with brown discolouration of root and stem surface (scale x 6). **B**) Close-up of same seedling (x 30) showing pycnidia of *Phoma*. **C**) Pycnidia and hyphae of *Phoma* on the surface of another seedling (x 30).

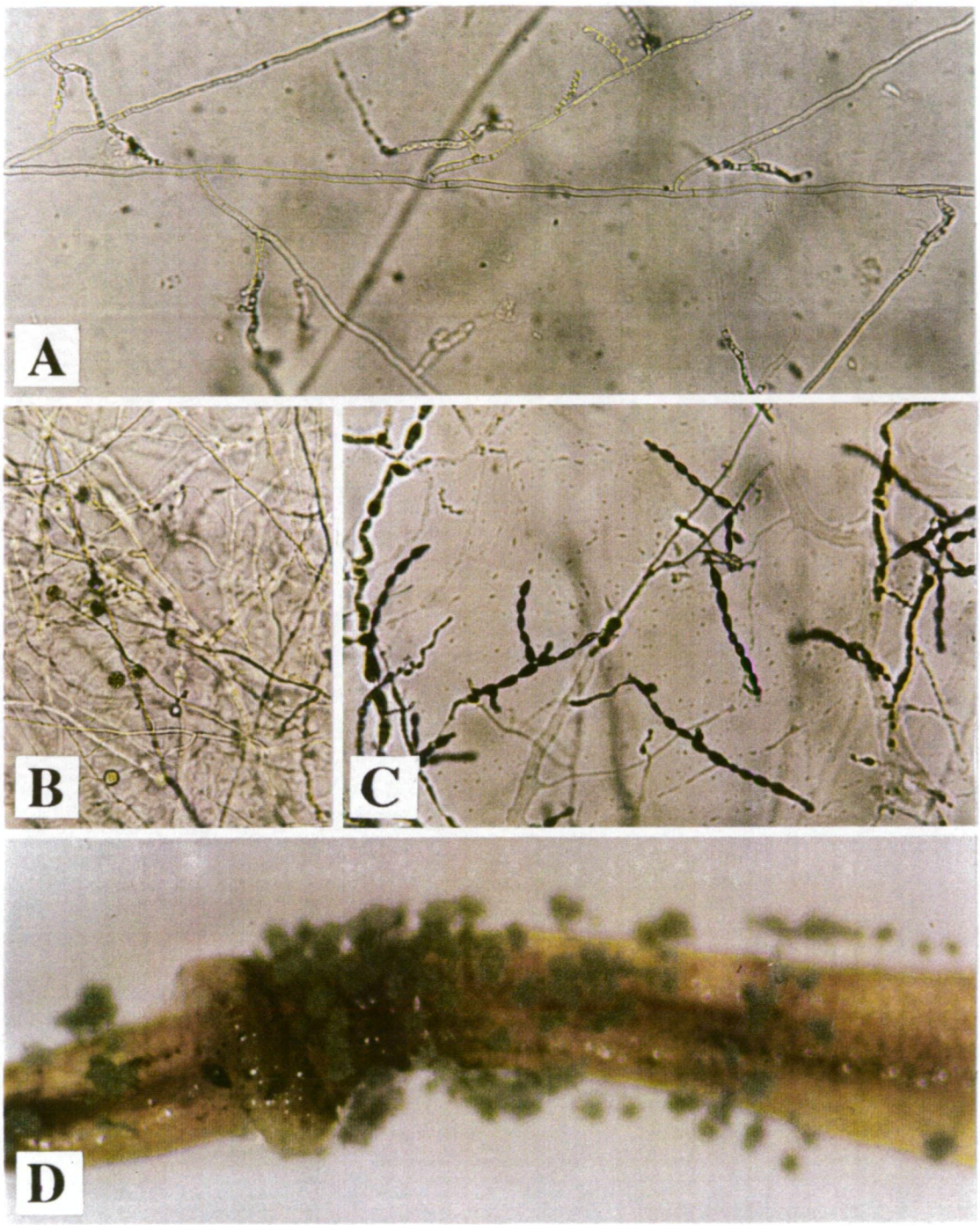


Figure 4.15.2.2 Some fungi commonly associated with seedling mortality. **A**) *Rhizoctonia* (showing hyphal branching pattern, x ~120). **B**) Pythiaceae (with aseptate hyphae and spherical sporangia, x ~90). **C**) *Alternaria* (showing catenulate conidia, x ~60). **D**) Conidia and conidophores of *Cladosporium* on dead *E. regnans* seedling (x 30).

4.16 Electrophoretic comparison of pectic enzymes produced by *Rhizoctonia* isolates and determination of number of nuclei per cell

Fourteen isolates from dead seedlings grown on field soil in the glasshouse and identified as *R. solani* were compared in this study and were found to be multinucleate and included three distinct zymogram groups. Ten isolates from *E. regnans* seedlings and four from seedlings of *Rorippa dictyosperma*, a cruciferous weed species were examined. All of the isolates from *R. dictyosperma* and two from *E. regnans* (Figure 4.16.1) conformed to zymogram group ZG5 (= anastomosis group AG2-1), a group which is a common cause of seed rot and damping-off of crucifers. Seven other isolates from *E. regnans* seedlings growing on one soil sample had an unfamiliar zymogram pattern. However a match to anastomosis group AG3 has been independently determined*. This group has been previously recorded as

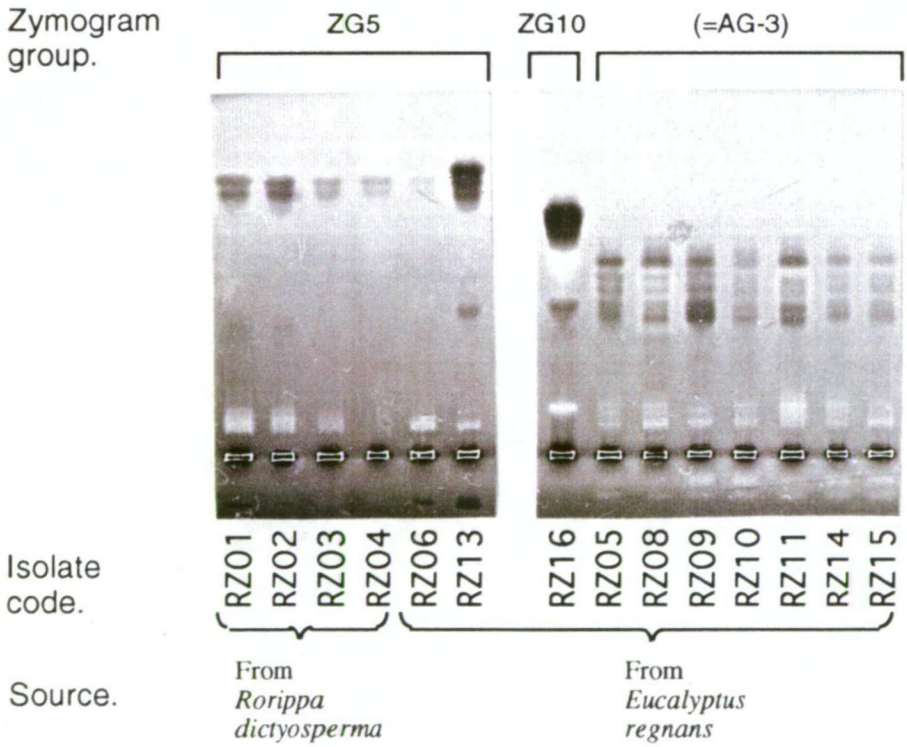


Figure 4.16.1 Pectic zymogram of 14 *Rhizoctonia* isolates.

* Representative isolates (RZ08 and RZ11) have been identified by G.C. MacNish (Department of Agriculture, Western Australia) as matching AG3 (=ZG7) on the basis of zymogram pattern and anastomosis reaction with test strains.

a cause of root rot and scurf of potatoes in South Australia (Neate *et al.*, 1988; Cruickshank, 1990). Representatives of both groups were subsequently found to be pathogenic to *E. regnans* seedlings in *in vitro* studies. The zymogram of isolate RZ16 matched group ZG10 (=AG 2-2)[#] and was later observed to be non-pathogenic. Additional study would be needed to determine the identity of other *Rhizoctonia*-like isolates obtained from the field.

4.17 Pathogenicity of isolated fungi

Representative isolates of most fungal types from the field and glasshouse with the exception of *Cylindrocarpon* and Pythiaceae were evaluated. The majority of isolates tested were observed to have no apparent detrimental effect on seedlings (Table 4.17.1). The *Botrytis* isolate had the most obvious effect with all seedlings being killed at or shortly after germination. One *R. solani* isolate (RZ08) also killed all six germinants while three other *R. solani* isolates were associated with lower seedling mortality. All three *Phoma* isolates tested appeared to cause root stunting of some seedlings and all roots had a brown discolouration with poor root hair growth. Pycnidia were produced on some seedlings and ungerminated seeds. All *Phoma* isolates killed some seedlings. Isolates of *Cladosporium, Alternaria, Epicoccum, Trichoderma* and representative isolates of two unidentified fungal types were not observed to be pathogenic. Germinant numbers for uninoculated controls varied between replicates, indicating possible variability in other treatments.

[#] Identity of isolate RZ16 has been independently confirmed by G.C. MacNish as matching AG2-2IIIB on the basis of zymogram pattern and anastomosis reaction.

Table 4.17.1 Effect of fungal isolates on *E. regnans* germination and seedling mortality *in vitro*

Fungal Isolate[a]	Number of germinants[b]	Number of dead seedlings[b]
Botrytis sp.	7	7
Rhizoctonia solani (RZ01)	8	5
R. solani, (RZ08)	6	6
R. solani, (RZ13)	7	2
R. solani, (RZ16)	10	1
Phoma sp., (B11)	10	1
Phoma sp., (14/9-1)	3	1
Phoma sp., (B42-2)	11	1
Epicoccum purpurascens, (B60)	9	0
Trichoderma sp.,(B14)	10	0
Cladosporium sp., (B11-1)	11	0
Cladosporium sp., (B20)	12	0
Alternaria sp., (14/9-12)	9	0
Alternaria sp., (B37)	11	0
Unidentified type 1, (B8)	6	0
Unidentified type 1, (B50)	7	0
Unidentified type 2, (B15)	6	0
Unidentified type 2, (14/9-5)	11	0
Uninoculated control	10	0
Uninoculated control	3	0
Uninoculated control	3	0
Uninoculated control	7	0

[a] Fungal isolate codes are shown in brackets

[b] Number of seedlings out of 20 seeds per isolate after 40 days at 10°C under constant fluorescent light.

4.18 Glasshouse testing of pathogenicity of selected isolates in field soil and investigation of the effect of steam-treatment of field soil

Final results for this experiment are shown in Table 4.18.1 ranked in order of number of seedlings. Standard errors were relatively constant across treatments so no transformation was applied before

analysis. The following observations can be made at the noted level of significance. Highest seedling counts were obtained for steam-treated field soil. On steamed soil to which 5% non-steamed soil had been added, seedling counts were significantly lower and were not different to those on non-steamed soil. With the addition of millet cultures of fungal isolates, seedling numbers were reduced compared to counts on steamed soil although this difference was less or not apparent when compared to numbers in non-steamed soil. *R. solani* isolate RZ08 was associated with a significantly lower seedling survival than any other isolate tested. The *Phoma* and *Cylindrocarpon* isolates were associated with higher seedling mortality than *R. solani* isolate RZ16. The addition of sterilised *Alternaria* millet culture to soil was associated with significantly lower seedling counts than most other treatments with the exception of those that had received *R. solani* isolate RZ08 or *Phoma*. Addition of sterilsied *Alternaria* millet culture may have resulted in lower seedling counts due to incomplete sterilisation of this culture, the production of toxins or subsequent colonisation by inhibitory biota.

Table 4.18.1 Mean survival of seedlings in glasshouse pot trial, comparing the effect of steamed and non-steamed soil and fungal inoculants*

Treatment details#	Mean number of seedlings at 30 days ±s.e.§
D) Steamed soil + *Rhizoctonia* (RZ08)	1.8 ±0.65 a
I) Steamed soil + sterilised *Alternaria* millet culture	4.5 ±0.89 ab
F) Steamed soil + *Phoma* (B11)	7.0 ±1.00 bc
G) Steamed soil + *Cylindrocarpon* (MCA005)	7.5 ±1.26 c
C) Steamed soil + 5% non-steamed soil	9.3 ±0.95 cd
H) Steamed soil + *Alternaria* (14/9-12)	9.8 ±0.91 cd
A) Non-steamed soil	10.0 ±0.93 cd
E) Steamed soil + *Rhizoctonia* (RZ16)	10.8 ±1.22 d
B) Steamed soil	13.7 ±0.76 e

* 6 replicate pots per treatment each sown with 0.25g of *E. regnans* seed.

\# Treatment details as outlined in Methods 3.5.8. Fungal isolate codes are shown in brackets.

§ Figures followed by the same letter are not significantly different according to Duncan's New Multiple Range test at $p<0.05$.

4.19 Fungicide studies

4.19.1 Fungicide phytotoxicity tests

Results for the evaluation of Raxil and Mancozeb are shown in table 4.19.1.1. All fungicide combinations tested appeared to be associated with some reduction in seedling establishment although only one of these (Raxil at 1.00 g a.i. [active ingredient] with Mancozeb at 2.0 g a.i. per kg of seed) was significantly different from the untreated control. Raxil also resulted in a delay in germination of several days (results not shown). Based on these results a combination of Raxil at 0.50 g a.i and Mancozeb at 4.0 g a.i. per kilogram of seed was chosen as a safe combination for field testing.

There was no significant difference (at $p<0.05$) in seedling establishment between any of the Sumisclex concentrations tested (Table 4.19.1.2).

Table 4.19.1.1 Establishment of *E. regnans* seedlings in glasshouse trial after seed dressing with various concentrations of Raxil and/or Mancozeb.

Raxil concentration, (g of active ingredient kg^{-1} of seed)#	Mancozeb concentration, (g of active ingredient kg^{-1} of seed)#	Mean number of seedlings at 45 days ±s.e.*
0	0	29.5 ±1.0 a
0	2.0	21.0 ±4.7 ab
0	4.0	21.5 ±4.3 ab
0	8.0	20.25 ±5.6 ab
0.25	0	19.0 ±3.2 ab
0.50	0	26.25 ±4.3 ab
1.00	0	17.25 ±3.8 ab
0.50	2.0	19.25 ±1.8 ab
1.00	2.0	16.5 ±2.4 b

Active ingredients: Raxil, 25 g/kg tebuconazole; Mancozeb 800 g/kg Zn&Mn bis-dithiocarbamate.

* Four replicate pots of steam sterilised field soil sown with 0.2 g of *E. regnans* seed per treatment. Figures followed by the same letter are not significantly different according to Duncan's New Multiple-Range test at $p<0.05$.

Table 4.19.1.2 Establishment of *E. regnans* seedlings in glasshouse trial after seed dressing with various concentrations of Sumisclex 500.

Sumisclex concentration, (g active ingredient kg^{-1} of seed)#	Mean number of seedlings at 60 days ±s.e.*
0	20.0 ± 2.3 a
2	24.4 ± 4.9 a
5	21.4 ± 2.6 a
10	22.4 ± 2.8 a
20	13.6 ± 3.7 a

Sumisclex 500 contained 500 g/kg procymidone as active ingredient.

* Five replicate pots of potting mix sown with 0.2 g of *E. regnans* seed per treatment. Figures followed by the same letter are not significantly different according to Duncan's New Multiple-Range test at $p<0.05$.

4.19.2 Effects of Raxil and Sumisclex on fungi *in vitro*

Very little inhibition of fungal growth by Sumisclex was noted at the concentration tested (Table 4.19.2.1), although Raxil at the same concentration of a.i. resulted in measurable inhibition of five of the 11 isolates. Representatives of the three most commonly isolated genera from field-killed *E. regnans* seedlings (*Phoma, Cladosporium* and *Alternaria*) were not measurably inhibited by these fungicides. Two out of the three *Rhizoctonia* isolates tested were inhibited by Raxil at this concentration.

Figures 4.19.2.1 and 4.19.2.2 show fungicide-containing plates inoculated with *Botrytis, Sclerotinia, Trichoderma* and *Rhizoctonia* .This unreplicated experiment was intended as a guide to determine the range of fungal species that were likely to be affected by the afore-mentioned fungicides. In order to obtain a more reliable measure of inhibition a range of concentrations would need to be tested against target species.

Table 4.19.2.1 Effects of Raxil and Sumisclex on growth of fungi *in vitro*.

	Inhibition zone around 8mm disc containing fungicide at initial concentration of 10ppm (w/v) active ingredient, (mm).#	
Fungal Isolate*	Raxil	Sumisclex
Sclerotinia sclerotiorum	6	0
Botrytis cinerea	18	8
Rhizoctonia solani, (RZ08)	14	0
R. solani, (RZ13)	0	0
R. solani, (RZ16)	12	0
Phoma sp., (B42-2)	0	0
Cylindrocarpon sp., (MCA005)	0	0
Epicoccum purpurascens, (B60)	0	0
Trichoderma sp., (B14)	8	1
Cladosporium sp., (B30)	0	0
Alternaria sp., (14/9-12)	0	0

Active ingredients: Raxil, 25 g/kg tebuconazole; Sumisclex, 500 g/kg procymidone. Inhibition zone was measured from edge of disc at 28 days after inoculation.

* Fungal isolate codes are shown in brackets.

Figure 4.19.2.1 Inhibition of *Botrytis*, *Sclerotinia sclerotiorum* and *Trichoderma* isolates at 13 days by Raxil (top) and Sumisclex (bottom) when fungicide was applied in 8mm disks at initial concentration of 10ppm (w/v) active ingredient.

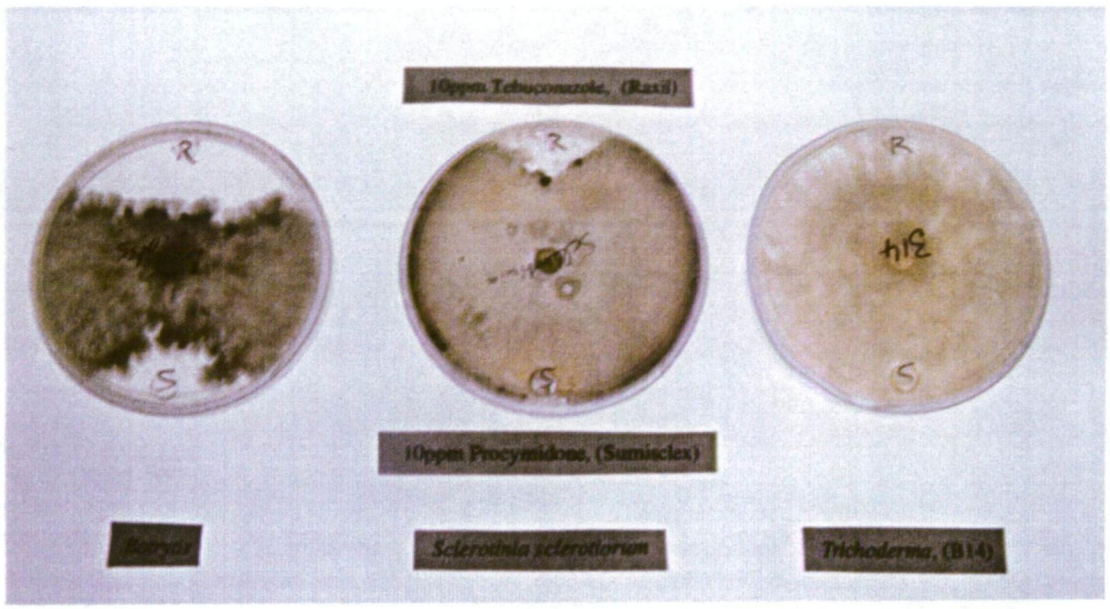
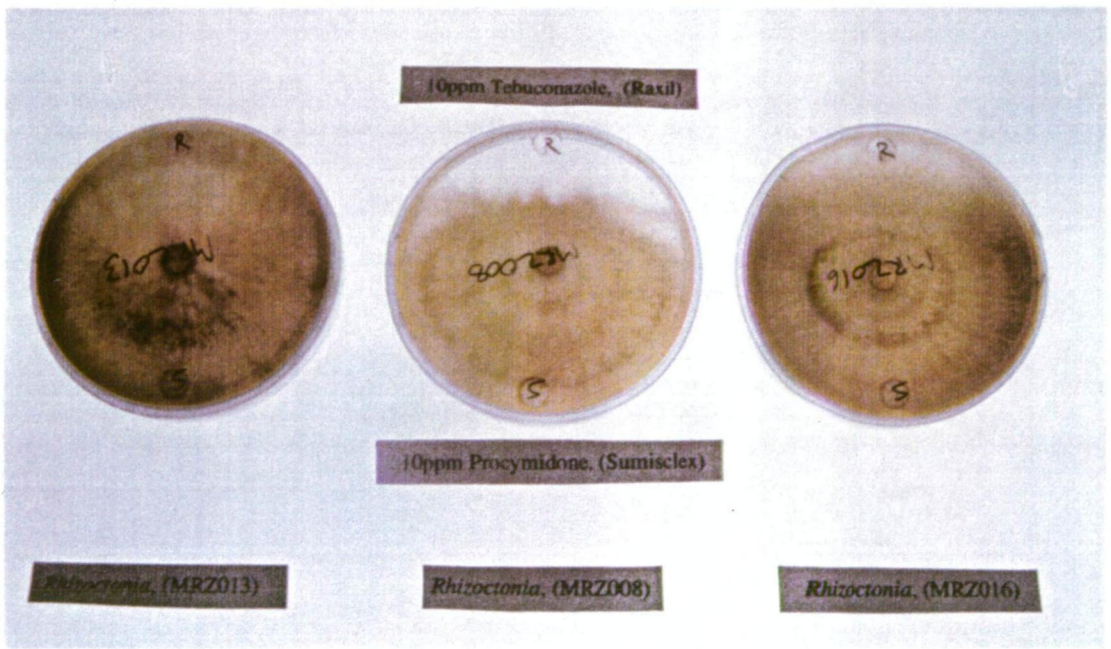


Figure 4.19.2.2 Inhibition of three *Rhizoctonia* isolates at 13 days by Raxil (top) and Sumisclex (bottom) when applied in 8mm disks at initial concentration of 10ppm (w/v) active ingredient.



4.20 Test of inhibition of fungal growth by bacterial isolates *in vitro*

Three out of 16 isolates produced a zone of inhibition on TSA (Figure 4.20.1,Table 4.20.1) while inhibition was less apparent on PDA (Figure 4.20.2,Table 4.20.1). Two of these 3 isolates (IS2 and IS6), showed reducing inhibition with inclusion of increasing concentrations of $FeCl_3$ to King's B agar (Table 4.20.2). Addition of $FeCl_3$ was also associated with increased growth of *R. solani* isolate RZ08.

Table 4.20.1 Inhibition of growth of *R. solani* isolate RZ08 by bacterial isolates after 5 days on TSA and PDA media.

	Zones of inhibition:	
Isolate	on TSA, (mm)	on PDA, (present)
IS2	5	+
IS4	5	+
IS6	2	+

Table 4.20.2 Effect of varying $FeCl_3$ concentration on inhibition of *R. solani* isolate RZ08 by bacterial antagonists after 5 days on King's B agar.[a]

$FeCl_3$ Concentration ,(μM)	IS2	IS4	IS6
0	+++	++	+
0.25	++	++	+
1.25	++	++	-
2.5	+	++	-
5.0	+	++	-

[a] Two replicates per determination.

Level of inhibition: +++, greatest; ++, intermediate; +, least; -, none

Figure 4.20.1 Inhibition of growth of *R. solani* isolate RZ08 (=MRZ008) by bacterial isolates after 5 days on TSA medium .

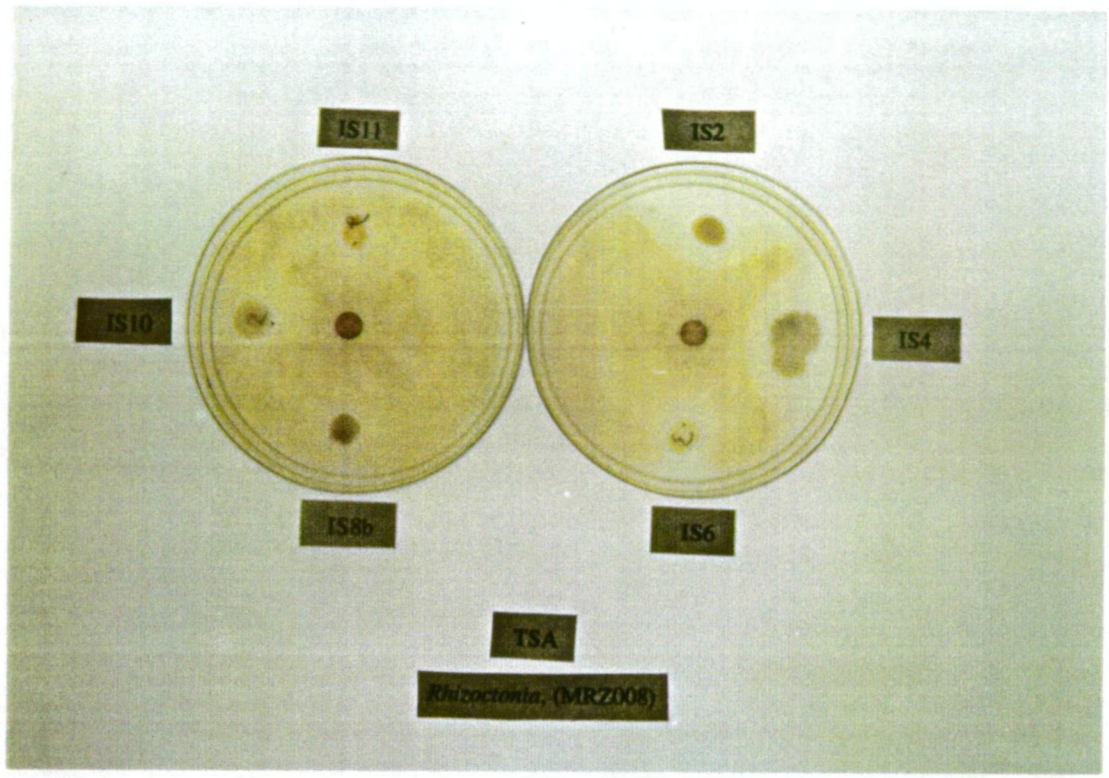
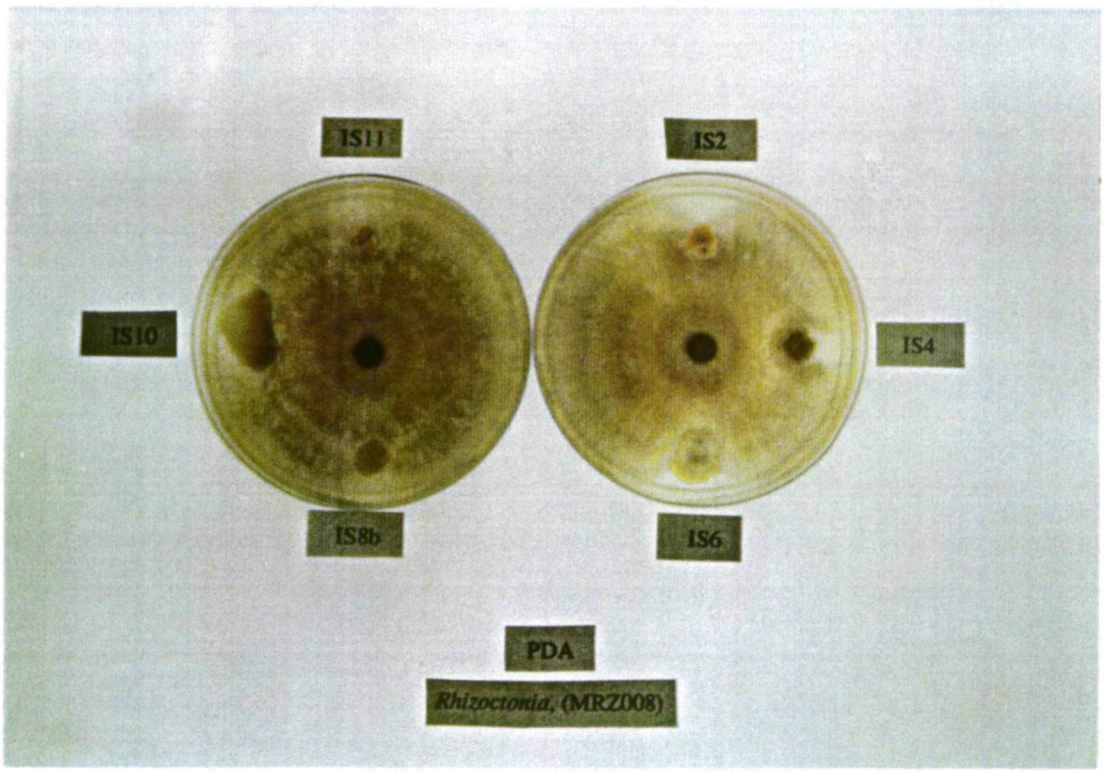


Figure 4.20.2 Inhibition of growth of *R. solani* isolate RZ08 (=MRZ008)by bacterial isolates after 5 days on PDA medium .



4.21 Identification of bacterial antagonists

Based on characteristics which were determined (Table 4.21.1.), bacterial antagonists were identified as *Pseudomonas fluorescens* (IS2 & IS6) and *Bacillus circulans* (IS4).

Table 4.21.1 Characteristics of bacterial isolates

Characteristics	Isolate:		
	IS2	IS4	IS6
Gram stain	-	+	-
Cell morphology	rods	rods	rods
Motility	+	+	+
Spore position	n.a.	terminal	n.a.
Spore shape	n.a.	oval	n.a.
Swollen sporangium	n.a.	+	n.a.
Cell diameter >1.0µm	-	-	-
O/F reaction	O	F	O
Oxidase	+	+	+
Catalase	+	+	+
Fluorescence	+	-	+
Citrate utilisation	+	-	+
Voges-Proskauer test	-	-	-
Methyl Red test (pH<6 in V-P broth)	-	+	-
Colony form(on TSA):			
Colour	cream	white	cream
Opacity	transparent	opaque	translucent
Form	circular	circular	circular
Elevation	convex	raised	umbonate
Margin	entire	undulate	undulate
Surface	glistening	rough	glistening
Hydrolysis of:			
Starch	-	+	-
Casein	+	+	+
Gelatin	+	+	+
Tyrosine degradation	+	-	+
Arginine dihydrolase	+	-	+
PHB accumulation	-	n.d.	-
Levan production	-	-	-
Indole production	-	-	-
Nitrate reduction	+	-	+
Hydrolysis of Tween 80	+	n.d.	+
Acid from glucose	-	+	-
Gas from glucose	-	-	-
Growth at:			
4°C	+	n.d.	+
25°C	+	+	+
40°C	-	-	-
50°C	-	-	-

n.d. = not determined

n.a. = not applicable

4.22 Test of inhibition of fungal growth by *E. purpurascens* isolates *in vitro*

Screening of five *E. purpurascens* isolates revealed variable level of antagonism against isolates of other selected fungal species (Table.4.22.1, Figures 4.22.1 to 4.22.3). Isolate B60 (Figure 4.22.4) showed greatest antagonism and was used in further studies.

Figure 4.22.1 Variable antagonism of *Sclerotinia sclerotiorum* by five *E. purpurascens* isolates.

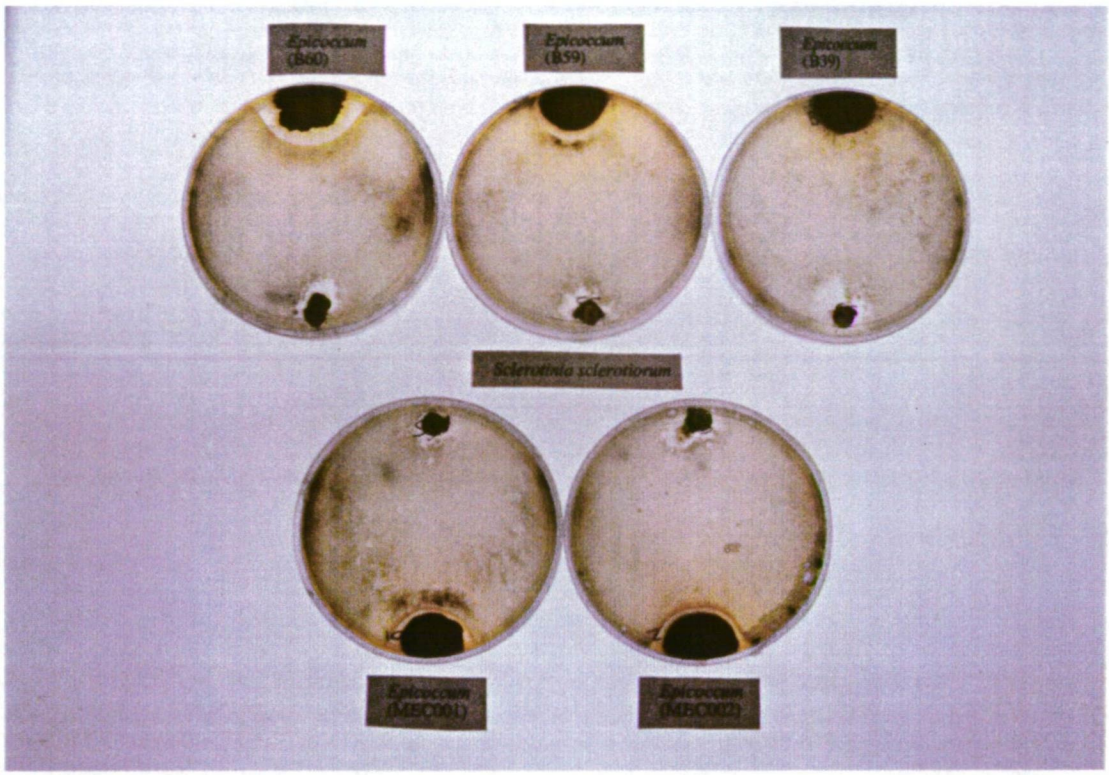


Figure 4.22.2 Variable antagonism of a *Rhizoctonia* isolate by five *E. purpurascens* isolates.

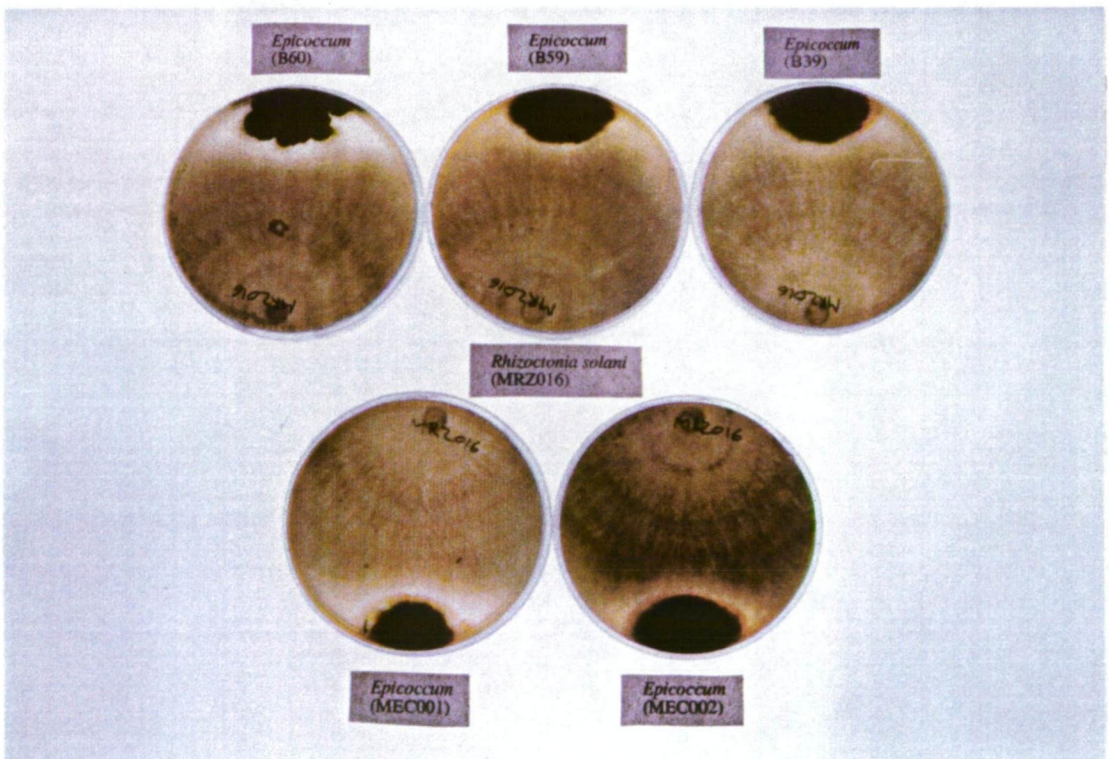


Figure 4.22.3 Variable antagonism of an *Alternaria* isolate by five *E. purpurascens* isolates.

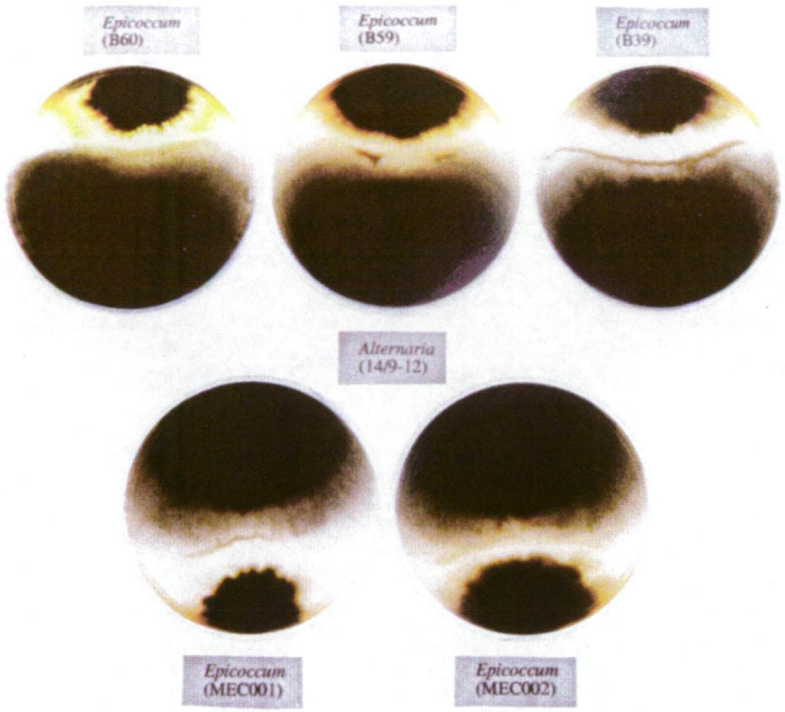


Figure 4.22.4 Comparative antagonism of five test fungi by *E. purpurascens* isolate B60 (antagonism of the slower growing *Phoma* and *Cylindrocarpon* isolates is not apparent at this stage).

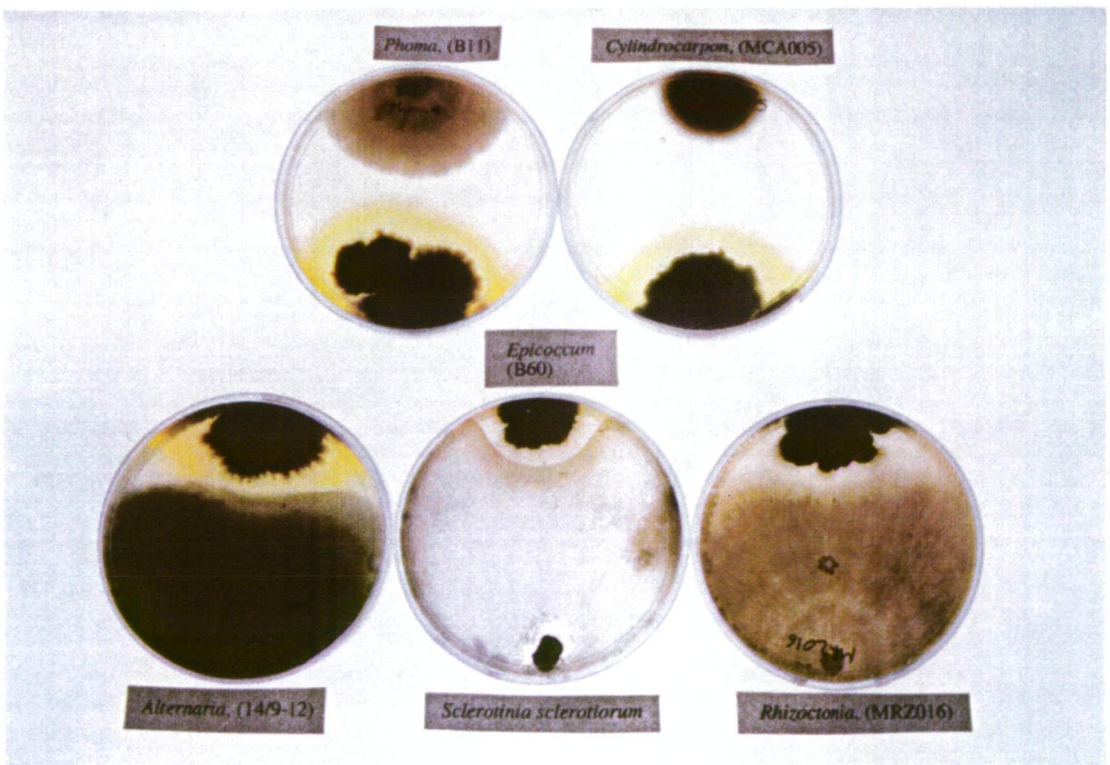


Table 4.22.1 Inhibition of fungal growth by five *E. purpurascens* isolates*

	Zones of inhibition, (mm)				
	EC01	EC02	B39	B59	B60
Phoma sp.,(B11)#	1	1	1	1	7
Cylindrocarpon sp., (MCA005)	0	0	0	0	3
Rhizoctonia solani, (MRZ016)	3	0	2	0	5
Alternaria sp., (14/9-12)	5	1	9	0	4
Sclerotinia sclerotiorum	2	2	0	0	5

* Isolates and pathogens were inoculated at opposite sides of PDA plates and incubated for 2 weeks at 25°C

\# Test fungus isolate codes are shown in brackets

4.23 Microbial antagonism to *R. solani* on *E. regnans* seedlings *in vitro*

Assays of seedling growth with and without antagonists showed none to be inhibitory to the plants (Table 4.23.1a). Two of the isolates (*B. circulans* & *E. purpurascens*) were associated with increased seed germination by 87% ($P<0.001$) and 55% ($P=0.023$) respectively relative to the control.

Germination rates of seedlings were similar in the presence and absence of *R. solani* (Table 4.23.1 a & b). All antagonists except *P. fluorescens* isolate (2) showed some degree of protection *in vitro* against seedling mortality caused by *R. solani,* particularly *E. purpurascens*, which gave total protection after 30 days, in comparison with 22% survival of control seedlings.

Table 4.23.1 Effect of microbial antagonists on germination and mortality of *E. regnans* seedlings *in vitro*: a) without *R. solani* and b) with *R. solani*.

a)Without *R. solani* §		Antagonist*			
	Control	P. fluor. (1)	P. fluor. (2)	B. circulans	E. purp.
Number of seeds used in test	102	104	100	103	102
Number of germinants at 30 days	31	34	24	58	48
χ^2 comparison to control, (df=1)		0.126	1.041	14.01	5.97
P (Monte Carlo)		0.769	0.350	<0.001	0.023
Number of dead germinants at 30 days	0	0	0	0	0

b)With *R. solani* §		Antagonist*			
	Control	P. fluor(1)	P. fluor(2)	B. circulans	E. purp.
Number of seeds used in test	104	104	100	104	103
Number of germinants at 30 days	36	47	23	63	52
χ^2 comparison to control, (df=1)		2.426	3.346	14.052	5.333
P (Monte Carlo)		0.163	0.094	<0.001	0.024
Number of dead germinants at 30 days#	28 (78%)	24 (51%)	13 (57%)	35 (56%)	0 (0%)
χ^2 comparison to control, (df=1)		6.217	2.991	4.889	59.32
P (Monte Carlo)		0.021	0.154	0.032	<0.001

§ *R. solani* isolate RZ08.

* Antagonists: P. fluor. (1), (=IS2) *Pseudomonas fluorescens* isolate 1;
P. fluor. (2), (=IS6) *P. fluorescens* isolate 2 ;
B. circulans, (=IS4) *Bacillus circulans*;
E. purp., (B60) *Epicoccum purpurascens* .

Percentages are shown in brackets.

4.24 Glasshouse testing of antagonists in field soil

A glasshouse trial of the protective effectiveness of the antagonists in steam sterilised field soil showed no significant effect of the bacterial isolates (Table 4.24.1). However inoculation with *E. purpurascens* was associated with increased seedling numbers both in the presence (6.8 compared to 1.2, P=0.066) and absence (31.0 compared to 21.7, P=0.027) of *R. solani* relative to controls. All treatments with *R. solani* had significantly less seedlings per pot than those without (P=<0.01 for all comparisons). An excessive amount of *R. solani* inoculum applied in the glasshouse trial meant that seedling mortality was too severe for comparisons to be made between treatments with and without this pathogen.

Table 4.24.1 Effect of microbial antagonists on *E. regnans* seedling establishment and pathogenicity of *R. solani* to *E. regnans* seedlings in field soil in glasshouse pot trial: mean seedling numbers per pot*

	Control	P. fluor. (1)	P. fluor. (2)	B. circulans	E. purp.
a)Treatments without *R. solani* §	21.7 (±2.87)	21.2 (±1.92)	28.0 (±4.57)	23.8 (±1.94)	31.0 (±3.41)
P(T<=t) for t-tests (paired comparisons to control)#		0.889	0.275	0.549	0.066
b)Treatments with *R. solani* §	1.2 (±0.79)	2.8 (±1.70)	3.3 (±1.02)	1.7 (±0.55)	6.8 (±1.78)
P(T<=t) for t-tests (paired comparisons to control with *R. solani*)#		0.404	0.128	0.620	0.027

* Six replicate pots were used per determination, assessed at 42 days. Standard errors are given in brackets.

Antagonists: P. fluor. (1), (=IS2) *Pseudomonas fluorescens* isolate 1;
P. fluor. (2), (=IS6) *P. fluorescens* isolate 2 ;
B. circulans, (=IS4) *Bacillus circulans*;
E. purp., (B60) *Epicoccum purpurascens* .

§ *R. solani* isolate RZ08

t-Test (two-sample assuming unequal variances)

5 DISCUSSION

5.1 Soil sterilisation by slash-burning and subsequent microbial recolonisation

Estimates of fungal and bacterial numbers in soil at 8 days after burning (Figure 4.1.1; Appendix 3) showed that partial sterilisation had occurred at the time of burning of surface material. The dilution plating technique used has inherent errors in that not all organisms will grow on the media used and fungal counts reflect numbers of propagules rather than actively growing hyphae (Theodorou and Bowen, 1982; Warcup, 1960). However these experimental results indicate that the sterilising effect of the fire was mainly restricted to the top 2cm of soil and that below 5cm there was little reduction in the microbiota. The apparent reduction in numbers of fungal propagules to depths of between 2 and 5 cm is in accordance with previous surveys of this type such as that of Theodorou and Bowen (1982). Variation in microbial counts is most likely to be a reflection of variation in intensity of heating. Previous researchers (Theodorou and Bowen, 1982; Renbuss *et al.*, 1973) have found that bacteria and in particular pseudomonads (Theodorou and Bowen, 1982) were the first to recolonise soil after burning. High bacterial counts for a few of the surface samples from the burnt area could have been due to bacterial recolonisation of favourable sites in the 8 days prior to sampling.

Presence of ascomycete growth on surface soil within a few weeks of burning is in accordance with findings of Warcup (1981,1990) and other researchers and is an example of the rapid microbial recolonisation of soil after partial sterilisation. It is possible that some of these fungi could become mycorrhizal, as was shown by Warcup (1990, 1991). The apparent disappearance of most of this fungal growth within about a month indicates a microbial succession attributable to competing microorganisms and to the depletion of nutrients.

5.2 Elevation in soil pH associated with burning and its influence on germination and survival of *E. regnans* seedlings

Elevation of soil surface pH was observed to occur as a consequence of slash-burning (Figures 4.3.1 & 4.6.1). This pH elevation was variable across burnt areas (Figure 4.3.2) and reflected intensity of burning.

Results of the *in vitro* studies (Figure 4.7.1; Table 4.8.1) show that high pH conditions can interfere with the germination of *E. regnans* seed at temperatures conducive to germination and this effect was accentuated with increasing pH. At temperatures sufficiently low to delay seed germination the influence of a transient elevated pH was reduced. There was no clear pH above which the effect was detrimental but it appeared to be in the pH range 8.4 to 9.0.

These findings indicate that if *E. regnans* seed is sown onto an alkaline ash-bed then a significant reduction in germination can be expected due to pH alone. Field and glasshouse results (Figures 4.4.1 & 4.4.2; Appendix 4) showed that some of the surface soil in the field was sufficiently alkaline to have some detrimental effect on seedling establishment, especially where seed was sown immediately after burning and if only light rain followed. Although observed effects could have been due to alkalinity alone, other soil and ash properties could have been partly responsible. Similar inhibition of plant growth by the top-most 5cm of soil from an ash-bed (produced by burning a log pile on the soil surface) was found by Renbuss *et al.* (1973), and this was also associated with an increase in pH.

Degree of soil alkalinity will depend on the amount of ash deposition (Table 4.5.1) which in turn depends on the amount and distribution of fuel and intensity of the fire (eg. Tomkins *et al.*, 1991). The persistence of surface ash and associated changes in soil chemistry will also depend on the pattern of rainfall following the fire. Ellis and Graley (1983) observed that after burning of a similar site, overall soil pH peaked at about two months followed by a gradual decline. The rise in pH was attributed to a gradual solubilisation of calcium oxides and carbonates that had been deposited in the ash. A similar pH trend after rain was noted by Tomkins *et al.* (1991).

Although pH reached potentially toxic levels in up to 10% of the presently described area, the accompanying shift in soil pH from acidic to near neutrality over the majority of the sites would be beneficial for plant growth. Such a pH shift may contribute to the 'ash-bed effect', in which marked stimulation of seedling growth can occur following a hot fire.

5.3 Suppression of seedling growth in field soil and response to fire, fertiliser addition and soil sterilisation

Suppression of *E. regnans* seedling growth in field soil in the glasshouse was apparent in comparison to seedlings grown in potting mix (Figure 4.9.1; Table 4.9.1). Burning was associated with some improvement in seedling growth rates although even this was much less than that seen on potting mix. A number of reasons for these differences were possible, including differences in soil structure, nutrient availability and microbiota. Of note was some improvement in the crumb structure of the clay field soil as a result of burning. Improved soil structure was a likely important factor allowing greater growth in the potting mix, with this much more open textured medium allowing improved root penetration. Also fertiliser (Osmocote) had been applied to the potting mix at the time of preparation while no fertiliser had been applied to field soil.

Improvement in seedling growth with the combined addition of N and P fertilisers to soil from the unburnt area (Figure 4.10.1; Table 4.10.1) showed that availability of nitrogen or nitrogen in combination with phosphorus was a limiting factor for growth of seedlings on this soil. In contrast, addition of N and P fertilisers to soil from the burnt area (Figure 4.10.2; Table 4.10.2) resulted in little if any improvement in seedling growth, indicating that these nutrients were already adequately available in this soil. Such an increase in nitrogen and phosphorus availability following burning has been observed by other workers. (Chandler *et al.*, 1983, Humphreys and Craig, 1981). Lack of response to fertiliser application in the 1993 field trial (Table 4.12.3.1) may have also been partly due to the enhanced availability of nutrients in the soil following burning.

In their study of factors affecting the growth of *E. delegatensis* in inhibitory soil, Ellis and Pennington (1992) found that poor growth could be partially overcome by the addition of N and P fertilisers or by partial soil sterilisation. In another aspect of the present study, seedling counts were found to be greater on steam-treated field soil than on non-steamed soil when sown in a glasshouse trial (Table 4.18.1). Mixing of 5% non-steamed soil with the steamed soil resulted in reduced seedling counts (which were not different to those on non-steamed soil) indicating a likely significant microbial component to this reduction.

5.4 Other factors limiting germination and survival of *E. regnans* seedlings

Factors affecting the establishment of *E. regnans* seedlings were elucidated in field and glasshouse studies, with availability of soil moisture being seen as the main factor limiting seedling establishment in the field. Although no quantitative study was made into the effects of soil moisture, the consistent association of poor seedling establishment with dry soil conditions indicates a causal relationship. The need for continuity in rainfall for seedling establishment was highlighted by the 1993 field trial (Table 4.12.3.1; Appendices 2 & 7). Higher germination following the April sowing-time appeared to be due to consistent rainfall during June and early July. Germination of July-sown treatments mainly followed a period of high rainfall in mid-August but was curtailed due to dry conditions that followed. Similar observations were made for other field trials. On drier sites, observed germination was confined to sheltered microsites. Battaglia and Reid (1992) have previously found that such microsite effects on germination and survival of *E. delegatensis* were prevalent under conditions of limited moisture availability.

Increased germination and reduced mortality rates noted in the glasshouse compared to the field (Table 4.14.1)were most likely a result of improved climate control afforded by growth under the glasshouse conditions. Continuity of soil moisture levels at or near field capacity was seen as the most important factor enabling seedling establishment since seed was present on the soil surface without a covering of soil. Also contributing to improved seedling survival in the glasshouse would have been temperatures close to optimal for plant growth, with extremes

being more moderate than those in the field. It is possible that airborne fungal inoculum from surrounding uncleared forest or the input of inoculum via soil percolation (both of which would have been absent in the glasshouse) or the presence of browsing animals may play a significant role in the inhibition of seedlings in the field. Altered soil conditions may also have influenced the behaviour of soil microbiota in the glasshouse. For example pathogenicity of *Pythium* may be influenced by glasshouse temperatures as was found in relation to effects on sugar beet by O'Sullivan and Kavanagh (1992).

Significantly greater germination rates were observed in burnt compared to unburnt areas (Table 4.12.1.1). Considering that it was shown that microorganisms isolated from these sites can stimulate germination *in vitro,* it is possible that such microbial stimulation may also occur in the field. Chemicals present in wood-derived smoke or charred wood may also stimulate germination of seeds (Baldwin *et al.* 1994; Brown, 1993).

Seedling mortality rates were also greater on burnt sites (Table 4.12.1.1) resulting in final seedling percentages that were not significantly greater than for unburnt sites. Reasons for this were not clear, but burning was likely to have reduced the moisture retention capacity of some of the surface soil by removal of organic matter and alteration of clay minerals (Humphreys and Craig, 1981). Blackening of the soil surface in some areas may also have promoted heating and drying of the soil. Possibly there were only a limited number of suitable microsites on the burnt sites for sustained seedling growth and microsite conditions suitable for germination were not necessarily favourable for continued seedling survival (Battaglia and Reid, 1993).

In contrast to previous claims by Gilbert (1958) and Cunningham (1960) who attributed a large proportion of seedling deaths to fungi, there was no strong evidence from field based assessments in the present study that fungi were primarily responsible for seedling mortality. Field observations were insufficient to ascribe death to fungal attack, as there was a general absence of features indicating the presence of fungi without an attempted isolation from affected seedlings prior to their death. Even on isolation of fungi from these seedlings, the extent to

which they were involved in mortality was not clear. Seed used in this study was not observed to be a source of pathogens.

Weeds were not observed to limit eucalypt seedling establishment on field sites in this study. On those plots where weeds were common, they were not observed to be in competition with eucalypt seedlings either in burnt or unburnt sites. Germination of seed and mortality of eucalypt seedlings were generally observed to occur before weeds had become large enough to have any influence. On the few occasions where weeds had overgrown eucalypt seedlings the affected seedlings appeared to benefit from the shelter provided.

Although no quantitative measurements were made of insect numbers or their effects, insects were observed to be of little consequence during a period of about six months following burning. Increase in insect activity following this time appeared to be associated with weed growth. Impact of insects may depend on opportunity to colonise from surrounding uncleared or revegetated areas.

Frost did not appear to be a primary cause of seedling mortality although it may have been a contributing factor. In practice it would appear to be difficult to separate the effects of frost damage from those of pathogen attack. Sub-lethal frost damage could encourage pathogens by leakage of cell contents or by allowing the entry of pathogens through wounds. In frost-prone areas with friable soil, frost-heave may have promoted desiccation by detaching seedlings from the soil.

Some limitations were imposed by the assessment procedures used in field studies. Time intervals between assessments may have resulted in imprecision, with under-reporting of seedlings that had germinated and died between assessments. However assessment times were sufficiently closely spaced to minimise this effect. The main 1992 field trial (Figure 4.12.1; Appendix 5) was assessed until January 1993 and extended over a time-span sufficient for all seed sown on experimental sites to germinate and for any seedling mortality at the time of seedling establishment to be observed. The time scale of the 1993 field trial may not have been long enough to record all germination and mortality, with a further assessment in November 1993 being

possibly of use (Appendix 7). However it is thought that any further change would have been minimal and unlikely to significantly alter the experimental findings. The sowing-time trial (1992) (Table 4.12.2.1; Appendix 6) was generally inconclusive. Sufficient time had probably been allowed for germination of the later sown treatments, however this was limited by the onset of drier weather and the probable influence of seed-feeding insects.

It is likely that rather than one single factor causing mortality of individual seedlings, the combined influence of a number of factors would be involved. Experimental sites were at, or about, the upper altitude limit of *E. regnans* in Tasmania (600m) and it is possible that ecological factors were intrinsically marginal for survival of *E. regnans* in this area. Also, while sites were in a high rainfall area, rainfall experienced during the experimental period was generally below average.

The influence of minor pathogens is difficult to prove (Salt, 1979) as such pathogens are ubiquitous and non-specialised, with pathogenicity often dependent on environmental conditions and host vigour. Additionally such species often act in consortia, with their pathogenicity being a combined effect.

5.5 Fungi associated with seedling mortality

Care had been taken to ensure that estimates of fungal incidence on seedlings were as accurate as possible (Tables 4.15.1 & 4.15.2). Chance of cross contamination was minimised in the collection of seedlings, while washing and surface-sterilisation would have removed any surface spores. Incubation on nutrient-free water agar would have allowed only those fungi that were already established on seedlings to sporulate within 2 days (5 days for pycnidia).

Fungi were found to be present on over 95% of dead and dying seedlings collected from the field and most could be identified to at least genus level. Identity of sterile hyphal isolates (mycelia sterilia) and a small number of uncommon isolates was not determined. Number

of fungal taxa present on individual seedlings increased with the amount of dead tissue present, possibly indicating that additional fungi were saprophytic colonisers. Any pathogens responsible for seedling mortality were more likely to be amongst those isolated at earliest stages of mortality.

Most common among fungi isolated from seedlings was a species of *Phoma,* found on 42 to 75% of dead and dying seedlings. Representative isolates caused discolouration and stunting of roots of *E. regnans* seedlings *in vitro,* but were otherwise not markedly pathogenic (Table 4.17.1). Although it is tempting to regard this species as a pathogen there was no strong evidence of pathogenicity *in vitro* or in the glasshouse (Table 4.18.1). It is possible that symptoms observed on seedlings *in vitro* were due to phytotoxin production as occurs with a number of *Phoma* species (Domsch *et al.*, 1980; Smith *et al.*, 1994). Under less optimal conditions such seedlings may have been prone to the adverse influence of climate and other pathogens. *Phoma* species have been implicated elsewhere as the cause of root and stem rots of other plants. The small conidia of this species are likely to have been readily dispersed by soil water. The association of a *Phoma* species with eucalypt seedling mortality does not appear to have been previously recorded in Australia.

Species of *Alternaria* and *Cladosporium* were among the most commonly isolated fungi from dying *E. regnans* seedlings, (Tables 4.15.1 & 4.15.2) but since their presence on such seedlings was in association with tissue that was already dead it was considered that these fungi were saprophytic. The saprophytic nature of these and of *Epicoccum purpurascens* was also indicated by their higher incidence on dead seedlings. Also none of the isolates tested was pathogenic *in vitro* (Table 4.17.1). Sources of inocula of these fungi may have been via airborne spores or from the surrounding soil. *Cladosporium* and *Alternaria* species are amongst the most common air-borne fungal spores, with their incidence being more common in wet weather (Domsch *et al.*, 1980). They are commonly found on dead and dying plant material and in soil (Domsch *et al.*, 1980).

Pythiaceous fungi were isolated from about 20 to 25% of seedlings. Identification to genus (*Pythium* or *Phytophthora*) was not

attempted for the majority of these isolates, while identity of eight representative isolates was not resolved using the technique of Waterhouse (1967). Use of pond water is believed to be of crucial importance for success of this procedure (T. Wardlaw pers. comm.) and substitution of stream water may have been the reason for absence of zoospore production. *Phytophthora* differs from *Pythium* in not forming an external evanescent vesicle during zoospore production. Some isolates were observed to have branched antheridia which are characteristic of *Pythium*.

Pathogenicity of pythiaceous fungi was not investigated. However their record of being the most common cause of damping-off disease of plants (Hendrix and Campbell, 1973; Neumann and Marks, 1976) indicates their probable significance as pathogens. It is possible that incidence of these fungi has been underestimated in this study as they can be out-competed by more aggressive or faster growing fungi (Hendrix and Campbell, 1973). Pythiaceous fungi have been previously found by Mwanza and Kellas (1987) to be involved in damping-off of *E. delegatensis* and *E. radiata* seedlings in a Victorian forest.

Seedling mortality due to *Phytophthora cinnamomi* is considered improbable as the soil type (a krasnozem) was not conducive to this species (Podger *et al.*, 1990a). Mean annual temperature experienced at the altitude (600m) is also not likely to be above the 7.5°C indicated by Podger *et al.*, (1990a) as being associated with increased damage due to this fungus.

Three distinct biotypes of *Rhizoctonia solani* have been isolated from *E. regnans* seedlings that had died on soil taken from field sites. One of these biotypes was identified as belonging to zymogram group ZG5 (Sweetingham and MacNish, 1991) which is commonly associated as a pathogen of cruciferous plants. Isolation of this same biotype from seedlings of an associated cruciferous weed species *Rorippa dictyosperma* indicated a host-pathogen association with this species. Another biotype isolated from *E. regnans* seedlings has been identified (G. MacNish, pers. comm.) as AG3 (=ZG7). Representative isolates from groups identified as ZG5 and ZG7 were found to be pathogenic to *E. regnans in vitro* (Table 4.17.1) and one isolate of group ZG7 (isolate RZ08) also appeared to be pathogenic when tested in a

glasshouse trial (Table 4.18.1). The single isolate identified as belonging to ZG10 (Sweetingham and MacNish, 1991) was not pathogenic to *E. regnans* either *in vitro* or in the glasshouse trial.

These findings may represent the first record of eucalypt seedling mortality due to *R. solani* in forest soil within Australia. However mortality due to this pathogen in the field was not proven. All identified *Rhizoctonia* isolates were obtained from seedlings growing on field soil in the glasshouse. *Rhizoctonia*-like isolates were also obtained from seedlings that had died in the field, but identity of these isolates has not been confirmed.

Cylindrocarpon species were isolated only from seedlings taken from unburnt areas. This supports the findings of Jehne (1982) that inoculum levels of this fungus in the soil surface were reduced in the year following burning.

Isolation of a *Botrytis* species from one dead seedling indicated the presence of this fungus in the field. This isolate was pathogenic to *E. regnans*, quickly killing seedlings when tested *in vitro*. However the low frequency of isolation would indicate that *Botrytis* was unlikely to be significant as a pathogen at the sites under investigation.

In vitro testing can only be indicative of pathogenicity as behaviour of pathogen and host may dependent on incubation conditions. Where pathogenicity was dependent on field conditions, such conditions would be difficult to recreate *in vitro*. Behaviour of pathogens may depend on many factors such as water activity, frost incidence, temperature fluctuations and interactions with other microbiota (Salt, 1979).

Glasshouse testing using field soil may give a more accurate measure of pathogenicity although this is dependent on imposed conditions. Use of millet culture of fungal isolates was one such imposed limitation. The amount of inoculum applied was arbitrarily determined and may have been too high or low to produce any effect. Possibly a range of pathogen concentrations could have been used in experiments where the appropriate level was unknown. It was not possible to say that reduced seedling numbers in the presence of millet cultures was due to the fungal action or due to the millet medium as

incorporation of sterile millet culture into soil as a control resulted in the second lowest seedling count (Table 4.18.1). Inoculated microorganisms were not re-isolated from the soil afterwards, thus Koch's postulates were not satisfied.

5.6 Fungicide Studies

If fungal pathogens were primarily responsible for seedling mortality then this should have been indicated by a positive response to fungicide treatment. However, lack of such a response in the 1993 field experiment (Table 4.12.3.1) could also indicate that the treatment applied was ineffective. Factors such as, timing, mode of application or choice of fungicides may have been inappropriate.

Choice of fungicides used was based on early evidence that *Rhizoctonia* was possibly a significant pathogen, with approximately one third of glasshouse *E. regnans* isolates from field soil being identified as *Rhizoctonia*. Triazole and dicarboximide fungicides (among others) have been shown to have high activity against *R. solani* and Kataria *et al.*, (1991) have found both cyproconazole (a triazole) and iprodione (a dicarboximide) were effective in the inhibition of all ten anastomosis groups of *R. solani*.

Two representatives of these fungicide groups, Sumisclex containing procymidone (a dicarboximide) and Raxil containing tebuconazole (a triazole) were selected for study. Sumisclex is normally used for control of *Botrytis* and *Sclerotinia* species against which it has a high level of activity (Hattori, 1976). As well as being also active against *Sclerotinia* and *Botrytis* species, Raxil (tebuconazole) has also been shown to control *Phoma lingam* and *Alternaria* species (Kaspers and Siebert, 1989). Mancozeb, an established broad spectrum fungicide, was included for control of *Pythium* and *Phytophthora* species.

Seed dressing was considered the most appropriate way to apply fungicide, allowing it to be most concentrated in the region of the seed and seedlings while not affecting unsown regions. Glasshouse testing established maximum non-phytotoxic concentrations of these fungicides for dressing on *E. regnans* seed (Tables 4.19.1.1 & 4.19.1.2). To keep the number of treatments to a minimum, only one fungicide

treatment was included in the 1993 field experiment and Raxil was chosen as it showed greater fungal inhibition (at 10 ppm active ingredient) than Sumisclex *in vitro* (Table 4.19.2.1). Although it is not possible to predict fungicide action in the field based on laboratory studies alone, fungi commonly found in the field were not noticeably inhibited by Raxil or Sumisclex at 10 ppm a.i., (which represented the level that might be expected in soil within 1cm of a seed treated at 0.5g a.i. per kilogram of seed, while higher concentrations would occur for a period at the seed surface).

5.7 Bio-control studies

Bacterial antagonists were obtained from the rhizosphere of healthy, year-old *E. regnans* and were initially isolated on a low nutrient medium which allowed colony development of slower-growing organisms. They were screened against the *Phoma* species as this fungus had been commonly isolated from dead *E. regnans* seedlings from field sites and because cultures of *Phoma* could be readily established from spores using the spread plate technique. Isolates were later screened against *R. solani* isolate RZ08 which had been previously shown to be pathogenic to *E. regnans* (Table 4.17.1). Reduced inhibition of *R. solani* by *Pseudomonas fluorescens* (IS2 and IS6) on addition of $FeCl_3$ (Table 4.20.2) to the medium indicates ferric siderophore production by the antagonist isolates although stimulation of the pathogen by increased Fe availability is possible.

Characteristics used to identify two antagonists (IS2 and IS6) which were Gram-negative, aerobic, motile, catalase-positive rods as *P. fluorescens* were their positive oxidase reaction, production of fluorescent pigment, presence of arginine dihydrolase, absence of poly-ß-hydroxybutyrate accumulation, absence of growth at 41°C and ability to denitrify (Table 4.21.1). Identity was also supported by absence of starch hydrolysis and growth at 4°C.

Antagonist IS4 a spore forming, motile, Gram-positive rod was identified as *Bacillus circulans* by positive catalase, negative Vogues-Proskauer, pH<6 in V-P broth, acid but no gas from glucose, facultative anaerobic growth, absence of growth at 50°C, hydrolysis of starch, casein and gelatin, cell diameter <1.0 μm with oval spores in

swollen sporangia and absence of parasporal bodies. Absence of tyrosine degradation, indole production and nitrate reduction were also consistent with identification as *B. circulans.*

The potential of bacteria as fungal antagonists has previously received much attention. *Pseudomonas* species are among the most widely studied of these (Campbell, 1989) as a result of their competitive ability and production of siderophores and antibiotics. Advantages of *Bacillus* species as antagonists include antibiotic production and the ability of spores to survive adverse conditions.

Epicoccum purpurascens was investigated as bio-control agent as antagonism by this species of a number of plant pathogenic fungi has been reported. Examples include: *Cochliobolus sativus* on wheat (Campbell, 1956); *Sclerotinia sclerotiorum* on beans (Zhou and Reeleder, 1989); *Monilinia laxa* on peach blossoms (Madrigal *et al.*, 1991); *R. solani* (Chand and Logan, 1984);*Ceratocystis ulmi* (Webber and Hedger, 1986)); *Phytophthora* and *Pythium* species (Brown *et al.*, 1987) and; *Cytospora cincta* (Royse and Ries, 1978).

A number of compounds with antifungal and antibacterial properties have been extracted from cultures of *E. purpurascens.* Flavipin, a yellow pigmented compound originally isolated from cultures of *Aspergillus flavipes* (Raistrick and Rudman, 1956) is also produced by *E. purpurascens* (Brown *et al.*, 1987). Flavipin was initially reported to have little antibacterial activity (Raistrick and Rudman, 1956; Bamford *et al.*, 1961), however its antibacterial activity against a range of bacterial species was reported by Madriagil *et al.*, (1991). Brown *et al.*, (1987) identified six antifungal compounds present in cultures of *E. purpurascens*. These were: flavipin, epicorazines A and B, two other compounds designated X and Y from the epicorazine fraction and, another unidentified compound.

E. purpurascens can be commonly found growing saprophytically on dead plant material (Schol-Schwarz, 1959) and is frequently reported to be present amongst the microbiota growing on leaf litter, including that of *Eucalyptus regnans* (Macauley and Thrower,

1966). It can also be found as a contaminant on eucalypt seed (Mwanza and Kellas, 1987).

Five *Epicoccum purpurascens* isolates investigated in the present study were found to have differing levels of antagonism to other fungi (Table 4.22.1; Figures 4.22.1 to 4.22.4) with one of the most promising of these being further evaluated.

In vitro testing of four antagonists on *E. regnans* seed (Table 4.23.1) revealed that two stimulated germination of seed as well as being antagonistic to the pathogen. The significantly greater seed germination rates in the presence of *B. circulans* and *E. purpurascens* may involve production of plant growth factors by the associated microorganisms as has been widely reported in other plant-microbial systems. Improved survival *in vitro* of seedlings inoculated with *P. fluorescens* isolate (1), *B. circulans* and particularly *E. purpurascens* in the presence of *R. solani* is likely to be due to microbial antagonism of the pathogen. Evidence of Fe-chelation has been shown by the *P. fluorescens* isolates, however antagonism by *B. circulans* and *E. purpurascens* most likely results from antibiotic production. *E. purpurascens* was associated with total protection of seedlings from pathogen attack, where 78% of controls lacking antagonist died.

Assay of the protective effectiveness of the antagonists against *Rhizoctonia* in steam-treated field soil in a glasshouse trial (Table 4.24.1) resulted in significant protection being provided only by the *E. purpurascens* isolate. A pathogen-free millet treatment should have been included in this experiment as a control to evaluate the effect of the millet preparation.

In the 1993 field trial no significant improvement in seedling establishment was noted where *E. purpurascens* was applied to the soil at the time of sowing seed (Table 4.12.3.1). This lack of improvement could reflect the increased complexity of microbial and other interactions in the field, or the limited involvement of pathogens in seedling mortality in this area.

This study demonstrated the potential of some root rhizosphere microbial associates of *E. regnans* seedlings to influence the germination of seed and to provide protection from a pathogen. The relative significance of pathogen attack on seedling mortality of *E. regnans* in field sites appears to be minor, with the primary cause(s) of this mortality remaining to be determined.

5.8 Overview of causes of seedling mortality

Although the primary causes of seedling mortality in the area studied remains to be conclusively ascertained, it is likely that mortality of individual seedlings was due to the combined effect of factors rather than individual factors acting alone. Although fungal pathogenicity was not observed to be severe under glasshouse or laboratory conditions the influence of fungi under the more complex field conditions may have been greater. Effects of any minor pathogens may be difficult to prove as pathogenicity can be dependent on environmental conditions and host vigour. Additionally such species often act in consort, with their pathogenicity being by combined action. Climate and soil properties may play a role in seedling mortality either by direct action on seedlings or through their influence on other soil biota. Frost may contribute to this process through possible tissue damage, while frost heave could promote desiccation by dislodging seedlings in some places.

Although it is clear that soil moisture was an important limiting factor in seedling establishment the extent to which this has occurred was not studied in detail as the primary aim of this project was to look at factors causing seedling mortality rather than those factors affecting germination. Influence of soil moisture on *E. regnans* establishment has been studied elsewhere (Cunningham, 1960) while a parallel study has shown a significant influence of soil moisture on *E. delegatensis* establishment in Tasmania (Battaglia and Reid, 1993). The limiting effect of soil moisture in the present study may have been to some extent an artefact of the unusually dry seasons over which field studies had been carried out. However influence of soil moisture on *E. regnans* seedling establishment may warrant further investigation.

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Appendix 1 Media and Reagents

1.1 Growth Media

All agar media were autoclaved for 15 minutes at 121°C unless otherwise specified.

1.1.1 Trypticase Soy Agar, (TSA)

Trypticase soya broth powder (BBL)	5.0 g
Yeast extract	1.0 g
K_2HPO_4	2.0 g
Glucose	2.0 g
Agar	15.0 g
Water	1000 ml

1.1.2 0.3% TSA Medium

Trypticase soya broth powder (BBL)	3.0 g
Yeast extract	1.0 g
Agar	15.0 g
Water	1000 ml

1.1.3 King's B Medium (King *et al.*, 1954)

Proteose peptone No 3, (Difco)	20.0 g
Glycerol	10.0 ml
K_2HPO_4	1.5 g
Mg $SO_4.7H_2O$	1.5 g
Agar	15.0 g
Water	1000 ml

Adjust pH to 7.2 before autoclaving.

1.1.4 Tap Water Agar, (TWA)

Agar	15.0 g
Tap water	1000 ml

1.1.5 0.3% TWA

As for TWA except with 3g agar litre^{-1}.

1.1.6 Rose Bengal Agar, (RBA; Johnson & Curl, 1972)

Agar	17.0 g
Glucose	10.0 g
Yeast extract	0.5 g
KH_2PO_4	0.5 g
K_2HPO_4	0.5 g
Mg $SO_4.7H_2O$	0.5 g
Peptone	0.5 g
Rose Bengal	0.05 g
Water	1000 ml
Streptomycin sulphate	0.03 g

Streptomycin sulphate was added to the media after it had been autoclaved and cooled to about 50°C.

1.1.7 Pectin medium (Sweetingham *et al.* 1986)

Citrus pectin	10.0 g
$(NH_4)_2SO_4$	2.64 g
KH_2PO_4	0.34 g
$MgSO_4.7H_20$	0.14 g
Distilled water	1000 ml

The pH adjusted to 5.5 and dispensed in 2ml aliquots into Bijoux bottles before sterilising.

1.1.8 **Hugh and Liefson Medium** (Smibert and Krieg, 1981)

Peptone	2.0 g
NaCl	5.0 g
KH_2PO_4	0.3 g
Bromothymol blue (1%)	3.0 ml
Agar	3.0 g
Distilled water	1000 ml

Dissolved ingredients were in water and adjusted to pH 7.1. After autoclaving and cooling to 60°C a filter sterilised 10% glucose solution was added to give a final concentration of 1%. Aliquots of 5.0 ml were then aseptically dispensed into sterile 13mm test tubes.

1.1.9 **Koser's Citrate Medium**

Sodium citrate	3.0 g
$NH_4NaHPO_4.4H_2O$	1.5 g
KH_2PO_4	1.0 g
$MgSO_4.7H_2O$	0.2 g
Distilled water	1000 ml

1.1.10 **V-P broth** (Smibert and Krieg, 1981)

Peptone (Oxoid)	7.0 g
NaCl	5.0 g
Glucose	5.0 g
Distilled water	1000 ml

The pH was adjusted to 7.6. Aliquots of 5ml were dispensed into tubes after autoclaving.

1.1.11 Thornley's semisolid arginine medium (Smibert and Krieg, 1981)

Peptone (Oxoid)	1.0 g
NaCl	5.0 g
K_2HPO_4	0.3 g
Phenol red	0.01 g
L-Arginine.HCl	10.0 g
Agar	3.0 g
Distilled water	1000 ml

L-Arginine HCl was omitted from control medium. The pH was adjust to 7.2 and transferred in 5ml aliquots to 13mm test tubes.

1.1.12 Nitrate reduction medium (Cruickshank *et al.*, 1975)

Peptone (Oxoid)	5.0 g
Beef extract (Difco)	3.0 g
KNO_3	0.2 g
Distilled water	1000 ml

1.1.13 Nutrient agar

Peptone (Oxoid)	5.0 g
Beef extract (Difco)	3.0 g
Agar	15.0 g
Distilled water	1000 ml

1.1.14 Nutrient gelatin (Cruickshank *et al.*, 1975)

Ingredients were as for nutrient agar except that 120g gelatine was substituted for agar.

1.1.15 Milk agar (Sneath *et al.*, 1986)

Skim milk powder	5.0 g
Agar	1.0 g

Each ingredient was dissolved separately in 50ml of distilled water. After autoclaving and cooling to 60°C these were mixed and poured into plates.

1.1.16 Starch agar

Trypticase soya broth without dextrose (BBL)	27.5 g
Soluble starch	2.0 g
Agar	15.0 g
Distilled water	1000 ml

1.1.17 **Tyrosine agar** (Sneath *et al.*, 1986)

L-tyrosine	0.2 g
Distilled water	10 ml
Nutrient agar	100 ml

Nutrient agar and tyrosine in water were sterilised separately. After cooling to 60°C these were mixed and poured into plates.

1.1.18 Glucose Peptone Broth (Skerman, 1967)

Peptone (Oxoid)	5%(w/v)
Glucose	5%(w/v)

Both in distilled water.

1.1.19 Palleroni and Doudoroff (1972) medium

NH_4Cl	1.0 g
$MgSO_4.7H_2O$	0.5 g
Ferric ammonium citrate	0.05 g
$CaCl_2$	0.005 g
0.033M Na-K phosphate buffer (pH 6.8)	1000 ml

The ferric ammonium citrate and $CaCl_2$ in a single stock solution were added (filter sterilised) after autoclaving.

1.2 Reagents

1.2.1 Tris (hydroxymethyl) aminomethane HCl, (Tris HCl) Buffer (Cruickshank *et al.*, 1975)

Stock solutions
A: 0.2M Tris (Hydroxymethyl) aminomethane
B: 0.2M HCl
Method
50ml of A + x ml of B, diluted to 200ml (gives a 0.1Mbuffer)

x	pH
5.0	9.0
8.1	8.8
12.2	8.6
16.5	8.4
21.9	8.2
26.8	8.0
32.5	7.8
38.4	7.6
41.4	7.4
44.2	7.2

1.2.2 Carbonate-Bicarbonate Buffer (Cruickshank *et al.*, 1975)

Stock solutions
A: 0.2M solution of Na_2CO_3
B: 0.2M solution of $NaHCO_3$

Method
x ml of A + y ml of B, diluted to a total of 200 ml (gives a 0.1M buffer)

x	y	pH
4.0	46.0	9.2
9.5	40.5	9.4
16.0	34.0	9.6
22.0	28.0	9.8
27.5	22.5	10.0
33.0	17.0	10.2
38.5	11.5	10.4
42.5	7.5	10.6

1.2.3 Methyl-Red Reagent

Methyl red	0.1 g
Ethanol (95%)	300 ml
Distilled water	200 ml

1.2.4 0.85% saline

NaCl	8.5 g
distilled water	1000 ml

1.2.5 **Kovac's reagent** (Smibert and Krieg, 1981)

p-Dimethylamiobenzaldehyde	3.0 g
iso-Amyl alcohol	75 ml
HCl (conc.)	25 ml

Appendix 2 Climatic data for the Jungle region (provided by ANM Forest Management)

Rainfall and temperature data for Jungle-19 for the period 13th April '92 to 18th January '93.

Time period	Number of days in period	Total rainfall, (mm)	Minimum temperature, (°C)	Maximum temperature, (°C)
13/04/92 - 11/05/92	28	36	2	16
11/05/92 - 8/06/92	28	45.5	-1	10
8/06/92 - 6/07/92	28	120	-3	8
6/07/92 - 20/07/92	14	20	-1.5	9
20/07/92 - 3/08/92	14	65	-2.5	7
3/08/92 - 17/08/92	14	50	-2	8
17/08/92 - 31/08/92	14	89	-2	9
31/08/92 - 14/09/92	14	40.5	-2	8
14/09/92 - 26/09/92	14	27	-1	12.5
26/09/92 - 12/10/92	14	50	0.5	14
12/10/92 - 26/10/92	14	32.5	-1	19
26/10/92 - 9/11/92	14	42	2.5	25
9/11/92 - 23/11/92	14	12.5	2	22
23/11/92 - 7/12/92	14	85.5	0.5	20
7/12/92 - 18/12/92	11	12.5	0	26.5
18/12/92 - 4/01/93	17	20	4.5	32
4/01/93 - 18/01/93	14	10	5	26

Rainfall and temperature data for Islet-12. (Approximately 1km east from Jungle-24) for the period 19th May '93 to 20th October '93.

Time period	Number of days in period	Total rainfall, (mm)	Minimum temperature, (°C)	Maximum temperature, (°C)
19/05/93 - 2/06/93	14	13.3	3	12
2/06/93 - 16/06/93	14	33	-2	10
16/06/93 - 30/06/93	14	85	-1	8
30/06/93 - 14/07/93	14	28	0.5	8.5
14/07/93 - 28/07/93	14	n.d.	0.5	8.5
28/07/93 - 11/08/93	14	18	1	8
11/08/93 - 25/08/93	14	83	0.5	11.5
25/08/93 - 8/09/93	14	16	-1.5	13
8/09/93 - 22/09/93	14	19.5	0.5	12
22/09/93 - 6/10/93	14	7.5	1	20
6/10/93 - 20/10/93	14	42.5	0	16.5

n.d. = not determined.

Appendix 3 Estimates of microbial numbers in soil from burnt and unburnt sites at 8 days after burning sites A and B*

* Colony forming units (CFU) per gram of soil with number of replicates being 10 for the burnt area (sites A and B combined) and 5 for the unburnt area (site C).

1) Estimates of Bacterial numbers.

	Depth range:		
	0-2cm	**2-5cm**	**5-10cm**
Burnt Area			
Mean	7.52×10^5*	7.15×10^5	2.55×10^6
Standard error	3.11×10^5	1.67×10^5	3.61×10^5
Unburnt Area			
Mean	3.05×10^5	4.66×10^5	2.50×10^5
Standard error	7.27×10^4	1.06×10^5	5.98×10^4

2) Estimates of fungal numbers.

	Depth range:		
	0-2cm	**2-5cm**	**5-10cm**
Burnt Area			
Mean	1.67×10^3	2.64×10^4	4.26×10^4
Standard error	4.91×10^2	7.42×10^3	1.20×10^4
Unburnt Area			
Mean	1.20×10^4	1.38×10^4	1.90×10^4
Standard error	3.98×10^3	4.23×10^3	3.53×10^3

Appendix 4 Soil pH measurements for experimental sites at 8 and 133 days after burning.*

	Soil sampled at 8 days after burning (27/4/92)			Soil sampled at 133 days after burning, (31/8/92)		
	Depth range			Depth range		
	0-2cm	2-5cm	5-10cm	0-2cm	2-5cm	5-10cm
Burnt area						
Mean pH (±s. e.§)	8.3 ±0.3	7.0 ±0.4	6.0 ±0.2	7.2 ±0.2	6.4 ±0.3	5.6 ±0.3
Minimum	7.0	4.8	4.7	5.9	5.1	3.9
Maximum	10.0	9.0	6.9	8.1	7.5	7.1
Unburnt area						
Mean pH (±s. e.)	5.2 ±0.1	5.2 ±<0.1	5.1 ±0.1	4.7 ±0.1	4.8 ±0.1	4.9 ±0.1
Minimum	4.9	5.1	5.0	4.4	4.5	4.6
Maximum	5.3	5.3	5.3	5.0	5.1	5.2

* 10 replicates for the burnt area (sites A and B combined) and 5 replicates for the unburnt area (site C). Samples comprised pooled duplicate sub-samples randomly collected from five $10m^2$ plots at each site.

§ s.e. = standard error.

Appendix 5

1992 Field Experiment - Seedling Data

Germination), mortality and survival of *E. regnans* seedlings following sowing in May '92 on burnt sites (A and B) and unburnt site C.

*5 replicate 1m^2 plots per site, each sown with 1.25 g of *E. regnans* seed (~244 viable seeds) .

	Assessment Date									
	13-May-92	24-Jun-92	12-Jul-92	4-Aug-92	31-Aug-92	14-Sep-92	5-Oct-92	27-Oct-92	25-Nov-92	14-Jan-93
Site A (Burnt)										
New germinants	0	1	11	82	256	26	14	3	0	0
Cumulative germination	0	1	12	94	350	376	390	393	393	393
Mean (5 replicates)*	0	0.2	2.4	18.8	70	75.2	78	78.6	78.6	78.6
Standard error	0	0.2	1.0	5.1	12.5	12.0	12.8	13.0	13.0	13.0
New seedling mortality	0	0	1	0	17	81	133	40	26	31
Cumulative seedling mortality	0	0	1	1	18	99	232	272	298	329
Mean (5 replicates)	0	0	0.2	0.2	3.6	19.8	46.4	54.4	59.6	65.8
Standard error	0	0	0.2	0.2	0.9	4.7	7.3	7.7	7.7	7.3
Total surviving seedlings										
Mean (5 replicates)	0	0.2	2.2	18.6	66.4	55.4	31.6	24.2	19	12.8
Standard error	0	0.2	1.1	5.1	12.6	10.9	11.7	12.1	11.9	7.4

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Appendix 5 (continued).

	Assessment Date									
	13-May-92	24-Jun-92	12-Jul-92	4-Aug-92	31-Aug-92	14-Sep-92	5-Oct-92	27-Oct-92	25-Nov-92	14-Jan-93
Site B (Burnt)										
New germinants	0	1	4	23	119	100	21	3	1	1
Cumulative germination	0	1	5	28	147	247	268	271	272	273
Mean (5 replicates)	0	0.2	1	5.6	29.4	49.4	53.6	54.2	54.4	54.6
Standard error	0	0.2	0.5	2.9	8.1	9.9	10.7	11.2	11.3	11.2
New seedling mortality	0	0	1	0	3	3	145	33	21	4
Cumulative seedling mortality	0	0	1	1	4	7	152	185	206	210
Mean (5 replicates)	0	0	0.2	0.2	0.8	1.4	30.4	37	41.2	42
Standard error	0	0	0.2	0.2	0.2	0.5	7.1	7.7	7.4	7.4
Total surviving seedlings										
Mean (5 replicates)	0	0.2	0.8	5.4	28.6	48	23.2	17.2	13.2	12.6
Standard error	0	0.2	0.6	3.0	8.0	9.6	8.7	8.5	7.0	7.3
Site C (Unburnt)										
New germinants	0	0	2	9	32	6	4	1	1	1
Cumulative germination	0	0	2	11	43	49	53	54	55	56
Mean (5 replicates)	0	0	0.4	2.2	8.6	9.8	10.6	10.8	11	11.2
Standard error	0	0	0.2	1.0	2.6	2.4	2.6	2.7	2.7	2.9
New seedling mortality	0	0	0	0	4	6	14	3	1	0
Cumulative seedling mortality	0	0	0	0	4	10	24	27	28	28
Mean (5 replicates)	0	0	0	0	0.8	2	4.8	5.4	5.6	5.6
Standard error	0	0	0	0	0.4	0.3	1.0	1.2	1.3	1.3
Total surviving seedlings										
Mean (5 replicates)	0	0	0.4	2.2	7.8	7.8	5.8	5.4	5.4	5.6
Standard error	0	0	0.2	1.0	2.6	2.4	2.0	1.6	1.4	1.6

Appendix 6 Sowing-Time Trial - Seedling Data

Germination, mortality and survival of *E. regnans* seedlings over a six month period following sowing on four dates (at six-week intervals) in May to September 1992.

* 4 replicate plots per sowing date, each sown with 1.50 g of *E. regnans* seed (~291 viable seeds).

§ n.a. = not applicable.

	Assessment Date (1992)							
	24-Jun	12-Jul	4-Aug	31-Aug	14-Sep	5-Oct	27-Oct	25-Nov
Sown 13/5/92								
New germinants	0	0	23	66	25	6	4	0
Cumulative germination	0	0	23	89	114	120	124	124
Mean (4 replicates)*	0	0	5.8	22.3	28.5	30.0	31.0	31.0
Standard error	0	0	2.6	4.3	4.0	4.7	4.7	4.7
New seedling mortality	0	0	0	14	9	52	5	5
Cumulative seedling mortality	0	0	0	14	23	75	80	85
Mean (4 replicates)	0	0	0.0	3.5	5.8	18.8	20.0	21.3
Standard error	0	0	0.0	2.0	2.6	4.4	4.8	4.6
Total surviving seedlings	0	0	23	75	91	45	44	39
Mean (4 replicates)	0	0	5.8	18.8	22.8	11.3	11.0	9.8
Standard error	0	0	2.6	3.3	1.7	3.3	3.5	2.9
Sown 24/6/92								
New germinants	0	3	1	24	67	25	10	3
Cumulative germination	0	3	4	28	95	120	130	133
Mean (4 replicates)	0	0.8	1.0	7.0	23.8	30.0	32.5	33.3
Standard error	0	0.8	0.7	2.8	2.6	1.5	0.6	0.9
New seedling mortality	0	0	0	2	9	48	8	13
Cumulative seedling mortality	0	0	0	2	11	59	67	80
Mean (4 replicates)	0	0	0	0.5	2.8	14.8	16.8	20.0
Standard error	0	0	0	0.3	1.5	1.4	0.9	0.7
Total surviving seedlings	0	3	4	26	84	61	63	53
Mean (4 replicates)	0	0.8	1.0	6.5	21.0	15.3	15.8	13.3
Standard error	0	0.8	0.7	2.6	1.7	1.7	1.3	1.3

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Appendix 6 (continued).

	Assessment Date (1992)							
	24-Jun	12-Jul	4-Aug	31-Aug	14-Sep	5-Oct	27-Oct	25-Nov
Sown 4/8/92								
New germinants	n.a[§]	n.a	0	0	2	18	24	4
Cumulative germination	n.a.	n.a.	0	0	2	20	44	48
Mean (4 replicates)	n.a.	n.a.	0	0	0.5	5.0	11.0	12.0
Standard error	n.a.	n.a.	0	0	0.3	2.3	5.3	6.1
New seedling mortality	n.a	n.a	0	0	0	1	1	17
Cumulative seedling mortality	n.a.	n.a.	0	0	0	1	2	19
Mean (4 replicates)	n.a.	n.a.	0	0	0	0.3	0.5	4.8
Standard error	n.a.	n.a.	0	0	0	0.3	0.3	3.0
Total surviving seedlings	n.a.	n.a.	0	0	2	19	42	29
Mean (4 replicates)	n.a.	n.a.	0	0	0.5	4.8	10.5	7.3
Standard error	n.a.	n.a.	0	0	0.3	2.5	5.4	3.4
Sown 14/9/92								
New germinants	n.a	n.a	n.a	n.a	0	0	2	0
Cumulative germination	n.a.	n.a.	n.a.	n.a.	0	0	2	2
Mean (4 replicates)	n.a.	n.a.	n.a.	n.a.	0	0	0.5	0.5
Standard error	n.a.	n.a.	n.a.	n.a.	0	0	0.5	0.5
New seedling mortality	n.a	n.a	n.a	n.a	0	0	0	0
Cumulative seedling mortality	n.a.	n.a.	n.a.	n.a.	0	0	0	0
Mean (4 replicates)	n.a.	n.a.	n.a.	n.a.	0	0	0	0
Standard error	n.a.	n.a.	n.a.	n.a.	0	0	0	0
Total surviving seedlings	n.a.	n.a.	n.a.	n.a.	0	0	2	2
Mean (4 replicates)	n.a.	n.a.	n.a.	n.a.	0	0	0.5	0.5
Standard error	n.a.	n.a.	n.a.	n.a.	0	0	0.5	0.5

Appendix 7 1993 Field Trial - Seedling Data

Germination, mortality and survival of *E. regnans* seedlings following sowing in April or June 1993.

Experimental design: Five treatments
Eight replicate 1 m^2 plots per treatment randomised in two blocks (four per block) with each plot sown with 1.25 gram of *E. regnans* seed (~208 viable seeds).

Blocks 1 and 2 were located at sites D and E respectively on Jungle-24.

* n.a = not applicable

Treatment A: Sown 20/4/93, untreated

	Assessment Date (1993)						
	22-May	2-Jun	30-Jun	28-Jul	25-Aug	21-Sep	20-Oct
New germinants							
Block 1	**0**	**27**	**52**	**38**	**14**	**1**	**0**
Block 2	**13**	**89**	**128**	**55**	**18**	**3**	**3**
Combined blocks	**13**	**116**	**180**	**93**	**32**	**4**	**3**
Cumulative germination							
Block 1	**0**	**27**	**79**	**117**	**131**	**132**	**132**
Mean (4 replicates)	0.0	6.8	19.8	29.3	32.8	33.0	33.0
Standard error	0.0	3.6	10.5	13.3	13.9	14.2	14.2
Block 2	**13**	**102**	**230**	**285**	**303**	**306**	**309**
Mean (4 replicates)	3.3	25.5	57.5	71.3	75.8	76.5	77.3
Standard error	3.3	7.9	11.5	11.4	9.9	9.8	9.5
Combined blocks	**13**	**129**	**309**	**402**	**434**	**438**	**441**
Mean (8 replicates)	1.6	16.1	38.6	50.3	54.3	54.8	55.1
Standard error	1.6	5.4	10.1	11.4	11.3	11.4	11.5
New seedling mortality							
Block 1	**0**	**0**	**7**	**38**	**48**	**22**	**7**
Block 2	**0**	**0**	**13**	**105**	**73**	**75**	**15**
Combined blocks	**0**	**0**	**20**	**143**	**121**	**97**	**22**
Cumulative seedling mortality							
Block 1	**0**	**0**	**7**	**45**	**93**	**115**	**122**
Mean (4 replicates)	0.0	0.0	1.8	11.3	23.3	28.8	30.5
Standard error	0.0	0.0	1.8	5.7	10.5	11.7	12.3
Block 2	**0**	**0**	**13**	**118**	**191**	**266**	**281**
Mean (4 replicates)	0.0	0.0	3.3	29.5	47.8	66.5	70.3
Standard error	0.0	0.0	1.1	7.8	10.0	10.3	10.6
Combined blocks	**0**	**0**	**20**	**163**	**284**	**381**	**403**
Mean (8 replicates)	0.0	0.0	2.5	20.4	35.5	47.6	50.4
Standard error	0.0	0.0	1.0	5.7	8.2	10.2	10.6
Total surviving seedlings							
Block 1	**0**	**27**	**72**	**72**	**38**	**17**	**10**
Mean (4 replicates)	0.0	6.8	18.0	18.0	9.5	4.3	2.5
Standard error	0.0	3.6	10.2	8.4	3.5	2.6	2.2
Block 2	**13**	**102**	**217**	**167**	**112**	**40**	**28**
Mean (4 replicates)	3.3	25.5	54.3	41.8	28.0	10.0	7.0
Standard error	3.3	7.9	10.7	5.3	0.9	0.7	1.1
Combined	**13**	**129**	**289**	**239**	**150**	**57**	**38**
Mean (8 replicates)	1.6	16.1	36.1	29.9	18.8	7.1	4.8
Standard error	1.6	5.4	9.7	6.4	3.9	1.7	1.4

Continued next page.

Appendix 7 (continued).

Treatment B: Sown 30/6/93, untreated

	Assessment Date (1993)						
	22-May	2-Jun	30-Jun	28-Jul	25-Aug	21-Sep	20-Oct
New germinants							
Block 1	n.a.*	n.a.	**0**	**0**	**23**	**5**	**0**
Block 2	n.a.	n.a.	**0**	**0**	**148**	**44**	**3**
Combined blocks	n.a.	n.a.	**0**	**0**	**171**	**49**	**3**
Cumulative germination							
Block 1	n.a.	n.a.	**0**	**0**	**23**	**28**	**28**
Mean (4 replicates)	n.a.	n.a.	0.0	0.0	5.8	7.0	7.0
Standard error	n.a.	n.a.	0.0	0.0	2.6	3.4	3.4
Block 2	n.a.	n.a.	**0**	**0**	**148**	**192**	**195**
Mean (4 replicates)	n.a.	n.a.	0.0	0.0	37.0	48.0	48.8
Standard error	n.a.	n.a.	0.0	0.0	3.4	3.2	3.3
Combined blocks	n.a.	n.a.	**0**	**0**	**171**	**220**	**223**
Mean (8 replicates)	n.a.	n.a.	0.0	0.0	21.4	27.5	27.9
Standard error	n.a.	n.a.	0.0	0.0	6.2	8.0	8.2
New seedling mortality							
Block 1	n.a.	n.a.	**0**	**0**	**0**	**19**	**6**
Block 2	n.a.	n.a.	**0**	**0**	**0**	**125**	**49**
Combined blocks	n.a.	n.a.	**0**	**0**	**0**	**144**	**55**
Cumulative seedling mortality							
Block 1	n.a.	n.a.	**0**	**0**	**0**	**19**	**25**
Mean (4 replicates)	n.a.	n.a.	0.0	0.0	0.0	4.8	6.3
Standard error	n.a.	n.a.	0.0	0.0	0.0	2.8	3.1
Block 2	n.a.	n.a.	**0**	**0**	**0**	**125**	**174**
Mean (4 replicates)	n.a.	n.a.	0.0	0.0	0.0	31.3	43.5
Standard error	n.a.	n.a.	0.0	0.0	0.0	3.5	3.3
Combined blocks	n.a.	n.a.	**0**	**0**	**0**	**144**	**199**
Mean (8 replicates)	n.a.	n.a.	0.0	0.0	0.0	18.0	24.9
Standard error	n.a.	n.a.	0.0	0.0	0.0	5.4	7.3
Total surviving seedlings							
Block 1	n.a.	n.a.	**0**	**0**	**23**	**9**	**3**
Mean (4 replicates)	n.a.	n.a.	0.0	0.0	5.8	2.3	0.8
Standard error	n.a.	n.a.	0.0	0.0	2.6	1.0	0.5
Block 2	n.a.	n.a.	**0**	**0**	**148**	**67**	**21**
Mean (4 replicates)	n.a.	n.a.	0.0	0.0	37.0	16.8	5.3
Standard error	n.a.	n.a.	0.0	0.0	3.4	2.5	1.5
Combined	n.a.	n.a.	**0**	**0**	**171**	**76**	**24**
Mean (8 replicates)	n.a.	n.a.	0.0	0.0	21.4	9.5	3.0
Standard error	n.a.	n.a.	0.0	0.0	6.2	3.0	1.1

Continued next page.

Appendix 7 (continued).

Treatment C: Sown 30/6/93, with seed dressed at 20g Raxil and 5g Mancozeb per kg.

	Assessment Date (1993)						
	22-May	2-Jun	30-Jun	28-Jul	25-Aug	21-Sep	20-Oct
New germinants							
Block 1	n.a.	n.a.	**0**	**0**	**11**	**18**	**2**
Block 2	n.a.	n.a.	**0**	**0**	**33**	**73**	**1**
Combined blocks	n.a.	n.a.	**0**	**0**	**44**	**91**	**3**
Cumulative germination							
Block 1	n.a.	n.a.	**0**	**0**	**11**	**29**	**31**
Mean (4 replicates)	n.a.	n.a.	0.0	0.0	2.8	7.3	7.8
Standard error	n.a.	n.a.	0.0	0.0	1.8	1.7	1.4
Block 2	n.a.	n.a.	**0**	**0**	**33**	**106**	**107**
Mean (4 replicates)	n.a.	n.a.	0.0	0.0	8.3	26.5	26.8
Standard error	n.a.	n.a.	0.0	0.0	2.3	6.0	6.0
Combined blocks	n.a.	n.a.	**0**	**0**	**44**	**135**	**138**
Mean (8 replicates)	n.a.	n.a.	0.0	0.0	5.5	16.9	17.3
Standard error	n.a.	n.a.	0.0	0.0	1.7	4.6	4.6
New seedling mortality							
Block 1	n.a.	n.a.	**0**	**0**	**0**	**8**	**12**
Block 2	n.a.	n.a.	**0**	**0**	**0**	**26**	**68**
Combined blocks	n.a.	n.a.	**0**	**0**	**0**	**34**	**80**
Cumulative seedling mortality							
Block 1	n.a.	n.a.	**0**	**0**	**0**	**8**	**20**
Mean (4 replicates)	n.a.	n.a.	0.0	0.0	0.0	2.0	5.0
Standard error	n.a.	n.a.	0.0	0.0	0.0	1.2	2.3
Block 2	n.a.	n.a.	**0**	**0**	**0**	**26**	**94**
Mean (4 replicates)	n.a.	n.a.	0.0	0.0	0.0	6.5	23.5
Standard error	n.a.	n.a.	0.0	0.0	0.0	1.8	5.5
Combined blocks	n.a.	n.a.	**0**	**0**	**0**	**34**	**114**
Mean (8 replicates)	n.a.	n.a.	0.0	0.0	0.0	4.3	14.3
Standard error	n.a.	n.a.	0.0	0.0	0.0	1.3	4.5
Total surviving seedlings							
Block 1	n.a.	n.a.	**0**	**0**	**11**	**21**	**11**
Mean (4 replicates)	n.a.	n.a.	0.0	0.0	2.8	5.3	2.8
Standard error	n.a.	n.a.	0.0	0.0	1.8	1.0	1.2
Block 2	n.a.	n.a.	**0**	**0**	**33**	**80**	**13**
Mean (4 replicates)	n.a.	n.a.	0.0	0.0	8.3	20.0	3.3
Standard error	n.a.	n.a.	0.0	0.0	2.3	4.5	1.3
Combined	n.a.	n.a.	**0**	**0**	**44**	**101**	**24**
Mean (8 replicates)	n.a.	n.a.	0.0	0.0	5.5	12.6	3.0
Standard error	n.a.	n.a.	0.0	0.0	1.7	3.5	0.8

Continued next page.

Appendix 7 (continued).

Treatment D: Sown 30/6/93 and millet culture of *E. purpurascens* isolate B60 applied to soil surface at 35cm^3 per plot

	Assessment Date (1993)						
	22-May	2-Jun	30-Jun	28-Jul	25-Aug	21-Sep	20-Oct
New germinants							
Block 1	n.a.	n.a.	**0**	**0**	**21**	**8**	**1**
Block 2	n.a.	n.a.	**0**	**0**	**161**	**55**	**7**
Combined blocks	n.a.	n.a.	**0**	**0**	**182**	**63**	**8**
Cumulative germination							
Block 1	n.a.	n.a.	**0**	**0**	**21**	**29**	**30**
Mean (4 replicates)	n.a.	n.a.	0.0	0.0	5.3	7.3	7.5
Standard error	n.a.	n.a.	0.0	0.0	1.0	1.3	1.2
Block 2	n.a.	n.a.	**0**	**0**	**161**	**216**	**223**
Mean (4 replicates)	n.a.	n.a.	0.0	0.0	40.3	54.0	55.8
Standard error	n.a.	n.a.	0.0	0.0	7.0	13.1	14.2
Combined blocks	n.a.	n.a.	**0**	**0**	**182**	**245**	**253**
Mean (8 replicates)	n.a.	n.a.	0.0	0.0	22.8	30.6	31.6
Standard error	n.a.	n.a.	0.0	0.0	7.4	10.7	11.3
New seedling mortality							
Block 1	n.a.	n.a.	**0**	**0**	**0**	**18**	**7**
Block 2	n.a.	n.a.	**0**	**0**	**0**	**141**	**49**
Combined blocks	n.a.	n.a.	**0**	**0**	**0**	**159**	**56**
Cumulative seedling mortality							
Block 1	n.a.	n.a.	**0**	**0**	**0**	**18**	**25**
Mean (4 replicates)	n.a.	n.a.	0.0	0.0	0.0	4.5	6.3
Standard error	n.a.	n.a.	0.0	0.0	0.0	1.2	1.6
Block 2	n.a.	n.a.	**0**	**0**	**0**	**141**	**190**
Mean (4 replicates)	n.a.	n.a.	0.0	0.0	0.0	35.3	47.5
Standard error	n.a.	n.a.	0.0	0.0	0.0	6.2	10.7
Combined blocks	n.a.	n.a.	**0**	**0**	**0**	**159**	**215**
Mean (8 replicates)	n.a.	n.a.	0.0	0.0	0.0	19.9	26.9
Standard error	n.a.	n.a.	0.0	0.0	0.0	6.5	9.3
Total surviving seedlings							
Block 1	n.a.	n.a.	**0**	**0**	**21**	**11**	**5**
Mean (4 replicates)	n.a.	n.a.	0.0	0.0	5.3	2.8	1.3
Standard error	n.a.	n.a.	0.0	0.0	1.0	1.1	0.5
Block 2	n.a.	n.a.	**0**	**0**	**161**	**75**	**33**
Mean (4 replicates)	n.a.	n.a.	0.0	0.0	40.3	18.8	8.3
Standard error	n.a.	n.a.	0.0	0.0	7.0	7.0	4.3
Combined	n.a.	n.a.	**0**	**0**	**182**	**86**	**38**
Mean (8 replicates)	n.a.	n.a.	0.0	0.0	22.8	10.8	4.8
Standard error	n.a.	n.a.	0.0	0.0	7.4	4.5	2.4

Continued next page.

Appendix 7 (continued).

Treatment E: Sown 30/6/93 and Osmocote (NPK 14:6.1:11.6) applied to soil surface at 40g per plot.

	Assessment Date (1993)						
	22-May	2-Jun	30-Jun	28-Jul	25-Aug	21-Sep	20-Oct
New germinants							
Block 1	n.a.	n.a.	**0**	**0**	**27**	**6**	**0**
Block 2	n.a.	n.a.	**0**	**0**	**82**	**20**	**1**
Combined blocks	n.a.	n.a.	**0**	**0**	**109**	**26**	**1**
Cumulative germination							
Block 1	n.a.	n.a.	**0**	**0**	**27**	**33**	**33**
Mean (4 replicates)	n.a.	n.a.	0.0	0.0	6.8	8.3	8.3
Standard error	n.a.	n.a.	0.0	0.0	1.1	1.7	1.7
Block 2	n.a.	n.a.	**0**	**0**	**82**	**102**	**103**
Mean (4 replicates)	n.a.	n.a.	0.0	0.0	20.5	25.5	25.8
Standard error	n.a.	n.a.	0.0	0.0	5.5	6.6	6.7
Combined blocks	n.a.	n.a.	**0**	**0**	**109**	**135**	**136**
Mean (8 replicates)	n.a.	n.a.	0.0	0.0	13.6	16.9	17.0
Standard error	n.a.	n.a.	0.0	0.0	3.7	4.5	4.6
New seedling mortality							
Block 1	n.a.	n.a.	**0**	**0**	**0**	**24**	**5**
Block 2	n.a.	n.a.	**0**	**0**	**0**	**65**	**29**
Combined blocks	n.a.	n.a.	**0**	**0**	**0**	**89**	**34**
Cumulative seedling mortality							
Block 1	n.a.	n.a.	**0**	**0**	**0**	**24**	**29**
Mean (4 replicates)	n.a.	n.a.	0.0	0.0	0.0	6.0	7.3
Standard error	n.a.	n.a.	0.0	0.0	0.0	1.1	1.3
Block 2	n.a.	n.a.	**0**	**0**	**0**	**65**	**94**
Mean (4 replicates)	n.a.	n.a.	0.0	0.0	0.0	16.3	23.5
Standard error	n.a.	n.a.	0.0	0.0	0.0	5.4	6.2
Combined blocks	n.a.	n.a.	**0**	**0**	**0**	**89**	**123**
Mean (8 replicates)	n.a.	n.a.	0.0	0.0	0.0	11.1	15.4
Standard error	n.a.	n.a.	0.0	0.0	0.0	3.2	4.3
Total surviving seedlings							
Block 1	n.a.	n.a.	**0**	**0**	**27**	**9**	**4**
Mean (4 replicates)	n.a.	n.a.	0.0	0.0	6.8	2.3	1.0
Standard error	n.a.	n.a.	0.0	0.0	1.1	0.9	0.6
Block 2	n.a.	n.a.	**0**	**0**	**82**	**37**	**9**
Mean (4 replicates)	n.a.	n.a.	0.0	0.0	20.5	9.3	2.3
Standard error	n.a.	n.a.	0.0	0.0	5.5	5.1	1.4
Combined	n.a.	n.a.	**0**	**0**	**109**	**46**	**13**
Mean (8 replicates)	n.a.	n.a.	0.0	0.0	13.6	5.8	1.6
Standard error	n.a.	n.a.	0.0	0.0	3.7	2.7	0.8

Appendix 8.

A) Fungal isolates used in this project

Isolate identity	Code	Source #	IMI Number§
Alternaria sp.	14/9-12	a	
	B37	a	
Botrytis sp.	-	a	
Cladosporium sp.	B11-1	a	
	B20	a	
Cylindrocarpon sp.	MCA005	b	
Epicoccum purpurascens	B39	a	
	B59	a	
	B60	a	
	EC01 (=MEC001)	b	
	EC02 (=MEC002)	d	
Phoma sp. ‡	B42-2	a	361246
	B11	a	
	14/9-1	a	
Rhizoctonia solani (ZG 5 ,AG 2.1)*	RZ01 (=MRZ001)	c	
	RZ02	c	
	RZ03	c	
	RZ04	c	
	RZ06	b	
	RZ13 (=MRZ013)	b	
R. solani (ZG 7, AG3)^	RZ05	b	
	RZ08 (=MRZ008)	b	
	RZ09	b	
	RZ10	b	
	RZ11	b	

....Continued next page.

Appendix 8 (continued).

Isolate identity	Code	Source #	IMI Number§
	RZ14	b	
	RZ15	b	
R. solani (ZG 10, AG2-2IIIB)Δ	RZ16 (=MRZ016)	b	
Sclerotinia sclerotiorum	-	e	
Trichoderma sp.	B14	b	
Unidentified type 1†	B8	a	
	B50	a	
Unidentified type 2¥	B15	a	
	14/9-5	a	

Sources: a) *Eucalyptus regnans* seedling from "Jungle-19";
b) *E. regnans* seedling grown in glasshouse on soil from "Jungle" area;
c) *Rorippa dictyosperma* seedling grown in glasshouse on soil from "Jungle" area;
d) *E. regnans* seed;
e) Agricultural Science Department culture collection.

§ International Mycological Institute identification

‡ See also Appendix 8 (B) . Description of isolate B42-2 by Dr E. Punithalingam of IMI: "...resembles *P. sydowii* Boerema, van Kesteren & Loerakker in producing pycnidia with blackened short necks but differs in having longer conidia 5-7 (-10) x 1.5 -2 μm. Cultures... ...also resemble *P. sorghina* (Sacc.) Boerema *et al.*, in producing pinkish sectors or mycelial tufts but differs in conidial morphology."

* Zymogram (ZG) and anastomosis (AG) groups.

Δ ZG / AG group has been confirmed by G.C. MacNish of the Western Australian Department of Agriculture.

† Sterile hyphae with pale brown colouration.

¥ Sterile hyphae with some yellow colouration to colony.

B) Description of a *Phoma* species commonly isolated from dead and dying *Eucalyptus regnans* seedlings taken from the "Jungle" area near Mt Thunderbolt, Tasmania.

On incubation of surface sterilised seedlings on water agar, pycnidia were observed to be present at about 5 days. Pycnidia were generally not found elsewhere other than on seedlings. Such pycnidia were occasionally visible on dead seedlings and on wetting dried seedlings spores were seen to be extruded in a cirrhus.

Fungal growth moderately slow on BBL potato dextrose agar (PDA) with cultures being pale pink and fluffy. Underside of colonies were usually straw coloured to pale pink. Grey patches were sometimes present within colonies and contained many 'chlamydospore-like' hyphal cells. Cultures produced a faint pink diffusible pigment which turned purple or blue on addition of NaOH. Pycnidia infrequently formed on PDA and were pale brown to black, osteolate without setae and were sometimes multi-osteolate or present in groups. Conidia were very small (5-7(10) x 1.5-2 μm) hyaline, single celled and immersed in a sticky substance.

On subculturing hyphal material, colonies of a number of isolates tended to be prone to sectoring, with development of a more diffuse colony type wit brick red colouring. Fresh cultures exhibiting the original characteristics could be readily established from conidia. Formation of pycnidia could be induced by incubating a small piece of PDA culture on water agar.