3. EXPERIMENTAL
PROCEDURES USED THROUGHOUT THE STUDY

ORGANIC SOLVENTS:
All solvents were of 'analytical reagent' grade and were further purified by fractional distillation through an all glass Vigreux column, collecting the:
61°C boiling point fraction for chloroform
65°C boiling point fraction for methanol
77°C boiling point fraction for ethyl acetate.

PLANT POTTING MEDIUM (UC MIX):
A plant potting medium, consisting of a 1:1 (v/v) mixture of fine sand and peat moss amended with 1.2 kg/m³ of CaCO₃ lime, 1.0 kg/m³ dolomite, and 1.0 kg/m³ of 'Osmocote' (slow release fertilizer; 15% N, 5.2% P, 12.5% K), was freshly prepared as required.

HOAGLANDS SOLUTION:
A plant nutrient solution was freshly prepared as required by adding volumes of the following stock solutions with distilled water as outlined below:

<table>
<thead>
<tr>
<th>Stock Solution</th>
<th>Volume of stock solution per litre of distilled water</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. KNO₃</td>
<td>161.0g/L 5ml</td>
</tr>
<tr>
<td>2. Ca(NO₃)₂</td>
<td>236.6g/L 5ml</td>
</tr>
<tr>
<td>3. MgSO₄·7H₂O</td>
<td>246.5g/L 2ml</td>
</tr>
<tr>
<td>4. KH₂PO₄</td>
<td>159.1g/L 1ml</td>
</tr>
<tr>
<td>5. Fe EDTA</td>
<td>3.28g/L 1ml</td>
</tr>
<tr>
<td>Compound</td>
<td>Amount (g/L)</td>
</tr>
<tr>
<td>-------------------</td>
<td>--------------</td>
</tr>
<tr>
<td>HBO₃</td>
<td>2.86</td>
</tr>
<tr>
<td>MnCl₂·4H₂O</td>
<td>1.81</td>
</tr>
<tr>
<td>ZnSO₄·7H₂O</td>
<td>0.22</td>
</tr>
<tr>
<td>NaMoO₄</td>
<td>0.025</td>
</tr>
<tr>
<td>CuSO₄·5H₂O</td>
<td>0.08</td>
</tr>
</tbody>
</table>
3.1 The Occurrence, Identification and Properties of Tasmanian Soil-Borne Cylindrocarpon species

3.1.1 Isolation and Identification

The aim of this section was to assess the frequency and distribution of Cylindrocarpon species in agricultural and forest soils as well as in glasshouse situations in Tasmania.

3.1.1.1 Development of a selective medium

My preliminary studies indicated that common soil-borne species of Pythium, Mucor, Trichoderma and Fusarium have a growth rate of two to five times that of Cylindrocarpon species on general mycological agar media at temperatures of 20-25°C. It was considered that this relatively slow growth rate may hinder the recovery of Cylindrocarpon from soil and plant roots. It was the aim of this work to develop an agar medium which would favour the growth of Cylindrocarpon in comparison to the normally faster growing soil-borne fungi.

Czapek Dox Agar (CDA) (Appendix 1.1) was chosen as the basal medium to which modifications were made. The initial strategy was to examine the effect of nutrient concentration and pH of the media. Taylor (1964) demonstrated that C. destructans grew well at a pH of 10 on solid media.

Materials and Methods

All media were prepared with distilled water and analytical grade chemicals and contained 1.2% agar (Davis, Grade J). Bottles containing 100ml of medium were autoclaved at 115°C for 20 minutes, cooled to 45°C and poured into 9cm diameter petri dishes (14ml/dish).

Antibiotics and fungicides were prepared as a suspension
in acetone-ethanol (1:1, v/v), and 0.5 ml pipetted into the media bottles just prior to pouring.

Several common soil-borne fungi from a range of genera and several isolates of *Cylindrocarpon*, maintained on CDA, were used to evaluate the different media. Plates were incubated in the dark and at 22°C unless otherwise stated. After the incubation period the radial extension of the colonies was determined as a measure of growth. The distance from the edge of the inoculum plug to the edge of the colony was measured in two directions (at right angles) to the nearest mm and the mean value was recorded. Measurements were made on three replicate plates.

The ability of media to recover *Cylindrocarpon* from soil and plant roots was also determined, using the soil plate and the sequential washing procedures outlined in section 3.1.1.2.

Results and Discussion

The growth of *Cylindrocarpon* was given a considerable selective advantage over the other soil-borne fungi by diluting Czapek Dox medium to one-fifth of its normal concentration and buffering it to pH 10 with NaHCO₃ and NaOH. *Trichoderma* and *Mucor* species were particularly sensitive to high pH (Table 1.1).

Antibiotic and fungicide additions to the dilute high pH medium further improved the growth rate of *Cylindrocarpon* relative to the other test fungi (Table 1.1). The addition of 20 ppm metalaxyl (as Ridomil 25% W.P.) was sufficient to give complete inhibition of pythiaceous fungi with no effect on the growth of *Cylindrocarpon*. Pentachloronitrobenzene (PCNB) at 20 ppm inhibited the growth of certain *Mucor* and *Mortiorella*
Table 1.1 The radial growth of *Cylindrocarpon* spp. and other soil-borne fungi on agar media incubated at 12°C for 10 days then 22°C for 4 days.

<table>
<thead>
<tr>
<th>Test Fungus</th>
<th>Radial Growth (mm) (Mean of 3 replicates)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>CDA</strong></td>
</tr>
<tr>
<td><em>Cylindrocarpon</em> sp. (E1)</td>
<td>23</td>
</tr>
<tr>
<td><em>C. destructans</em> (V4)</td>
<td>18</td>
</tr>
<tr>
<td><em>C. didymum</em> (S1)</td>
<td>17</td>
</tr>
<tr>
<td><em>C. tenue</em> (E9)</td>
<td>13</td>
</tr>
<tr>
<td><em>Cylindrocarpon</em> sp. (E8)</td>
<td>34</td>
</tr>
<tr>
<td><em>C. theobromicola</em> (B5)</td>
<td>11</td>
</tr>
<tr>
<td><em>Aspergillus</em> sp.</td>
<td>17</td>
</tr>
<tr>
<td><em>Fusarium culmorum</em></td>
<td>40</td>
</tr>
<tr>
<td><em>F. culmorum</em></td>
<td>45</td>
</tr>
<tr>
<td><em>F. oxysporum</em></td>
<td>27</td>
</tr>
<tr>
<td><em>F. oxysporum</em></td>
<td>21</td>
</tr>
<tr>
<td><em>F. solani</em></td>
<td>15</td>
</tr>
<tr>
<td><em>Mortiorella</em> sp.</td>
<td>45</td>
</tr>
<tr>
<td><em>Mucor</em> sp.</td>
<td>45</td>
</tr>
<tr>
<td><em>Penicillium</em> sp.</td>
<td>22</td>
</tr>
<tr>
<td><em>Pythium ultimum</em></td>
<td>45</td>
</tr>
<tr>
<td><em>Trichoderma</em> sp.</td>
<td>45</td>
</tr>
<tr>
<td><em>Verticillium</em> sp.</td>
<td>16</td>
</tr>
</tbody>
</table>

*Standard errors of the means were less than 1.8mm for all treatments.

**CDA** = Czapek Dox Agar (Appendix 1.1)

1/5 CDA = one-fifth strength Czapek Dox agar

1/5 CDA, pH 10 = one-fifth strength Czapek Dox agar buffered to pH 10 with 420mg NaHCO₃ and 88mg NaOH per litre of medium.

CSA = *Cylindrocarpon* selective agar medium (Table 1.2).

***Cylindrocarpon isolate code (Appendix 1.2):
species. However, at higher concentrations PCNB favoured the growth of Fusarium relative to Cylindrocarpon. Neomycin sulphate at 200 ppm reduced bacterial contamination and favoured the growth of Cylindrocarpon relative to Fusarium.

The constituents of the adopted selective medium are listed in Table 1.2.

Table 1.2 Cylindrocarpon selective agar medium (CSA).

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>6.0g</td>
</tr>
<tr>
<td>Sodium nitrate</td>
<td>0.4g</td>
</tr>
<tr>
<td>Magnesium glycerophosphate</td>
<td>0.1g</td>
</tr>
<tr>
<td>Potassium sulphate</td>
<td>0.1g</td>
</tr>
<tr>
<td>Sodium bicarbonate</td>
<td></td>
</tr>
<tr>
<td>Sodium hydroxide</td>
<td>420mg</td>
</tr>
<tr>
<td>*Pentachloronitrobenzene (PCNB)</td>
<td>20mg</td>
</tr>
<tr>
<td>*Ridomil (25% metalaxyl)</td>
<td>80mg</td>
</tr>
<tr>
<td>*Neomycin sulphate</td>
<td>200mg</td>
</tr>
<tr>
<td>Agar</td>
<td>12g</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>1 litre</td>
</tr>
</tbody>
</table>

*Dissolved in 5ml of ethanol-acetone (1:1, v/v) and added to the autoclaved medium when cooled to 50°C.

The CSA greatly increased the frequency with which Cylindrocarpon was recovered from soil and plant roots (Plates 1.1 and 1.2). However, when CSA plates were incubated at 20-25°C Fusarium frequently grew as a contaminant.

The relative growth rate of Fusarium in comparison to Cylindrocarpon was shown to be less at lower temperatures (Figure 1.1). This supported the findings of Taylor (1964). Fusarium contamination could be reduced and Cylindrocarpon recovery further enhanced on CSA by incubating plates at 10-12°C. However, at these temperatures certain Cylindrocarpon
Plate 1.1

The increased recovery of *C. destructans* from soil using CSA. Soil, amended with *C. destructans*, plated onto CDA (left) and CSA (right) and incubated for five days at 20°C. *C. destructans* colonies are easily recognizable by their orange-brown pigmentation.
Plate 1.2

The increased recovery of *C. destructans* from the rhizoplane of strawberry plants using CSA. Washed strawberry root segments plates onto CDA (left) and CSA (right) and incubated for five days at 20°C. Orange-brown pigmented *C. destructans* colonies developed on CSA, whereas a *Penicillium* species grew predominantly on CDA.
Figure 1.1

Effect of temperature on the growth of Cylindrocarpon and Fusarium on Czapek Dox agar in the dark.

*Mean of three replicates, standard errors of means less than 1.2 mm for each treatment.
isolates failed to sporulate and produce their characteristic pigments making recognition of colonies more difficult. Subsequently, it was found that incubating plates at 12°C for ten days, then transferring them to 20°C for four days, produced pigmented and sporulating Cylindrocarpon colonies as well as reducing Fusarium contamination.

3.1.1.2 Recovery from soil and plant tissue

Materials and Methods

Cylindrocarpon selective agar (CSA) (Table 1.2) or Czapek Dox agar (CDA) (Appendix 1.1) were used throughout these studies. Plates were incubated in the dark at 12°C for ten days then 20°C for four days, or for seven days at 20°C.

Isolation from soil

Conventional soil dilution plates and soil plates (Warcup, 1950) were prepared from samples taken at depths of 2 to 15 cm.

Isolation from the rhizoplane

A quick and convenient technique was developed to isolate Cylindrocarpon from the surfaces and cortical tissues of plant roots using the sequential washing principle of Harley and Waid (1955). Roots were superficially washed free of adhering soil material under running tap water and cut into approximately 5mm length segments. About 30 segments were transferred into 50ml sterile water in a 100ml beaker and vigorously agitated with the aid of magnetic stirring for two minutes. The washings were discarded through a 500μ sieve, enabling the root segments to be collected and transferred to
a second beaker of sterile water. This washing procedure was repeated four times. After the final wash the root segments were individually removed from the sieve with flamed forceps, touched onto sterile filter paper to remove excess moisture, and pressed lightly into solidified agar plates.

Isolation from the vascular tissue of stems and roots

The stem or root to be examined was thoroughly washed under running tap water to remove soil or other debris. The specimen was then surface sterilised by dipping in absolute ethanol and flaming. Pieces of the desired tissue were then excised with a sterile scalpel blade and transferred to solidified agar plates.

Isolation from forest litter

The sequential washing procedure for isolation from the rhizoplane was employed.

Results and Discussion

The recovery of *Cylindrocarpon* from soil dilution plates was extremely rare even using the selective medium. Recovery from soil plates was somewhat more frequent with *Cylindrocarpon* growing out from colonised organic fragments in the sample. *Cylindrocarpon* was recovered from vineyard soils (medium-clay) at Bream Creek on Tasmania's east coast and from eucalypt forest soil (sandy-loam) near Hastings in southern Tasmania. *Cylindrocarpon* was frequently isolated from washed eucalypt forest litter segments.

*Cylindrocarpon* was frequently isolated from the rhizoplane of apparently healthy strawberry, grapevine, boronia and peppermint plants. *Cylindrocarpon*
would appear to have a particular affinity for the rhizoplane of strawberry plants whether grown in the field or in glasshouse conditions. No mature strawberry plant, examined from a wide range of locations in Tasmania, had a rhizoplane free of this fungus. *Cylindrocarpon* was also frequently isolated from the vascular tissue of the crowns of apparently healthy strawberries.

*Cylindrocarpon* was consistently isolated from the black necrotic lesions on the roots and rhizomes of diseased peppermint crops in southern Tasmania. However, the fungus *Phoma strasseri* Moesz. and the nematode *Paratylenchus* sp. appear to be the primary pathogens involved in this disease (Nik Yahaya, 1980).

*Cylindrocarpon* was recovered from the decaying roots of glasshouse-grown cyclamen, begonia, kangaroo paw and *Streptocarpus* sp. plants. Several potentially pathogenic fungi, including *Fusarium* and *Thielaviopsis* species, were also recovered from these roots.

*Cylindrocarpon* was also recovered from discoloured wood in the basal stem region of diseased grapevines, lavender and boronia plants, and one-year-old almond and radiata pine seedlings. All these plants had gum deposits in the xylem vessels. The role of *Cylindrocarpon* in these disorders was only examined in detail in the case of the grapevines (Section 3.3).

Thus *Cylindrocarpon* appears to be widely associated with diseased and healthy tissue from a wide range of plant species and geographical locations throughout Tasmania.
Subcultures (of single spore isolates) from different sources, and those from the same source but considered morphologically distinct, were maintained on one-fifth strength Czapek Dox agar plates at 10°C and subcultured at three-monthly intervals for the remainder of the study. The full list of the 80 isolates maintained, and their respective sources, is given in Appendix 1.2.

3.1.1.3 Description and taxonomy of the isolates

Booth (1966) completed the most recent full revision of the taxonomy of the genus *Cylindrocarpon*. Since this revision several new species of *Cylindrocarpon* have been reported in the literature and a further revision of the genus is currently being prepared by Dr. C. Booth (pers. comm.).

The production of chlamydospores in the mycelium and a microconidial state are consistent characters for the different species. The size, shape and septation of macroconidia are the other characters used by Booth (1966) to distinguish species. Domsch *et al.* (1980) also provide useful descriptions of the more common soil-borne species of *Cylindrocarpon*.

Materials and Methods

Cultures were grown on CDA at 20°C in the dark. The presence of a microconidial state was determined by preparing a slide from the advancing edge of four- to six-day-old cultures. Fourteen-day-old cultures were observed for the presence of chlamydospores and the examination of macroconidia. Culture pigmentation was described using the mycological colour chart of Rayner (1970).

All mounts for observation and photomicrography were made in aqueous trypan blue (0.05%, w/v). Photographs were
taken with a Zeiss 35mm camera attachment through a Zeiss Research Compound Microscope, using Ilford FP4 black and white film. Prints were made on high contrast photographic paper (Ilfospeed 5.1M).

All the isolates from Section 3.1.1.2 were observed and identified to species using the key of Booth (1966). Some atypical isolates were sent to Dr. C. Booth at the Commonwealth Mycological Institute for verification.

Results and Discussion

Culture pigmentation

All the isolates examined produced pigmented cultures on CDA. By viewing the agar of 14 or more day old cultures from below, all the isolates were observed to produce either bay (moderate reddish-brown), fulvour-umber (moderate brownish-orange) or buff (pale yellow) pigmentation.

Chlamydospires

All the isolates observed produced chlamydospires in the mycelium of mature cultures. All the isolates producing fulvous-umber pigmented cultures formed hyaline to weakly pigmented, barrel-shaped chlamydospires (Plate 1.3). These were occasionally produced singly but more often in chains. All the isolates producing bay pigmented cultures formed strongly pigmented, globose chlamydospires (Plate 1.4) which often appeared rough or tuberculate. These were produced singly or in chains, but in certain isolates were produced tightly clumped together to form microsclerotial type structures up to 250μ in diameter (Plate 1.5). The isolates producing buff pigmented cultures produced weakly pigmented, smooth
Plate 1.3

Barrel-shaped chlamydosporos (C. obtusisporum, B1).

Plate 1.4

Globose chlamydosporos (C. destructans, P1).
Plate 1.5

Developing microsclerotia in a 14-day-old CDA culture of _C. destructans_ (Pn1).
or rough globose chlamydospores, singly or in chains.

**Culture odour**

Several fulvous-umber and two buff pigmented cultures produced a scent suggesting toilet soap. One buff pigmented culture produced an actinomycete-like odour. All the bay pigmented cultures were considered odourless (Table 1.3).

**Table 1.3** The association between *Cylindrocarpon* culture odour and pigmentation.

<table>
<thead>
<tr>
<th>Culture Odour</th>
<th>Bay (number of isolates)</th>
<th>Fulvous-umber</th>
<th>Buff</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toilet Soap</td>
<td>0</td>
<td>27</td>
<td>2</td>
<td>29</td>
</tr>
<tr>
<td>Actinomycete</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Neither</td>
<td>41</td>
<td>9</td>
<td>0</td>
<td>50</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>41</strong></td>
<td><strong>36</strong></td>
<td><strong>3</strong></td>
<td><strong>80</strong></td>
</tr>
</tbody>
</table>

Data from Appendix 1.3.

Domsch et al. (1980) reported that *C. destructans* isolates frequently produce cultures with an odour suggesting toilet soap and that *C. olidum* isolates commonly produce an actinomycete-like odour.

**Microconidia**

Several isolates produced a distinct microconidial state in young cultures (Plates 1.6 and 1.7). Other isolates produced a few conidia of microconidial dimensions (6-14 x 3-4μ) which were presumably abortive macroconidia.

**Macroconidia**

The size and shape of macroconidia and the proportion
Plates 1.6 and 1.7

A comparison of the distinct microconidial and macroconidial states of *C. destructans* (Br3) cultures.

Observations of open petri dishes under the low power objective lens of a compound microscope.
Plate 1.6

Microconidial state (advancing edge of a four-day-old culture).

Plate 1.7

Macroconidial state (centre of a ten-day-old culture).
with zero, one, two, three or more septa was quite variable between isolates. However, certain isolates had macroconidia which were considered to be characteristic of a particular species. Isolates E3 and E9 had macroconidia less than 20μ long (Plate 1.8) characteristic of C. tenue Bugn. and isolate B5 had distinctively large, slightly curved, three septate macroconidia (Plate 1.9) characteristic of C. theobromicola Booth (Booth, 1966). Isolates S1, S6 and V7 commonly produced macroconidia with asymmetrical apical cells (Plate 1.10) indicative of C. didymum (Hartig) Wollenw. (Domsch et al., 1980).

Species identification [after Booth (1966)]

Fifty-six of the 80 isolates examined were identified as C. destructans (Zins.) Scholten. Isolates that key out to this species vary considerably in macroconidial appearance (Plates 1.11 to 1.13) and include those with bay and fulvousumber pigmented cultures. This species appears to be widespread throughout Tasmania and was isolated from soil, the rhizoplane of healthy plants, diseased roots, and discoloured vascular tissue.

Isolates B1, S2, T1 and V16 produced pale fulvousumber pigmented, odourless cultures. Microconidial production was rare, macroconidia were straight with three or less septa (Plate 1.14), and barrel-shaped chlamydospores were produced in the mycelium of mature cultures. Isolate T1 was identified as C. obtusisporum (Cooke and Harkness) Wollenw. (Booth pers. comm.) which would appear to fit all four of these isolates. Isolate E4 produced cultures of similar appearance to these isolates, but the macroconidia were somewhat larger
Plate 1.8

Macroconidia of *C. tenue* (E3): small and one septate.

Plate 1.9

Macroconidia of *C. theobromicola* (B5): large, slightly curved and three septate.
Plate 1.10

Macroconidia of *C. didymum* (S1): mostly 1-2 septate with apical cell often asymmetrically curved.

Plate 1.11

Macroconidia of *C. destructans* (V4): long, mostly straight, 0-4 septate.
Plate 1.12

Macroconidia of *C. destructans* (St1): straight to slightly curved, wide, 1-3 septate.

Plate 1.13

Macroconidia of *C. destructans* (Pn1): short, straight and mostly aseptate. [Atypical strain of *C. destructans* (Booth, pers. comm.).]
Plate 1.14

Macroconidia of *C. obtusisporum* (T1): straight, 1-3 septate.

---

Plate 1.15

Macroconidia of *Cylindrocarpon* sp. (E1) (IMI 196141): straight, mostly 1-3 septate.
and slightly curved. This isolate was identified as an atypical isolate of *C. candidum* (Link) Wollenw. (Booth pers. comm.).

Three isolates were identified as *C. didymum*. Two of these, isolated from the rhizoplane of the feeder roots and the vascular tissue in the crown respectively of a strawberry plant, produced buff pigmented cultures. The other, isolated from vineyard soil, produced a fulvous-umber pigmented culture. All three isolates produced toilet soap-scented cultures.

Two isolates, one from eucalypt forest soil and litter respectively, were identified as *C. tenue*. These both produced odourless, bay pigmented cultures.

One isolate from the decaying roots of a glasshouse-grown begonia was identified as *C. theobromicola*. This isolate produced a buff pigmented culture with a weak actinomycete-like odour.

The remaining isolates belong to an undescribed species of *Cylindrocarpon* (Booth, pers. comm.) representatives of which have been deposited with the Commonwealth Mycological Institute Herbarium (IMI 196141, IMI 260246). Isolates of this species produce bay pigmented cultures with tuberculate globose chlamydospores (10-14μ diameter) which commonly aggregate into microsclerotia. They do not produce a definite microconidial state. Macroconidia are commonly 0-3 septate (Plate 1.15).

This species was frequently isolated from *Eucalyptus obliqua* L'herit forest litter samples collected from a forest suffering 'regrowth dieback' near Hastings in southern Tasmania (Appendix 1.2b). Jehne (1976) reported that this species of *Cylindrocarpon* was consistently isolated from the cortical tissues of the feeder roots of *E. obliqua* trees suffering
'regrowth dieback' in southern Tasmania. This species was also recovered from the discoloured vascular tissue in the basal stem portion of diseased boronia and lavender bushes grown at Richmond and Lilydale respectively (Appendix 1.2b).

3.1.2 Toxin Production in vitro

The aim of this work was to determine the frequency and distribution of Tasmanian soil-borne Cylindrocarpon isolates capable of producing the toxins breveldin A and radicicol.

3.1.2.1 Rapid Qualitative Assay

A technique was developed to enable large numbers of isolates to be screened rapidly for their ability to produce breveldin A and radicicol using a single agar plate culture.

Materials and Methods

Cultural

Isolates to be tested were grown on 15ml of Czapek Dox agar, modified by the addition of 0.05% yeast extract (Oxoid), in 9cm diameter petri dishes.

Plates were inoculated centrally with a single 4mm² plug cut from the advancing edge of a CDA culture, and incubated at 22⁰C in the dark. Two petri dish cultures were grown for each isolate to be tested.

The cultures were harvested when the radial growth had just reached the edge of the petri dish (12 to 14 days for most isolates).

Extraction

The entire contents of each petri dish culture were
placed into separate 100ml erlenmeyer flasks containing 40ml of redistilled ethyl acetate and capped with aluminium foil to prevent solvent evaporation. After 24 hours extraction the solvent was decanted from each flask, dried with 2g anhydrous Na₂SO₄, and concentrated to 0.5ml by rotary vacuum evaporation at 45°C. Extracts were stored in screw-capped glass vials at room temperature.

Standards

Authentic samples of brezelfdin A and radicicol were supplied by Professor T.R. Watson (Department of Pharmacy) and Dr. W.C. Taylor (Department of Organic Chemistry) of the University of Sydney. Standard solutions of these authentic samples were prepared in ethyl acetate (2mg ml⁻¹).

Thin Layer Chromatography (TLC)

Extracts and standards were chromatographed on 10 x 10cm Silica Gel 60 HPTLC plates (Schleicher and Schüll G1570/LS25). Plates were activated at 110°C in a vertical position and stored in a desiccator. Plates were cleaned just prior to use by developing them fully in ethyl acetate-methanol 95:5 (v/v). The solvent was evaporated and the plates covered with another clean 10 x 10cm glass plate, leaving a 15mm strip of layer exposed at the base of the plate. The second glass plate prevented the absorption of moisture from the air and provided a straight reference edge from which to load the samples.

Disposable glass micropipettes were used to apply 0.1μl samples of the extracts and standards, 1mm behind the edge of the overlying glass plate. Spots of less than 1mm in diameter could be achieved by this method and 24 samples
spaced 4mm apart were loaded per plate.

The loaded plates were equilibrated in an atmosphere of ethyl acetate-methanol 95:5 (v/v) for 15 minutes then developed vertically in a glass tank (20 x 8 x 16cm) lined with chromatography paper wicks containing 100ml of the same solvent. The ethyl acetate-methanol solvent was prepared freshly just before each run. Plates were developed until the solvent front travelled 4.0cm from the origin (approximately five minutes).

After evaporation of the solvent, detection was achieved by spraying plates with p-anisaldehyde reagent (Appendix 1.4) and heating at 110°C for ten minutes. Plates were observed under natural and 366nm light.

Results and Discussion

Brefeldin A was visualised as a violet-grey spot under normal light and as a dark pink spot under 366nm light, and had an $r_f$ of 0.45 to 0.50. Radicicol was visualised as a dark grey spot under natural light and a yellow spot under 366nm light, and had an $r_f$ of 0.65 to 0.70. Other lipid soluble components of the extracts were well separated from these two metabolites (Plate 1.16).

The sensitivity of the assay was shown to be sufficient to detect 10μg of brefeldin A or radicicol per petri dish.

Appendix 1.3 contains the results of this assay for all the Tasmanian soil-borne isolates examined. Of the 80 isolates tested, 41 produced brefeldin A, 21 produced radicicol, and 18 produced neither of these metabolites at the level of sensitivity of the assay. No isolates produced both of the metabolites. These results support the view of Evans (1964)
TLC assay of ethyl acetate extracts of CDA cultures of Cylindrocarpon for brefeldin A and radicicol.

(Plates treated with p anisaldehyde reagent and photographed under visible light.)

The first and second spots from the left were samples of pure radicicol and brefeldin A respectively. The remaining samples were extracts of different Cylindrocarpon isolate cultures, some of which can be seen to contain either brefeldin A or radicicol.

Brefeldin A \( r_f = 0.47 \)
Radicicol \( r_f = 0.69 \)
that nectrolide (brefeldin A) producing strains of C. destructans were more widespread than radicicol producing strains.

Some interesting correlations exist between the ability of isolates to produce these metabolites, and cultural pigmentation and odour (Tables 1.4 and 1.5).

**Table 1.4 The association between Cylindrocarpon culture pigmentation and toxin production.**

<table>
<thead>
<tr>
<th>Toxin produced</th>
<th>Bay (number of isolates)</th>
<th>Fulvous-umber</th>
<th>Buff</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brefeldin A</td>
<td>7</td>
<td>32</td>
<td>2</td>
<td>41</td>
</tr>
<tr>
<td>Radicicol</td>
<td>21</td>
<td>0</td>
<td>0</td>
<td>21</td>
</tr>
<tr>
<td>Neither</td>
<td>13</td>
<td>4</td>
<td>1</td>
<td>18</td>
</tr>
<tr>
<td>Total</td>
<td>41</td>
<td>36</td>
<td>3</td>
<td>80</td>
</tr>
</tbody>
</table>

Data from Appendix 1.3.

**Table 1.5 The association between Cylindrocarpon culture odour and toxin production.**

<table>
<thead>
<tr>
<th>Toxin produced</th>
<th>Soapy (number of isolates)</th>
<th>Actinomycete-like</th>
<th>Non-distinct</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brefeldin A</td>
<td>29</td>
<td>0</td>
<td>12</td>
<td>41</td>
</tr>
<tr>
<td>Radicicol</td>
<td>0</td>
<td>0</td>
<td>21</td>
<td>21</td>
</tr>
<tr>
<td>Neither</td>
<td>0</td>
<td>1</td>
<td>17</td>
<td>18</td>
</tr>
<tr>
<td>Total</td>
<td>29</td>
<td>1</td>
<td>50</td>
<td>80</td>
</tr>
</tbody>
</table>

Data from Appendix 1.3.

All isolates producing cultures with a 'soapy' odour produced brefeldin A. Most of the isolates producing fulvous-
umber pigmented cultures produced brefeldin A. However, there were five isolates which produced bay pigmented, non-odoriferous cultures that also produced brefeldin A.

All isolates that produced radicicol had bay pigmented, non-odoriferous cultures.

3.1.2.2 The Isolation of Pure Brefeldin A and Radicicol

Sufficient quantities of pure brefeldin A and radicicol were required for subsequent experiments on the properties of these substances. The methods used for the production and isolation of brefeldin A were modified from those of Evans (1964). The radicicol isolation procedure was modified from that of Nozawa and Nakajima (1979).

Materials and Methods

Cultural

Still surface cultures of Cylindrocarpon isolates, known from the HPTLC assay to produce brefeldin A or radicicol, were grown on Czapek Dox liquid medium supplemented with 0.05% (w/v) yeast extract (Oxoid). Cultures were grown in either 4L penicillin flasks or 500ml erlenmeyer flasks containing 1L or 100ml aliquots of medium respectively.

The flasks were inoculated with a 1cm² plug cut from a mature sporulating CDA culture, gently shaken to disperse the conidia, and incubated at 22°C in the dark. Ten flasks were inoculated with each isolate.

After 14 days the mycelium was removed by filtration, dried at 80°C for 24 hours, and weighed.

Extraction and purification of brefeldin A

Culture filtrates were bulked and extracted with an
equal volume of chloroform. The chloroform was then sequentially washed with an equal volume of 0.1M NaHCO₃ and 0.1M NaOH, dried with anhydrous Na₂SO₄, and concentrated to dryness by rotary vacuum evaporation. The residue consisted of crystalline brefeldin A slightly contaminated by a brown gum. Pure brefeldin A could be recovered as colourless needle-shaped crystals by successive recrystallization from ethyl acetate.

Extraction and purification of radicicol

Culture filtrates were extracted in 1L portions with an equal volume of chloroform. The chloroform was then washed with an equal volume of 0.1M NaCl, dried with anhydrous Na₂SO₄, and concentrated to 10ml by rotary vacuum evaporation. The 10ml of concentrated extract was loaded onto a 20 x 1.0cm column of silica gel (100-200 mesh) and eluted with chloroform-methanol 40:1 (v/v). The first 35ml of eluent was discarded and the next 20ml collected. The final column fractions from several 1L extractions were bulked and concentrated to dryness by rotary vacuum evaporation. The residue was recrystallized from ethyl acetate to yield pure radicicol.

Confirmation of identity

Melting points were determined using an electrothermal melting point apparatus with capillary melting point tubes. The values presented are uncorrected for thermometer expansion. Infrared spectra (IR) were determined on a Beckman IR spectrophotometer using samples prepared in nujol.

Mass spectra were obtained on a VG7070F double focussing quadrapole mass spectrometer using an ionization potential of 70eV and an accelerating voltage of 4kV.
Mass spectra were also obtained from the authentic samples supplied by Professor T.R. Watson (Department of Pharmacy) and Dr. W.C. Taylor (Department of Organic Chemistry) at the University of Sydney.

Results and Discussion

Of the several isolates of *Cylindrocarpon* tested, yields of brefeldin A under these conditions ranged from approximately 80 to 130 mg per litre of culture filtrate. Yields of radicicol were of the order of 40 to 50 mg per litre culture filtrate.

The melting point of brefeldin A was determined as 204-205 °C [reported 204-205 °C, (Sigg, 1964); 208-209 °C, (Evans et al., 1967)]. The melting point of radicicol was determined as 192-193 °C [reported 195 °C, (Evans, 1964); 191 °C Nozawa and Nakajima, 1979]).

The IR spectra were consistent with the spectra published for brefeldin A by Harri et al. (1963) and Evans (1964) and the spectral data for radicicol supplied by McCapra et al. (1964) and Mirrington et al. (1966).

The mass spectra of the two metabolites (Appendix 1.5) were identical to those of the authentic samples and consistent with the spectral data reported by Taniguchi et al. (1973) for brefeldin A, and Nozawa and Nakajima (1979) for radicicol.

3.1.3 Pectic Enzyme Production in vitro

Enzymes capable of degrading plant cell wall and membrane constituents have been shown to be produced in vitro by many phytopathogenic fungi and have been hypothesized to have a role in pathogenesis (Bateman and Basham, 1976). Of these, the pectin depolymerizing enzymes have been
the most studied and are the best understood in relation to pathogenicity. Based on the current concepts of cell wall structure (Keegstra et al., 1973), it would seem quite reasonable that pectin depolymerizing enzymes would have a key role in cell wall decomposition. These enzymes are capable of acting on intact cell walls and probably loosen their structure rendering the other wall polymers susceptible to enzymic degradation (Bateman and Basham, 1976).

The primary role of pectic enzymes is particularly evident in the 'soft-rot' type of tissue maceration of storage organs and the damping-off of young seedlings (Wood, 1967; Mount, 1978).

The production of pectic enzymes by Cylindrocarpon species has been demonstrated by Milko and Melnik (1960), Jackson (1965), Lyr and Kluge (1968) and Domsch and Gams (1969).

A study of the pectic enzymes produced in vitro by Tasmanian soil-borne Cylindrocarpon isolates was undertaken using the electrophoretic method of Cruickshank and Wade (1980). The electrophoretic patterns of fungal isoenzymes (zymograms) have been shown to be of taxonomic value by several workers (Wong and Willetts, 1973, 1975; Lawson et al., 1975; Willetts et al., 1977). Pectic zymograms have shown promise in distinguishing between species of Fusarium (Scala et al., 1981) and Sclerotinia (Cruickshank, 1983). Taxonomic studies per se were not intended to become a major part of this thesis. However, it was hoped that the pectic zymograms might enable pathogenic and non-pathogenic strains of Cylindrocarpon to be distinguished.
Preliminary studies showed that the medium on which the Cylindrocarpon isolates were grown and the physiological age of the cultures greatly influenced the number of isozymes detected. The cultural conditions described below resulted in the detection of a large number of isozymes for most isolates.

Materials and Methods

Cultural

Cultures were grown in 9cm diameter petri dishes containing 20ml medium per dish. The medium consisted of:

- Citrus Pectin (Sigma, P9135), 10g;
- ammonium sulphate, 2.0g;
- magnesium glycerophosphate, 0.5g;
- potassium chloride, 0.2g;
- yeast extract (Oxoid, L21), 0.5g;

in 1L of distilled water.

Inoculum consisted of a 1cm² plug cut from the advancing edge of a CDA culture of the test fungus. Cultures were incubated for ten days at 22°C in the dark.

Electrophoresis

The method of Cruickshank and Wade (1980) was employed with slight modifications. Pectin-acrylamide gels 160mm long by 82mm wide by 2mm thick with 4.5µl sample wells were used. The pectin acrylamide mixture was the same as that used by Cruickshank and Wade (1980) (Appendix 1.6) but gels were prepared the same day as electrophoresis was to be conducted.

To improve electrophoretic separation, culture filtrates were sampled and mixed with a one-fifth volume of superfine Sephadex G-150 to form pipettable slurries which were loaded into the gel wells (Cruickshank, 1983). Electrophoresis was conducted at 4°C with the gels supported by efficient cooling plates. Gels received a constant current of 16mA until the
bromophenol tracker dye had migrated 50mm from the front of the sample wells.

**Enzyme detection**

After electrophoresis the gels were incubated in 0.1M malic acid for 90 minutes at 18°C. Gels were then stained in aqueous 0.03% (w/v) Ruthenium Red at 4°C overnight, then rinsed in several changes of chilled water for one hour. A direct photographic print of the gel was made onto high contrast photographic paper (Ilfochrome 5.1M).

Dark bands on the prints correspond to polygalacturonase activity and white bands to pectin esterase activity (Cruickshank and Wade, 1980). Rf values of the bands were calculated as the ratio of the distance from the leading edge of the well to the centre of the band, to the distance the tracker dye migrated from the leading edge of the wells.

**Results and Discussion**

The pectic zymograms produced by all the isolates tested appear in Plates 1.17-1.20.

Several isolates had virtually identical zymograms. Isolates V1 and V4 were considered identical, as were V3, V5, V6, V8, V9, V12, V15, V17 and V18 (Plate 1.17). All these isolates came from diseased tissue or the rhizoplane of grapevines. However, identical zymograms do not necessarily indicate a host specialization of the isolates. Isolates V10 and V11 (Plate 1.17) from grapevine rhizoplane had an identical zymogram to isolates P2 and P8 (Plate 1.20) from diseased peppermint roots.

All of the isolates identified as *C. obtusisporum* (B1, S2, V20 and T1) had an identical zymogram. However, isolate E4, identified as an atypical strain of *C. candidum* (Booth,
Plates 1.17 to 1.20

Pectic zymograms of Tasmanian soil-borne *Cylindrocarpon* isolates.

Dark zones on the photographic prints correspond with polygalacturonases.

White zones on the photographic prints correspond with pectin esterases (see Cruickshank and Wade, 1980). Wells labelled with the *Cylindrocarpon* isolate code (Appendix 1.2).
Isolates B2 and B4 were subsequently identified as *Cylindrocladium parvum* Anderson.
pers. comm.), also had this zymogram (Plate 1.21). The two isolates of *C. tenue* (E3 and E9) had identical zymograms which were distinct from the zymograms of all the other isolates (Plate 1.18). Two of the isolates of *C. didymum* (S1 and S6) had an identical zymogram and the third isolate of this species, (V7), produced many of the same complement of isozymes (Figure 1.2). The single isolate of *C. theobromicola* (B5) had a zymogram distinct from all the other isolates (Plate 1.18). However, for isolates identified as *C. destructans*, considerable variability between zymograms was evident (Plate 1.21).

Correlations appear to exist between the cultural characteristics and toxin production of isolates and their zymograms (Tables 1.6 and 1.7). All isolates producing a pectin esterase at $R_f = 0.10$ have fulvous-umber pigmented cultures. All isolates with this pectin esterase band and a polygalacturonase at $R_f = 0.48$ produce toilet soap-scented cultures and brefeldin A.

**Table 1.6** The association between *Cylindrocarpon* culture pigmentation and pectin esterase production.

<table>
<thead>
<tr>
<th>Esterase $R_f$</th>
<th>Culture Pigment</th>
<th>Total (number of isolates)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bay</td>
<td>Fulvous-umber</td>
</tr>
<tr>
<td>0.10</td>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td>0.08</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Neither</td>
<td>41</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>41</td>
<td>36</td>
</tr>
</tbody>
</table>

Data from Appendix 1.3.
Plate 1.21

Pectic zymograms of C. candidum, C. obtusisporum, and several C. destructans isolates. (Wells labelled with *Cylindrocarpon* isolate code [Appendix 1.2].)

*E4 = atypical strain of C. candidum* (Booth, pers. comm.)

Interpretation of zymograms as for Plates 1.17-1.20.
Figure 1.2

Interpretative drawing showing the similarity between the pectic zymograms of *C. didymum* isolates S1 and V7.

Photographic print of stained gel (full size)

Interpretative drawing (x2)

--- polygalacturonase
--- pectin esterase
Table 1.7 The association between toxin and pectin esterase production by *Cylindrocarpon* isolates.

<table>
<thead>
<tr>
<th>Esterase R_f</th>
<th>Brefeldin A (number of isolates)</th>
<th>Radicicol</th>
<th>Neither</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.10</td>
<td>26</td>
<td>0</td>
<td>4</td>
<td>30</td>
</tr>
<tr>
<td>0.08</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Neither</td>
<td>12</td>
<td>21</td>
<td>14</td>
<td>47</td>
</tr>
<tr>
<td>Total</td>
<td>41</td>
<td>21</td>
<td>18</td>
<td>80</td>
</tr>
</tbody>
</table>

Data from Appendix 1.3.

Isolates with identical zymograms usually have macroconidia of similar size, shape and septation.

The analysis of pectic zymograms would appear to be a potentially powerful taxonomic tool for the genus *Cylindrocarpon*. Pectic isozymes produced under standardised cultural conditions provide a stable and reproducible character by which isolates may be compared. Pectic zymograms remain reproducible for isolates maintained for several years in culture, even in cases where morphological characters have altered.

Information obtained from amylase and ribonuclease zymograms was found complementary to pectic zymograms in this regard. All isolates with an identical pectic zymogram also had an identical amylase and ribonuclease zymogram (see Appendices 1.7 and 1.8 for methods and results).

A substantially larger culture collection, containing type specimens of a range of *Cylindrocarpon* species, would require analysis before an attempt could be made to correlate zymogram patterns with the species concept of Booth (1966).
3.1.4 The Effect of Temperature on Growth in vitro

A knowledge of how temperature influences the growth of Cylindrocarpon species was considered relevant to a study of their pathogenicity. Preliminary experiments indicated that the optimum temperature for growth of several Tasmanian soil-borne Cylindrocarpon species on CDA was between 18 and 25°C. None of the isolates grew above 30°C but some continued slow growth at 1.5°C.

The behaviour of Cylindrocarpon species at low temperature has been studied by several workers. The incidence of C. destructans on the rhizoplane was shown to be greater at lower than at higher temperatures (Rouatt et al., 1963; Taylor and Parkinson, 1964; Thorton, 1965), and Taylor (1964) showed that the "competitive saprophytic ability" of this fungus was greater at 10°C than at 20°C. The data of Taylor (1964) showed that the growth rate of C. destructans was double that of Fusarium oxysporum at 10°C, but at 25°C the converse was true. A similar result was recorded in this study (Figure 1.1, Section 3.1.1.1). Cormack (1937) reported that C. ehrenbergii could grow at temperatures as low as -2°C in vitro. This fungus caused a root rot of alfalfa in Alberta, Canada, when the soil temperatures were low. Pathogenicity tests showed the fungus to be more virulent at 1.5°C than at 17 or 21°C.

It was therefore decided to study the growth rate of a broad spectrum of Tasmanian soil-borne Cylindrocarpon species at temperatures below the optimum for growth.

Materials and Methods

Fifty-eight Cylindrocarpon isolates were cultured on CDA at 22°C in the dark for seven days. Petri dishes (9cm
diameter) containing 15ml of CDA were inoculated at three positions equidistant from each other and the edge of the dish with 4mm\(^2\) plugs cut from the advancing edge of the CDA cultures of the isolates. The petri dishes were incubated in the dark at either 6\(^\circ\)C for 13 days, 12\(^\circ\)C for 9 days, 18\(^\circ\)C for 6 days, or 22\(^\circ\)C for 6 days. Each isolate was grown at each incubation temperature on one petri dish.

The radial extension of the colonies was determined as a measure of growth. The distance from the edge of the inoculum plug to the edge of the colony was measured in two directions at right angles for each of the three inoculum plugs and the mean value was recorded. From this value the mean radial growth rate over the period of incubation was determined.

Results and Discussion

The radial growth of each of the *Cylindrocarpon* isolates at each of the temperatures studied are recorded in Appendix 1.3. These results show that the isolates can be categorised as either "fast" or "slow" growing types at low temperature. Furthermore, the growth rate of isolates at low temperature appeared to be correlated with cultural pigmentation type (Table 1.8). Temperature-growth rate curves for two isolates of *C. destructans* (V1 and V14) are presented in Figure 1.3 as representative examples of "slow" and "fast" growing types.
Figure 1.3
Temperature-growth rate characteristics for two isolates of *C. destructans*

V1 'slow' type at low temperature.
V14 'fast' type at low temperature.

*Data from Appendix 1.3.*
Table 1.8. The association between *Cylindrocarpon* culture pigmentation and growth rate at low temperature.

<table>
<thead>
<tr>
<th>Culture Pigmentation</th>
<th>Slow*</th>
<th>Fast*</th>
<th>Total (Number of Isolates)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fulvous-umber</td>
<td>23</td>
<td>1</td>
<td>24</td>
</tr>
<tr>
<td>Bay</td>
<td>8</td>
<td>24</td>
<td>32</td>
</tr>
<tr>
<td>Buff</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>25</td>
<td>58</td>
<td></td>
</tr>
</tbody>
</table>

Data from Appendix 1.2.

*Slow: <0.46mm/day at 6°C and <1.17mm/day at 12°C.

*Fast: >0.46mm/day at 6°C and >1.17mm/day at 12°C.

The "fast" growing isolates at low temperatures (as defined in Table 1.8) all produced bay pigmented cultures except for isolate K1. Most of the eight other bay pigment producing isolates had growth rates which very nearly put them in this "fast" growing category. All but one of the isolates producing fulvous-umber pigmented cultures were "slow" growing isolates at low temperature. In Sections 3.1.2 and 3.1.3 toxin and pectin esterase production were shown to be associated with cultural pigmentation types. Thus a correlation also appears to exist between toxin and pectin esterase production and growth rate at low temperature. Most "slow" growing isolates produce brefeldin A and pectin esterase whereas most "fast" growing isolates do not.

The "fast" growing types are presumably particularly well adapted to growth and colonization of organic matter and plant root surfaces at low soil temperatures. However, even the "slow" growing types are probably better adapted to low soil temperatures than many other common soil saprophytes.
Isolate V1 (a "slow" type) for example, has a faster growth rate on CDA than *Fusarium oxysporum* and *F. solani* at 5°C (Figure 1.1, Section 3.1.1.1).