EFFECTS OF ROOT-INVADING FUNGI ON THE GROWTH OF RED CLOVER (*Trifolium pratense* L.)

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ABSTRACT

Methods to monitor invasion of red clover roots by soilborne fungi, and assess effects on clover growth and persistence, were developed and tested using soil from a plant breeder's red clover evaluation block at DSIR Grasslands Division, Palmerston North, which was known to contain several fungal species pathogenic to red clover. A quantitative method employing tissue maceration and plating was used to determine the internal microflora of red clover roots from the evaluation block. Effects of environmental factors, and of application of fungicide drenches to the soil, on root invasion under controlled environment and field conditions were also studied using the root maceration method. Fungi isolated from roots of red clover from the evaluation block were tested for their effects on establishment, growth, and persistence of red clover in the glasshouse and in field microplots. Light microscopy and transmission electron microscopy were used to study invasion of red clover roots by Trichocladium basicola, and the effects of the fungicides benomyl and prochloraz on this fungus in vitro and in vivo.

The root maceration method detected a similar range of fungi to that found by plating 1 - 2 mm long segments, but yielded more colonies and showed less variation. Using standardised amounts of tissue and blending times (2 g and 60 or 120 sec.) differences in fungal populations in roots subjected to different treatments were readily detected.

Verticillium dahliae, Trichocladium basicola, and Cylindrocladium scoparium were the major components of the root-invading mycoflora of red clover in the evaluation block, which consisted of 40 fungal species. Other major invaders were Fusarium solani, F. oxysporum, Cylindrocarpon destructans, and Gliocladium roseum, which are the fungi most commonly isolated from roots of red clover and other forage legumes worldwide.

Fungal invasion of red clover roots was affected by plant age, soil temperature and moisture. Generally, numbers of fungal colonies isolated progressively increased from the seedling stage onwards and more colonies were

isolated from roots of plants grown at 20 and 25°C than at 10 and 15°C, and from 60 and 80% WHC than at 40% WHC.

Prochloraz was the most suitable of 11 fungicides tested for use as a soil drench to study effects of root-invading fungi on red clover growth in the field. It showed a broad spectrum of antifungal activity, controlled the major root-invading fungi encountered in the experimental soil, was not toxic to Rhizobium trifolii, and was least retardant to red clover growth.

The numbers of fungal colonies recovered per gram of roots was 60 - 80% lower from plants from field plots receiving a single application of prochloraz drench at 3.46 g/m^2 than from plants from untreated plots. Yields from treated plots harvested 4 times over a period of 45 weeks were 28 - 95% higher than those from untreated plots.

The major root-invading fungi isolated from red clover plants grown in the plant breeder's evaluation block, Cy. scoparium, T. basicola, F. solani, and F. oxysporum, reduced survival of red clover plants in field plots and microplots by 20 -75%, and dry matter yield by 20 -60%, over a periods of 62 and 76 weeks. V. dahliae, C. destructans, and G. roseum, also reduced plant growth in field plots but to a lesser extent. Seedling establishment, and nitrogen fixation and nodulation, were affected adversely by some fungal isolates.

T. basicola was found to penetrate roots of red clover directly and colonise tissue by means of "beaded hyphae" (intracellular hyphae which were constricted at their septa) then "straight hyphae" (unconstricted hyphae growing parallel to the root axis). The fungus is hemibiotrophic. Invaded host cells in the epidermis and cortex of the root are apparently unaffected at first then degenerate and die. Papillae are often found at sites of penetration through cell walls but these rarely obstruct fungal development. Pre-treatment of seedlings with benomyl or prochloraz reduced fungal penetration and growth within tissues. Changes in fungal ultrastructure resulting from benomyl treatment were an increased frequency of lomasome production and occasionally a disorganisation of cell contents. Changes resulting from prochloraz treatment included thickening and fragmentation of cell walls, and necrosis of hyphal cells.

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CHAPTER 1
INTRODUCTION

Red clover (Trifolium pratense L.) is an important legume in world agriculture. It produces large quantities of high quality forage for grazing or conservation, has wide geographic adaptation, and improves soil fertility through symbiotic nitrogen fixation. Red clover is currently attracting even greater attention as it, and other forage legumes, become increasingly used as alternatives to nitrogen fertilisers in both developing and developed countries, in response to growing concern about the effect on fresh water quality of a burgeoning use of fertiliser nitrogen for grassland farming.

Red clover is a perennial, but it is not uncommon for stands to die out in one or two years (Fergus and Hollowell, 1960; Rumball, 1983). Root deterioration caused by a complex of soilborne fungi is one of the major causes of decline in red clover stands (Kilpatrick et al., 1954; Fezer, 1961; Ylimäki, 1967; Leath et al., 1971; Rufelt, 1986; Skipp et al., 1986; Marten, 1989).

Despite recognition of the existence of root rot problems of red clover from as long ago as the 17th century (Fergus and Valleau, 1926), and the occurrence of root disease wherever the crop is grown, the cause and effects of the disease have not been fully understood, and suitable experimental techniques have not been developed to analyse effects of root disease on red clover growth. Consequently, there is not enough knowledge available to optimise disease management practices, as is done with many other crops.

In New Zealand, persistence has been an important criterion used by Grasslands Division, DSIR, for evaluating red clover cultivars (Claydon and Rumball, 1982). Evaluation of persistence of red clover at Palmerston North has been carried out in a particular block of land which has been maintained as a red clover monoculture for several years. Root rot and poor persistence in this block are associated with a complex of pathogenic, root-invading fungi (Skipp et al., 1986), many of which are present in red clover in New Zealand farms (R.A. Skipp and M.J. Christensen, pers. comm.). This suggested that the plant breeder's

evaluation
block was a good disease nursery and could be a suitable experimental site to develop generally applicable methods to study the cause and effect of red clover root rots.

The present study was started in October 1986 with the particular aims of : -

1. developing appropriate experimental techniques for monitoring fungal invasion of red clover roots and the response of plants to infection;

2. using these methods to assess effects of soilborne pathogens on establishment, survival, and growth of red clover; and

3. studying effects of environmental factors on fungal invasion of red clover roots.

CHAPTER 2
REVIEW OF LITERATURE

2.1 INTRODUCTION

The destruction of forage legumes by root diseases has long been recognised. The causal agents have been investigated extensively, together with their relationship with the hosts and the effect of environmental conditions on disease development. Several methods have been developed to produce diseased plants under controlled environmental conditions and some aspects of the effects of root diseases on plant growth have been assessed.

Although knowledge of the mechanisms and significance of root diseases of forage legumes is far from complete, progress has been made towards understanding and overcoming this problem.

Literature published in English since the early 1900's was surveyed. Some basic information about red clover is briefly documented, and the major achievements in the research of root diseases of forage legumes are reviewed and summarised.

2.2 RED CLOVER PLANT

2.2.1 Taxonomy, origin and distribution

Red clover belongs to the genus Trifolium L. The genus is in the tribe Trifolieae of the subfamily Papilionoideae, family Leguminosae (alternate name, Fabaceae). According to Zohary and Heller (1984) there are approximately 240 species of Trifolium divided into eight sections. The section Trifolium, containing about 70 species, has been further divided into 17 subsections. Red clover along with T. mazanderanicum Rech fil., T.noricum Wulf., T.pallidum Waldst.and Kit., and T.diffusum Ehrh., was assigned to the Trifolium subsection.

Within the species there are two main types of red clover: 1. an erect, open, early-flowering form known as Broad, medium, or double-cut red clover. 2. a prostrate, dense, late-flowering, and more persistent form known as Montgomery or single-cut red clover. Between these two types, there is a large number of intermediate types, which are mainly the result of breeding programmes. Of the New Zealand bred cultivars, 'Grasslands Hamua' is a Broad type, and 'Grasslands Turoa' is a Montgomery type (Rumball, 1983).

Red clover is thought to have originated in Asia Minor and south-eastern Europe, and is known to have been cultivated in Europe as forage by the third century A.D.(Rumball,1983). It was widely used in Spain during the sixteenth century. From there it spread to Holland and Germany, and was taken to England in 1650 and to the USA about 1700. Today, it is an important forage legume in the temperate regions of Europe, USSR, Australia, New Zealand, Argentina, Chile, Canada, Japan, Mexico, Columbia, eastern and central USA (Taylor and Smith, 1979), southern China (Ren J. Z. pers comm.), as well as high altitudes of subtropical regions (Rumball, 1983).

2.2.2 Importance in world agriculture

Red clover is of value because of its wide climatic range, high yield, and the nutritional value and digestibility of its herbage (Frame,1976). In addition, through symbiotic nitrogen fixation, red clover furnishes nitrogen for growth of companion grasses and subsequent crops. Estimated amounts of nitrogen fixed range from 125 kg/ha/year to 220 kg/ha/year (Smith et al., 1985). Because of these qualities red clover is valued in the temperate zones of both hemispheres for pasture, hay, and silage, for soil improvement, and for use in cropping rotations (Fergus and Hollowell, 1960).

Approximately 20 million ha of red clover are grown in the world (Smith et al., 1985). The overall economic importance of red clover to world agriculture is difficult to assess. However, information is available in some countries. Red clover is grown on about 7 million ha in the USA, mainly in mixed clover/grass pastures. In 1976, the hay crop of 4t/ha was worth about $ 1.5 billion, and the

seed crop about $ 25 million (Smith et al., 1985). In the UK, red clover is included in general purpose seed mixtures to improve hay yield in the first year, and to boost the quality and quantity of winter forage. Red clover is of lesser importance than white clover (Frame, 1976). In countries of northern Europe, such as Sweden and Finland, red clover is the dominant forage legume. It is normally grown in a mixture with forage grasses (Ylimäki, 1967; Rufelt, 1986).

In New Zealand agriculture, red clover is much less important than white clover in agriculture. It is used far more in systems of forage conservation and in short-term leys than in permanent pasture. Over recent years, about 400 t of Broad red clover seed and 300 t of Montgomery red clover seed have been harvested annually (Rumball, 1983).

2.2.3 Effects of soil temperature on red clover

Red clover is grown in areas where the annual mean temperature ranges from 4.9-20.3°C (mean of 91 cases = 10.6°C) (Duke, 1981). The minimum, optimum, and maximum temperatures for growth are 7, 20-25, and 35-38°C, respectively (Kendall, 1958; Fergus and Hollowell, 1960; Bowley et al., 1984). However, Gist and Mott (1957) and Smith (1970) reported that growth of red clover in plant growth chambers decreased with increasing temperature above 15°C. The number of shoots per plant increases as temperature decreases (Smith, 1970). Root growth is affected more than shoot growth when temperature is increased (Gist and Mott, 1957; Kendall et al., 1962).

At temperatures above 40°C, seedlings become chlorotic and die (Fergus and Hollowell, 1960). Therefore, it is likely that under a continental climate, red clover plants are adversely affected by high temperatures during summer months. However, the temperature at which stress begins varies with available soil moisture which may facilitate cooling of the plant (Kendall and Stringer, 1985).

Generally, the adverse effects of low temperature on red clover have been expressed as "winter killing". This catch-all term covers all phases of plant death in spring when new growth is initiated. There are many references related to this subject. More comprehensive information is available in the reviews by Fergus

and Hollowell (1960), Bowley et al. (1984), and Kendall and Stringer (1985).

Temperature also affects the effectiveness of the red clover/Rhizobium symbiosis. Roughley et al. (1981) found that the effectiveness of red clover and Rhizobium symbiosis was much less at 16/11 and 27/22°C (day/night) than at 22/17°C.

Smith (1970) described the effects of temperature on some inorganic and organic constituents of red clover. The concentration of potassium in the shoots was extremely low in plants grown at cool temperatures (15/10°C day/night), and the concentration of total available carbohydrates (TAC) in the roots was very low in the plants grown at high temperature (35/27°C). The poor persistence of red clover in summer months may be related to low levels of carbohydrate reserves associated with high temperature (Kendall, 1958; Fergus and Hollowell, 1960).

2.2.4 Effects of soil moisture on red clover growth

In the experiments involving the status of soil water content, different terms, such as "water content", "field capacity", "permanent wilting percentage", "readily available water content", "water holding capacity", and "water potential", have been used. No attempt is made here to define each term or compare one with another. Full definitions and discussions are available in Griffin (1966) and Kramer (1983).

Geographically, red clover is adapted within the area with annual rainfall of 310 - 1910 mm (mean of 91 cases = 860 mm) (Duke, 1981). Results from both controlled environment and field experiments showed that shoot and root weight were generally positively related to available soil moisture (Gist and Mott 1957, Kilmer et al., 1960). Highest yields of shoot and root were obtained from plants growing at nearly 100% of water holding capacity (WHC) (Gist and Mott, 1957). Shoot and root growth was reduced with decreasing soil moisture content (Kilmer et al., 1960; Bowley et al., 1984). Rooting depth decreased as soil moisture level increased from 20% to 70% of WHC (Bennet and Doss, 1960).

The most extreme drought condition which red clover plants survived was 9.4% of WHC in a pot trial conducted by Pohjakallio and Antila (1955). From the

same experiment they concluded that removal of shoots reduced the drought resistance of red clover plants. Under water stress red clover infected with vesicular-arbuscular mycorrhizae (Glomus mossae Nicol and Gerd.) extracted water from soil more effectively, and after watering, recovered turgidity more rapidly than non-mycorrhizal plants (Hardie and Leyton, 1981). However, Fitter (1988) reported that uptake of water by mycorrhizal plants was no better than by non-mycorrhizal plants.

Red clover tolerates up to 15 days of flooding, which is similar to alsike clover (T.hybridum L.) and lucerne (Medicago sativa L.) (Heinrichs, 1970).

An interesting interaction between soil moisture level and light intensity is that seedlings growing at low light failed to show a growth response when given adequate moisture, so that root development became restricted to the upper few cm of the soil where there was much less water than at greater depths. Therefore, plants growing in shade can suffer water stress more easily than those grown in full light (Dougherty, 1972; Kendall and Stringer, 1985).

Soil moisture stress can cause severe reduction in nitrogenase activity of red clover plants and thus affect N fixation (Rice, 1980).

The effects of soil moisture on inorganic and organic constituents in red clover have also been shown. Kilmer et al. (1960) showed that the concentration of phosphorus in the shoots increased as soil moisture increased, but concentrations of nitrogen, sulfur and boron in the plants were not significantly affected by variations in soil moisture. Heinrichs (1970) found that the concentration of crude protein in the shoot decreased in plants grown in a flooding situation, which suggested that nitrogen fixation may have been reduced.

2.3 ROOT DISEASES OF FORAGE LEGUMES, AND ASSOCIATED FUNGI

2.3.1. Fungi associated with root diseases

The results of a systematic literature survey on fungi associated with root diseases of forage legumes, with particular reference to red clover, showed that approximately 180 fungal species have been recorded (Table 2-3-1). About 112 of them have been reported from roots of red clover, 75% (84) were deuteromycetes. The remainder were plasmodiophoromycetes, oomycetes, zygomycetes, ascomycetes and basidomycetes, which accounted for 0.9% (1), 8% (9), 3.6% (4), 9.8% (11), and 2.7% (3), of the total number fungal species isolated from red clover roots, respectively (Table 2-3-1).

The fungal species found in roots, and the frequency of their occurrence, probably depends on geographical location, plant species, plant age, time of year, cultural practice, type of root tissue, and type of root. Some fungi, such as the fusaria, appear to be ubiquitous and were predominant among isolates obtained in most studies (Kilpatrick et al., 1954; Kilpatrick and Dunn, 1961; Fezer, 1961; Willis, 1965; O'Rourke and Millar, 1966; Aubé and Deschenes, 1967; Ylimäki, 1967; Leath et al., 1971; Richard, 1981; Skipp and Christensen, 1981; Wong et al., 1985 a; Skipp et al., 1986). Other fungal species commonly found to be associated with root diseases of forage legumes were species of Alternaria, Cylindrocarpon, Gliocladium, Phoma, Rhizoctonia, and some common rhizosphere fungi, such as species of Aspergillus, Penicillium, Rhizopus, and Trichoderma (Table 2-3-1).

Table 2-3-1 Fungi isolated from roots of forage legumes.

Fungi	Hosts and References
Myxomycota	
Physoderma alfalfae (Lagerh.) Karling (Urophlyctis alfalfae (Lagerh.) Magh.)	AF[1] 8[2], 27
Polymyxa graminis Ledingh.	RC 14 WC 39
Mastigomycotina	
Aphanomyces euteiches Drechs.	AF 27
Phytophthora clandestina Taylor,Pascoe & Greenhalgh	SC 4
P. megasperma Drechs.	AF 8 SC 4
P. megasperma f.sp.medicaginis Kuan & Erwin	AF 26
Pythium acanthicum Drechs.	BM 5 SC 4
P. arrhenomanes Drechs.	RC 17
P. coloratum Vaartaja	SC 46
P. debaryanum Hesse	AF 27 RC 17, 47 SC 4
P. echinulatum Matthews	SC 46
P. hypogynum Middleton	AF 27
P. irregulare Buism.	BM 5 RC 17 SC 4, 46
P. mamillatum Meurs.	SC 4
P. middletonii Sparrow	SC 4
P. oligandrum Drechs.	BM 5
P. paroecandrum Drechs.	AF 27 RC 17
P. rostratum Butler	BM 5 RC 17
P. spinosum Sawada	SC 4
P. splendens Braun	RC 17 SC 46
P. sylvaticum Campbell & Hendrix	AF 27
P. torulosum Coker & Patterson	AF 27
P. ultimum Trow	AF 27 BM 5 RC 17, 40
Pythium spp.	AF 8 RC 12, 21, 31, 40, 43 SC 46 WC 22, 23
Zygomycotina	
Absidia sp.	AF 33 RC 45 WC 22,23
Mortierella spp.	SC 46
Mucor spp.	AF 1, 33 RC 1, 21, 26, 45, 43 SC 46 WC 22, 23
Rhizopus stolonifer (Ehrenb.) Vuillemin (R. nigricans Ehrenb.)	RC 47 WC 22, 23
Rhizopus spp.	AF 1, 33 RC 1, 12, 21, 26, 40, 45
Ascomycotina	
Bimuria novae-zelandiae Hawksw., Chea & Sheridan	RC 40 WC 38, 39

Chaetomium aureum Chivers	RC 47
C. cochliodes Pall.	AF 1 RC 1, 47
C. crispatum (Fuckel) Fuckel	AF 1 RC 1
C. diliochotrichum Ames	AF 1 RC 1
C. globosum Kunze	AF 1 RC 1
Chaetomium spp.	AF 33 RC 10, 21, 26, 40, 45, WC 22, 38
Gibberella zeae (Schwe.) Petch	RC 21 WC 23
Sclerotinia trifoliorum Eriksson	AF 8, 10 RC 10, 22, 43, 47, WC 22
Sclerotinia spp.	AF 1 RC 1
Thielavia sp.	RC 21

Basidiomycotina

Ceratobasidium cornigerum (Bourd.) Rogers	RC 40 WC 38, 39
Ceratobasidium sp.	SC 4, 46
Marasmius graminum (Lib.) Fries	RC 47
Unidentified spp.	RC 11, 40

Deuteromycotina

Acremonium spp.	RC 40 WC 39
Alternaria alternata (Fries) Keissler	RC 21, 47 WC 22, 23
Alternaria spp.	AF 1, 10, 33 BT 32 RC 1, 10, 26, 45 SC 46
Aspergillus flavus Link & Gray	WC 22, 23
A.terreus Thom.	WC 22, 23
Aspergillus spp.	AF 10 BT 32 RC 10, 11, 21, 26, 31, SC 46
Aureobasidium pullulans (deBary) Arnaud	SC 46
Botrytis cinerea Pers.	RC 47 WC 28
Botrytis sp.	AF 1, 10 RC 1, 45
Cepedonium sp.	RC 26
Cephalosporium spp.	AF 1 BT 32 RC 1, 10, 26
Chrysosporium sp.	RC 40 WC 38, 39
Cladosporium herbarum (Pers.) Link & Gray	WC 23
Cladosporium sp.	AF 1 RC 1, 45 WC 22, 23
Codinaea fertilis Hughes & Kendrick	RC 40 WC 6, 29, 38, 39
Colletotrichum destructivum O'Gara	RC 22 WC 22, 23
C. trifolii Bain & Essary	RC 22 WC 23
Colletotrichum sp.	AF 1 BT 32 RC 1, 11 WC 39
Camposporium sp.	RC 45
Coniothyrium (Coniothyrinula) sp.	AF 1, 33 RC 1, 10, 21, 26
Coprinus psychromorbidus Redhead & Traquair	AF 27
Curvularia sp.	RC 21 SC 46

Cylindrocarpon destructans (Zins.) Sch. (C. radicicola Wr.)	AF 1, 27 RC 1, 40, 43, 47 SC 46 WC 38
C. didymum (Hartig.) Wollenw.	SC 4
C. ehrenbergi Wr.	AF 1, 27 RC 47
C. lucidum Booth	BM 5
C. obtusisporum (Cke. & Hark.) Wr.	AF 27 RC 47
C. olidum (Wollenw.) Wollenw.	AF 27 SC 46
Cylindrocarpon sp.	RC 11, 40, 41, 45 WC 39
Cylindrocladium clavatum Hodges & May	AF 30
C. crotalariae (Loos) Bell & Sobers	AF 30
C. floridanum Sobers & Seymour	AF 30
C. scoparium Morgan	AF 30 RC 37, 40
Dendryphion nanum (Nees & Gray) Hughes	RC 40
Diplodia sp.	AF 1 RC 1, 10
Drechslera sp.	SC 46
Epicoccum purpurascens Ehrenb. (E. nigrum Link & Link)	RC 31
Epicoccum sp.	AF 1, 10 BT 32 RC 21, 26
Exophiala sp.	RC 40
Fusarium acuminatum Ell. & Everh.	BM 5 RC 15, 47 SC 46, 4
F. arthrosporioides Sherb.	AF 33 RC 47
F. avenaceum (Fr.) Sacc.	AF 8, 33 BM 5 RC 15, 41, 43 SC 4, 46 WC 39
F. culmorum (W.G.Smith) Sacc.	AF 27 BM 5 RC 41, 43, 47 SC 4, 46 WC 39
F. equiseti (Corda) Sacc.	BM 5 SC 4, 46
F. graminearum Schwabe	BM 5 SC 4, 46
F. heterosporum Nees.	SC 46
F. moniliforme Sheld.	AF 7, 10 RC 7, 9, 10 11, 21, 26, SC 4
F. oxysporum Schlecht.	AC 25 AF 1, 7, 8, 10, 27, 33 BM 5 RC 1, 7, 9, 10, 11, 12, 21, 22, 26, 31, 40, 41, 44 SC 4, 46 WC 22, 23, 38, 39
F. poae (Pk.) Wr.	BM 5 RC 47
F. roseum Lk.	AC 25 AF 1, 7, 10, 27 RC 1, 7, 9, 10, 11, 12, 21, 26, 44, 47 WC 22, 23
F. sambucinum Fuckel	AF 8 BM 5 RC 41, 47 SC 46
F. solani (Mart.) App. & Wr.	AC 25 AF 1, 7, 8, 10, 27, 33, RC 1, 7, 9, 10, 11, 12, 21, 26, 31, 40, 47 WC 22, 23
F. tabacinum (vanBeyma) W.Gams	SC 46
F. tricinctum (Corda) Sacc.	AF 27
Fusarium spp.	AC 25 AF 8, 33 BT 32 RC 31, 45
Geotrichum sp.	RC 21, 40 WC 23

Gliocladium roseum Bainier	AF 33 RC 10, 11, 12, 21, 26, 31, 40 SC 47 WC 22, 23
Gliocladium spp.	AF 1 BT 32 RC 1, 10, 12, 45 WC 38, 39
Gloeosporium sp.	AC 25
Harknessia (Chaetomella) sp.	AF 1 RC 1, 21
Helminthosporium sp.	AF 1 RC 21
Hormodendrum sp.	AF 1 RC 21
Humicola. sp.	RC 40
Mycoleptodiscus terrestris (Gerd.) Ostazeski (Leptodiscus terrestris Gerd.)	AF 7, 10, 27 BT 32 RC 7, 31 WC 22, 23
Mycoleptodiscus sp.	RC 10
Macrophomina phaseolina (Tassi) G.Goid	AF 27 BT 32 SC 4, 46
Macrosporium sp.	RC 45
Monilia sitophila (Mont.) Sacc.	AF 1 RC 1
Monilia spp.	RC 21 WC 22 33
Mycogone sp.	RC 10, 26
Myrothecium verrucaria (Fr.) Sacc.	SC 4
Neurospora sp.	WC 22
Nigrospora sp.	RC 21, 45 WC 22, 23, 28
Paecilomyces sp.	RC 40
Papularia sp.	AF 1 RC 1, 45
Penicillium sp.	AF 1, 33, 10 BT 32 RC 1, 10, 21, 26, 40, 43, 45 SC 46 WC 22, 23, 38
Periconia sp.	WC 39
Pestalotia truncata Lev.	RC 47
Peziza ostracoderma Korf	AF 1 RC 1
Phialophora sp.	SC 46
Phoma chrysanthemicola Hollós.	RC 40 WC 38
P. fimeti Brun.	WC 38
P. herbarum Westend.	AF 33
P. leveillei Boerema & Bollen.	SC 46
P. medicaginis Malbra. & Roum.	AF 8, 27 BM 5 SC 4, 46
P. pinodella (Jones) Morgan-Jones & Burch (P. medicaginis var. pinodella (Jon.) Boerema	RC 47
Phoma spp.	AF 1, 33 RC 1, 10, 26, 40, 41, 43, 45 WC 22, 23
Phomopsis sp.	WC 39
Phymatotrichum omnivorum (Shear) Dug. (Ozonium omnivorum Shear)	AF 27
Pithomyces sp.	SC 46
Plenodomus meliloti Dearn. and Sandf.	AF 27 RC 35 SC 35
Plenodomus sp.	AF 1
Pullularia sp.	AF 1
Pyrenochaeta terrestris (Hans.) Gorenz, Walker & Larson	RC 1, 31
Pyrenochaeta spp.	AC 25 AF 1 BT 32 RC 1

Rhizoctonia cerealis van der Hoe.	SC 4
R. crocorum (Pers.) DC.	AF 27 RC 47
R. endophytica Sak. & Vaa.	RC 47
R. leguminicola Gough & Elliott	AF 16 RC 12, 16, 21
R. solani Kühn.	AF 8 BM 5 BT 32 RC 22, 31, 47 SC 4, 46 WC 22, 23, 28
Rhizoctonia spp.	AC 25 AF 1, 10 BM 5 RC 1, 10, 11, 12, 26, 45, SC 4
Sclerotium bataticola Taub.	RC 18 WC 22, 28
S. rolfsii Sacc.	AF 27 WC 28
Scopulariopsis sp.	RC 40
Sepedonium sp.	RC 10
Sphaeropsis sp.	RC 21
Spicaria sp.	RC 11
Stagonospora meliloti (Las.) Petr.	AF 8, 27
Stemphylium botryosum Wr.	RC 47
Stemphylium spp.	AF 1 RC 1
Stysanus medius Sacc.	AF 1 RC 1
Tetracoccosporium paxianum Szabo	RC 44
Trichocladium basicola (Berk. & Br.) Car. (Chalara elegans Nag Raj & Kendrick, Thielaviopsis basicola (Berk. & Br.) Fer.)	AF 8 RC 40 SA 36
Trichocladium spp.	BT 32 RC 42 WC 22, 23
Trichoderma album Preuss	AF 1 RC 1
T. lignorum (Tode) Harz	WC 22, 23
T. viride Pers.	AF 1 RC 1, 10, 26
Trichoderma spp.	AF 10, 33 BT 32 RC 11, 12, 21, 40, 43, 45 SC 46 WC 39
Trotteria sp.	RC 21
Truncatella sp.	SC 46
Ulocladium sp.	SC 46
Verticillium albo-atrum Reinke & Bert.	AF 2, 8, 27 WC 2
V. dahliae Kleb.	AC 24, 25 AF 20 RC 2, 34, 40 SA 19
V. lateritium (Ehre.) Rabenh.	AF 1
V. nigrescens Pethybr.	AF 1
V. tenerum	RC 40
Verticillium spp.	AF 33 BT 32 RC 21,41 WC 23
Volutella sp.	RC 40
Waitea circinata War. & Tal.	SC 4, 46
Wardomyces anomala Brooks & Hansford	RC 1
Zygorhynchus sp.	AF 1 RC 10, 45
Zythia sp.	RC 21

[1]: **AC**, alsike clover; **AF**, lucerne; **BM**, barrel medick; **BT**, birdsfoot trefoil; **RC**, red clover; **SA**, sainfoin; **SC**, subterranean clover; **WC**, white clover.

[2]: **1**. Aubé and Deschenes, 1967. **2**. Aubé and Sackston, 1964. **3**. Baldwin, 1962. **4**. Barbetti, et al. 1986 a. **5**. Bretag, 1985. **6**. Campbell, 1980. **7**. Carroll and Elliott, 1964. **8**. Close et al., 1982. **9**. Crall, 1951. **10**. Elliott et al., 1969. **11**. Fezer, 1961. **12**. Fulton and Hanson, 1960. **13**. Garren, 1955.

14. Gerdemann, 1955. **15**. Gordon, 1959. **16**. Gough and Elliott, 1956. **17**. Halpin et al., 1952. **18**. Henson and Valleau, 1937. **19**. Isaac, 1946. **20**. Isaac 1957. **21**. Kilpatrick et al., 1954. **22**. Kilpatrick, 1959. **23**. Kilpatrick and Dunn, 1961. **24**. Leach et al., 1961. **25**. Leach et al., 1963. **26**. Leath et al., 1971. **27**. Leath et al., 1988. **28**. McCarter and Halpin, 1962. **29**. Menzies, 1973. **30**. Ooka and Uchida, 1982. **31**. Ostazeski, 1957. **32**. Pettit et al., 1966. **33**. Richard, 1981. **34**. Roberts, 1963. **35**. Sanford, 1933. **36**. Sears et al., 1975. **37**. Sherbakoff, 1928. **38**. Skipp and Christensen, 1981. **39**. Skipp and Christensen, 1983. **40**. Skipp et al., 1986. **41**. Sundheim, 1970. **42**. Tverskoi et al., 1950. **43**. Wanson and Maraite, 1984. **44**. Watson and Guthrie, 1964. **45**. Willis, 1965 **46**. Wong et al., 1985 a. **47**. Ylimäki, 1967

2.3.2 Seedling damping-off

Damping-off, caused by species of Pythium, Phytophthora, and other fungi, is the most widespread and destructive seedling disease of clovers and lucerne (Hanson and Kreitlow, 1953; Leath et al., 1988). Damping-off can destroy seedlings before (pre-emergence damping-off) or after (post-emergence damping-off) they emerge from the soil (Hanson and Kreitlow, 1953).

Resistance to seedling pathogens usually increases gradually as seedlings get older (Halpin and Hanson, 1958; Chi and Hanson, 1962; Latch and Skipp, 1987). Temperature, soil type, and soil moisture content influence the development of resistance (Chi and Hanson, 1962) as well as the severity of disease (Leach, 1947; Graham et al., 1957; Latch and Skipp, 1987). An exception to this general rule is infection by Phytophthora megasperma f.sp.medicaginis Kuan & Erwin, because this fungus can also cause root rot on adult plants of lucerne (Leath et al., 1988).

2.3.3 Root rot

Root rot is a severe and often limiting problem on red clover everywhere it is grown. It also occurs in varying degrees on other forage legumes. Damage to forage legumes resulting from root rot has been known in Europe since at least 1669 and in America since 1747 (Fergus and Valleau, 1926). The root rot problem has been studied extensively since the 1920s, particularly in the USA. Root rot is thought primarily to be caused by fungi, although physiogenic disorders, such as

internal breakdown in the crown of red clover (Graham et al., 1960; Cressman, 1967), and bacteria have now been also implicated in root deterioration (Shinde and Lukezic, 1974; Lukezic et al., 1983; Leath et al., 1989),

Many fungi can attack roots of forage legumes (Table 2-3-1). Some, such as Cylindrocladium spp.(Ooka and Uchida, 1982), and Codinaea fertilis Hughes & Kendrick (Menzies, 1973), are highly virulent pathogens. They can act alone, each causing a particular type of rot. Such cases have been well documentated and reviewed by Leath (1985), Latch and Skipp (1987), and Leath et al. (1988). In contrast, a complex of several relatively weak pathogens contribute to root deterioration, causing what Hanson and Kreitlow (1953) called "common root rot". This kind of root rot can occur at all stages of plant development. The general symptoms of the disease have been summarised by Hanson and Kreitlow (1953), Latch and Skipp (1987), Barbetti et al. (1986 a).

The rate of development of disease is often dependent on the environment and the type of management to which the crop has been subjected. Physical environment and stress of many kinds can enhance the development of root rot. These stress factors will be discussed in another section of this chapter.

Species of Fusarium are the fungi most commonly isolated from diseased roots of forage legumes (Table 2-3-1). This is probably the reason common root rot is also often called "Fusarium root rot complex" in North America (Leath et al., 1971) and Europe (Ylimäki, 1967; Sundheim, 1970; Rufelt, 1986). However, research in New Zealand suggests that Trichocladium basicola (Berk. & Br.) Car., Cy. scoparium Morgan, and Verticillium dahliae Kleb. should be considered as potentially important pathogens of red clover in addition to the Fusarium spp. normally associated with root rot in other parts of the world (Skipp et al., 1986).

Besides Fusarium spp., many other fungi have been reported as components of the root rot complex (Table 2-3-1). Many can colonise, invade, and cause root rot of red clover seedlings (Kilpatrick et al., 1954; Fulton and Hanson, 1960; Skipp et al., 1986). Some pathogenic similarities between fungal species isolated from roots of lucerne and red clover have been reported (Carroll and Elliott, 1964; Elliott et al., 1969; Leath et al., 1988).

2.3.4 Wilt

Wilt disease can destroy entire stands of lucerne within a few weeks (Kreitlow, 1962; Christen and Peaden, 1981), and limit production of clovers and other forage legumes (Isaac, 1946; 1957; Pratt, 1982). Although bacterial wilt of lucerne caused by Clavibacter michiganese subsp. insidiosum (McCulloch 1925) Davis, Gillaspie, Vidaver, & Harris 1984 (Corynebacterium insidiosum (McCulloch) Jensen) can be a serious problem in some areas, most wilt diseases of forage legumes are caused by Verticillium albo-atrum Reinke & Bert., V. dahliae, and Fusarium oxysporum Schlecht., (Isaac, 1946; 1957; Leach et al., 1963; Aubé and Sackston, 1964; Milton and Isaac, 1976; Pratt, 1979; 1982; Skipp et al.,1986; Latch and Skipp, 1987. For details see Table 2-3-1).

Details of individual wilt disease of forage legumes are summarised by Weimer (1928), Isaac (1946, 1957), Kreitlow (1962), Christen and Peaden (1981), and Pratt (1982).

Verticillium albo-atrum is the major causal agent of verticillium wilt in lucerne. It was reported first in Sweden in 1918 and in Germany in 1938 (Leath et al., 1988). It was established throughout Europe in the 1940's and 1950's (Kreitlow, 1962). It was first found in North America in Canada in 1962 (Aubé and Sackston, 1964), and in the USA in 1976 (Graham et al., 1977). The disease is one of the most destructive diseases of lucerne in Great Britain and Europe (Kreitlow, 1962), and occurs wherever the crop is grown. The pathogen has also been isolated from Ladino clover (Aubé and Sackston, 1964).

Verticillium dahliae has been isolated from lucerne (Isaac, 1957; Aubé and Sackston, 1964), red clover (Aubé and Sackston, 1964; Skipp et al., 1986), alsike clover (Leach et al., 1963), and sainfoin (Isaac, 1946).

Generally, verticillium wilt is much less of a problem on clovers than on lucerne. However, it has been reported that isolates of V. dahliae from lucerne caused wilt of red clover and other clovers, and vice versa (Aubé and Sackston, 1964; Milton and Isaac, 1976).

Different form species of F. oxysporum (that have differing host specificity) have been described on lucerne and clovers. F. oxysporum f.sp.

medicaginis (Weimer) Sny. and Hans., causal agent of Fusarium wilt in lucerne, was first reported by Weimer in 1928 in the USA, and has since been found in many other areas of the world. Some other forms have also been reported to be pathogenic to lucerne (Leath et al., 1988). A new form species of F. oxysporum has been reported from Mississippi which causes a wilt disease of crimson clover (Pratt, 1982).

A Fusarium wilt disease of red clover was reported from Russia in 1917 (Pratt, 1982), from Switzerland in 1928 (Neuweiler, 1928), and later from Iowa, USA (Crall, 1951). Fusarium oxysporum was thought to be the causal agent, but no detailed information was given by the authors.

2.4 EFFECTS OF SOIL TEMPERATURE AND MOISTURE, AND OTHER FACTORS ON ROOT ROT COMPLEX OF FORAGE LEGUMES

2.4.1 Effects of temperature and moisture on mycelial growth of root-rotting fungi from forage legumes in culture

The relationship between temperature, moisture, and fungal growth has been reviewed by Yarwood (1965), Griffin (1963), and Cook and Duniway (1981). The optimum temperature and minimum water potential for mycelial growth of some root-rotting fungi from forage legumes are listed in Table 2-4-1.

Optimum temperatures for fungal growth in culture ranged from 20° - 30°C (Table 2-4-1). Trichocladium basicola, C. destructans, and two Verticillium species have relatively low optimum temperatures (20 - 25°C), similar to those of red clover (see section 2.2.). The remaining fungi in Table 2-4-1 have a higher optimum temperature than red clover. The results indicate that, in most regions the temperature at some time (seasonally or diurnally) will be favourable for the fungi. Yet, diseases caused by T. basicola and Verticllium species occur more often at relatively low temperatures, whereas Fusarium diseases tend to be favoured by higher temperatures.

The minimum moisture requirements for fungal growth in culture are markedly different (Table 2-4-1). Rhizoctonia solani, T. basicola, and the two

Verticillium species require a higher moisture content than Fusarium species. Alternaria alternata can grow under extremely dry conditions. Since the minimum moisture requirements of the fungi (-60 to -210 bars) are well below those of their hosts (permanent wilting point at -15 bars), they would be expected to continue growing when host plants had wilted through lack of moisture. Most fungi will thus be less adversely affected than their hosts by decreased moisture content.

In practice, temperature, moisture, and other environmental factors can seldom be treated separately, because changes in one environmental factor can affect another. For example, the experiment carried out by Manandhar and Bruehl (1973) showed that the effects of temperature on the growth of V. dahliae in culture were influenced by moisture content. At 35°C, the fungus did not grow on ordinary corn meal dextrose agar, but when the water potential was reduced to -30 to -40 bars, considerable growth occurred.

Table 2-4-1. The optimum temperatures (°C) and minimum water potentials (-bars) for mycelial growth in culture of some selected fungi isolated from roots of forage legumes.

Fungi	Temperature	Moisture
Alternaria alternata	25-28 (4)[1]	210 (4)
Cylindrocarpon destructans	20-21 (4)	
Cylindrocladium scoparium	25-30 (4)	
Fusarium culmorum	25-30 (4)	130 (4)
F. moniliforme	28 (6)	180 (4)
F. oxysporum	28 (1, 4)	125-155 (4)
F. solani	28-29 (1, 4)	140 (4)
Gliocladium roseum	24-28 (6)	
Rhizoctonia solani	25-30 (8)	60 (5)
Trichocladium basicola	20-24 (4)	<80 (3)
Verticillium albo-atrum	20-24 (4)	<100 (3)
V. dahliae	20-25 (2)	<100 (7)

[1]: 1. Chi and Hanson, 1964. 2. Congly and Hall, 1976. 3. Cook and Papendick, 1972. 4. Domsch et al., 1980. 5. Dube et al., 1971. 6. Fulton and Hanson, 1960. 7. Manandhar and Bruehl, 1973. 8. Smith, 1946.

2.4.2 Effects of temperature and moisture on the development of root rot

The effects of temperature on root rot of forage legumes have been investigated in glasshouse experiments, and monitored in field plots.

The severity of root rot of red clover caused by Fusarium spp. (Kreitlow and Hanson, 1950; Chi and Hanson, 1959; Siddiqui and Halisky, 1968 a), or combinations of Fusarium spp. with other soilborne pathogens (Fulton and Hanson, 1960), increased with increased temperature in the range of 12 - 32°C. The most severe root rot developed at 28 - 32°C.

Similar results were reported by McCarter and Halpin (1962) on white clover, but they found that temperature-related effects varied with the individual fungal species. Wong et al. (1984) also found that the effect of temperature on the development of root rot of subterranean clover plants inoculated with a range of soilborne pathogens (both alone and in combinations) differed with the fungal species used. They demonstrated that the most severe root rot occurred at 10°C, with less at 15 and 25°C, and least at 20°C.

The effect of temperature on invasion and damage of forage legumes by root-invading fungi has also been investigated in field plots. O'Rourke and Millar (1966) found that the recovery of root-invading fungi (mainly F.oxysporum) from field grown lucerne, both as total numbers and percentage of isolates, was highest during the summer.

In most of the above studies when Fusarium spp. were the main component of root invading mycoflora, the influence of temperature on root rot was similar to that on the growth of Fusarium spp. in culture. This suggests that the Fusarium species dominate the interaction of plant and pathogen at high temperatures. In addition to direct effects on fungal growth, high temperatures may disturb physiological processes of the plant (see section 2.2.3).

The effect of soil moisture on root rot development has been less well studied than the effect of temperature and results show less agreement between one another. Root rot of red clover incited by Fusarium spp. was more severe

(Chi and Hanson, 1959; Ylimäki, 1967), and more plants died (Fezer, 1961), in dry soil than in very wet soils. However, Fezer (1961) found that poor aeration under high soil moisture conditions caused physiological deterioration of red clover roots.

Wong et al. (1984) reported that the most severe root-rotting of subterranean clover occurred at 65% of water holding capacity (WHC), with less at 45% of WHC, and least under flooding conditions.

However, in other reports, soil moisture content did not influence root rot development in lucerne (Leath et al., 1971; Emberger and Welty, 1983) or subterranean clover (Barbetti and MacNish, 1978).

Differences between the results of the above studies may partly be due to the use of different host plants and pathogens in each experiment, and to the use of different types of soil, and of different criteria for measurement of water status of the soils. It seems necessary for both water content and water potential to be measured in studies on the relationship between soil moisture and root rot development.

2.4.3 Effects of other factors on root rot development

Publications resulting from both field and glasshouse experiments have shown that there is a close connection between severity of root rot and the effects of various environmental, biological and management factors which put a stress on the plant. This topic has been reviewed by Leath et al. (1971) and Latch and Skipp (1987), so these factors will be only briefly discussed here.

Damage caused by insects and nematodes not only reduces the vigour of plants, but also, and perhaps more importantly, provides an avenue of entry for the root-rotting fungi through their feeding wounds on the root surface (Graham and Newton, 1960; Kilpatrick and Dunn, 1961; Leach et al., 1961; 1963; Willis and Thompson, 1979). Physiological changes taking place as a result of root injury can alter the host-pathogen interaction to favour fungal development in the root (Stutz et al., 1985). Consequently, root rot development can be enhanced by the presence of insects and/or nematodes.

The insects most often associated with root rots of clovers and lucerne are clover root curculio (Sitona hispidulus F.) and clover root borer (Hylastinus obscurus Marsham) (Leath et al., 1971). Increased root rot caused by the presence of the root curculio and/or root borer have been reported for red clover (Graham and Newton, 1959; Thompson and Willis, 1968), alsike clover (Leach et al., 1963), white clover (Graham and Newton, 1960; Kilpatrick and Dunn, 1961; James et al., 1980), and lucerne (Hill et al., 1969). Dunn et al. (1964), however, failed to find a link between Sitona spp. and root rot of white clover.

Other insects that have been shown to enhance the development of root rot included a root feeding weevil (Calomycterus setarius Roelofs) on red clover (Newton and Graham, 1963), and a fungus gnat, (Bradysia sp.) on red clover and lucerne (Leath and Newton, 1969).

Insects feeding on growing shoots also can affect root health. Leath and Byers (1977) reported that both pea aphid (Acyrthosiphon pisum Harris), and potato leafhopper (Empoasca fabae Harris) can increase root rot of red clover, white clover and lucerne.

Information on nematode damage to clovers can be found in a review by Skipp and Gaynor (1987). Root rot of lucerne has been reported to be enhanced by root knot nematode (Meloidogyne spp.) (McGuire et al., 1958; Kushner and Crittenden, 1967; Welty et al., 1980; Griffin and Thyr, 1988;), and root lesion nematode (Pratylenchus penetrans (Cobb) Filipjev & Schuurmans Stekhoven) (Mauza and Webster, 1982). In a field study, Wills and Thompson (1979) demonstrated that Fusarium infection of lucerne was least in plots with the lowest number of nematodes. In contrast, Leach et al. (1963) found no evidence that root rot of alsike clover increased in the presence of a Pratylenchus sp..

Infection by leaf and stem pathogens can also predispose plants to greater damage from root-rotting fungi. Diseases which have been reported to enhance the severity of root rot are spring black stem and leaf spot of lucerne caused by Ascochyta imperfecta Peck (Phoma medicaginis Mal. and Roum.) (O'Rourke and Millar, 1966), bacterial diseases of lucerne caused by Pseudomonas marginalis var. alfalfae Shinde & Lukezic 1974 or an unidentified bacterium (WB-3)) (Shinde and Lukezic, 1974), and diseases of red clover caused by red clover vein

mosaic virus (Denis and Elliott, 1967), clover yellow mosaic virus, white clover mosaic virus (Watson and Guthrie, 1964), of arrowleaf clover caused by bean yellow mosaic virus (Pratt et al., 1982), and of white clover by clover yellow vein virus (Campbell and Moyer, 1983).

Management practices have an important influence on root rot development of forage legumes. They either increase the severity of root rot or slow down disease development. Cutting regime and plant nutrient levels are two important considerations.

Clipping reduces carbohydrate reserves in the roots (Nelson, 1925; Virtanen and Nurmia, 1936; Tesar and Ahlgren, 1950; Colville and Torrie, 1962; Lukezic et al., 1969 b; Hay, 1985). Consequently, it can greatly influence the severity of root rot and plant survival. It has been found that frequent and severe clipping increases damage by root-invading fungi. This has been demonstrated in experiments with red clover (Fulton and Hanson, 1960; Fezer, 1961; Siddiqui et al., 1968; Elliott et al., 1969; Sundheim, 1970; Rufelt, 1986), white clover (Moody et al., 1967; Menzies, 1973), and lucerne (O'Rourke and Millar, 1966; Lukezic et al., 1969 a). However, experiments in which clipping did not increase root rot severity of red clover have also been reported (Leath and Kendall, 1978).

Addition of fertilizers, particularly potassium, can reduce root rot development as demonstrated experimentally on red clover (Chi and Hanson, 1964), and lucerne (Stivers et al., 1956; O'Rourke and Millar, 1966). Fezer (1961), however, suggested that nutrient balance had a greater influence on root rot of red clover than did the absolute level of individual nutrients.

2.5 METHODS USED TO OBTAIN PLANTS WITH DIFFERENT LEVELS OF ROOT DISEASE

2.5.1 Glasshouse studies

2.5.1.1 Soil (growth substratum) infestation method

This method has been used extensively in glasshouse studies on the effects of pathogens on seed germination, seedling damping-off, wilt, root rot, and the interactions of fungi with insects, nematodes, or viruses.

Various plant growth media have been used including sterilised soil, sand, or mixtures of sterilised soil with sand or vermiculite. Non-sterile soil has rarely been used because of difficulties in establishing pathogens in a medium which often contains micro-organisms antagonistic to the pathogens.

Media used to grow fungi before incorporation into the plant growth substrate often include agar media (Halpin et al., 1952; Benedict, 1954; Gerdemann, 1954; McDonald, 1955; Graham et al., 1957; Fulton and Hanson,1960; Fezer, 1961; Chi and Hanson, 1962; Ylimäki, 1966; McGee and Kellock, 1974), liquid media such V-8 juice (Lukezic et al., 1969 a; Welty et al., 1980; Ooka and Uchida, 1982; Gray and Wofford, 1987), cereal grains or the seeds of other plants (Cormack, 1937; Smith, 1943; Menzies, 1973; Milton and Isaac, 1976; Barbetti and MacNish, 1978; Hancock, 1983; Millis, 1984; Wong et al., 1984; Barbetti et al., 1986 b), mixtures of sand with ground cereal products such as cornmeal, wholemeal etc. (Graham and Newton, 1960; Newton and Graham, 1963; Thompson and Willis, 1968; Aubé and Gagnon, 1970; Sherwood et al., 1970; Burgess et al., 1973; Campbell,1980; Skipp and Christensen,1982; Pratt et al., 1982; Bretag,1985) or vermiculite moistened with nutrient solution (Watson and Guthrie, 1964; Greenhalgh and Taylor,1985).

Fungal cultures on agar or liquid media are usually macerated before incorporation into the soil medium, whereas inoculum on grains or from sand or ground cereal products may be added directly. Soil also can be inoculated by

spraying a spore or mycelial suspension on to the seed bed before or after sowing (Gerdemann,1954; Irwin and Jones,1977; Gray et al.,1980).

Few studies have compared different inoculation methods. Gray and Wofford (1987) found that incorporation of inoculum into the soil gave less satisfactory results than by placing inoculum in the seed furrow just before planting. Material added to the soil with the fungal inoculum, such as PDA or grains, may also provide a good substrate for development of air-borne pathogens or saprophytes which may mask the effect of the test fungi (Ostazeski, 1957; Pettit et al., 1969). However, Pratt (1982) reported that results were similar whether obtained by dipping roots, or adding inoculum grown on agar or cornmeal/sand media to the soil via a hole.

Various methods have been used to infest medium containing the established root system of older or mature plants. Many researchers have added inoculum to holes made in the soil around the plants (Smith,1943; Graham and Newton, 1959; Fulton and Hanson,1960; Newton and Graham, 1963; Thompson and Willis, 1968; Sundheim,1970; Irwin and Jones, 1977; Pratt et al., 1982; Gray and Wofford,1987; Griffin and Thyr,1988). Others have simply poured the inoculum suspension into the pots (Leath and Newton, 1969; Lukezic et al., 1969 a; Menzies,1973; Ooka and Uchida,1982), or injected it into the substratum around the roots with a hypodermic syringe (Schmitthenner and Williams, 1957). Disturbance of roots due to transplanting or hole-making can be avoided by inserting a centrifuge tube into the soil in the pot before seeding or transplanting and leaving it there until the time of inoculation (Halpin and Hanson, 1958). On the other hand, to optimise the contact between the inoculum and the root surface, roots can be dipped in inoculum suspension prior to transplantation (McDonald,1955; Chi and Hanson, 1961; Chi, 1965).

2.5.1.2 Root dip method

This method has been used to study wilt diseases as well as other root diseases of forage legumes.

Seedlings have been grown in a sterilised substratum, e.g. soil, potting mix,

mixtures of soil and peat or vermiculite, then washed free of substratum. Some workers have directly immersed the roots in inoculum suspension (Aubé, 1966; Pegg and Parry, 1983), while others have cut the roots prior to inoculation (Auld et al., 1976; Christen and Peaden, 1981; Richard et al., 1982; Emberger and Welty, 1983; Hawthorne, 1987). Skipp et al., (1986) found that trimmed and non-trimmed roots infected equally well by several soilborne fungi. After inoculation the seedlings are re-planted in a sterilised substratum.

2.5.1.3 Root-injuring method

This method has been mainly used for studying effects of root-invading fungi on several-month-old or mature plants.

In most reported work, plants were inoculated in situ. The crown and upper part of the tap roots were exposed by removing the growth substratum and wounds made by severing roots with a sterile needle (Pettit et al., 1969; Sundheim, 1970; Auld et al., 1976; Johnson et al., 1982), by cutting with a scalpel (Isaac, 1946; Graham and Newton, 1960; Ylimäki, 1967; Leath and Byers, 1977), by splitting the crown (Staten and Leyendecker, 1949), or by drilling with a sterilised bit (Fezer, 1961). Inoculum was then applied to the wound, and the growth substratum replaced (Jones and Weimer, 1938; Kreitlow and Hanson, 1950).

2.5.1.4 Slant-board method

This is a nutrient solution culture method in which the roots grow between layers of polyester cloth (Kendall and Leath, 1974). Inoculum is prepared by placing sterile polyester strips, 1 x 1.5 cm, on the surface of agar near the advancing edge of a fungal colony. When the strips were covered with the fungal mycelium, they were removed and positioned on a root. The method affords frequent, non-injurious, direct observation of inoculated sites. It also provides a means to determine the degree of root rot, and its effect on the growth of individual plants (Leath and Kendall, 1978; Stutz and Leath, 1983; Stutz et al., 1985; Leath et al., 1989).

2.5.1.5 Fungicide application method

This method has mainly been used to study effects of multiple pathogens on plants which may be controlled selectively or non-selectively by an appropriate fungicide. Field soil taken from pasture with a history of root problems is used as the plant growth substratum. Intact (undisturbed) soil cores have also been used (Smiley et al., 1986; Barbetti et al., 1987).

Fungicide has been applied as a seed treatment (Chilton and Garber, 1941; Allison and Torrie, 1944; Gerdemann, 1951; Fulkerson, 1953; Mead, 1955; Skipp et al., 1986; Smiley et al., 1986; Hwang, 1988), mixed with soil prior to sowing (Faechner and Bolton, 1978; Bretag and Kollmorgen, 1986), or drenched before or after sowing (Hancock, 1983; Skipp et al., 1986; Smiley et al., 1986, Skipp and Watson, 1987; Barbetti et al., 1987).

This method has the advantage that disease develops from natural soilborne inoculum. However, because of the complex interactions which can occur in soil, the fungicide may not have its major effect on the target fungus.

2.5.2 Field plot methods

2.5.2.1 Fungicide application method

Three ways of fungicide application have been reported i.e. applying fungicide to seed, to the sward, or to soil.

Seed treatment with fungicide prior to sowing has mainly been used to study effects of soilborne fungi on establishment (Falloon, 1981). Field experiments have been carried out with treated seed sown into 1 - 2 m long rows at a rate of 100 - 200 seeds per row (Allison and Torrie, 1944; Vlitos and Preston, 1949; Gerdemann, 1951; Mead, 1955; Athow, 1957; Falloon and Skipp, 1982), into small plots of 30 - 45 cm^2 (Kreitlow et al., 1950) or into larger field plots (Tyler et al., 1956).

Applying fungicide to the sward has not often been used to study root disease. It has been employed mainly to examine effects in established pasture

with fungicide being sprayed after each harvest of herbage and effects assessed over one or more growing seasons (James et al., 1980; Rufelt, 1986).

Applying fungicide directly to the soil has been used to study disease effects on all stages of plant growth. Fungicide has been incorporated into soil before (Sheaffer et al., 1982) or during seed sowing (Bretag and Kollmorgen, 1986), or while transplanting seedlings (Willis and Thompson, 1975). Fungicides have also been drenched into established pasture (Leath et al., 1973; Irwin and Jones, 1977; Taylor et al., 1985 b; Greenhalgh and Clarke, 1985).

All the studies cited above have demonstrated the benefits of using fungicides as experimental tools to study effects of root diseases on forage legumes under field conditions. There are, however, some disadvantages which include secondary effects resulting from phytotoxicity, or beneficial effects, such as improvement of nutrition of plants by release of ammonium nitrogen or increase in populations of beneficial soil microorganisms (Agnihotri, 1971; Ridge and Theodorou, 1972; Rovira and Ridge, 1979).

2.5.2.2 Plant inoculation method

A few field experiments have been reported using a plant inoculation method. Inoculation of established plants in the field has been carried out in a similar way to that used in glasshouse trials (see above). Plants have been inoculated by root dipping in the greenhouse before transplantation to the field (Johnson et al., 1982), by injection of inoculum to the crown of field-growing plants with a syringe (Turner and VanAlfen, 1983), by placing a small portion of oat hull bearing inoculum against the tap root (Cormack, 1937), by placing inoculum into small-bore openings of the roots and covering with plastic film to maintain high humidity (Kushner and Crittenden, 1967), or by pouring inoculum suspension directly around the crown of each plant (Faris and Sabo, 1981).

Although satisfactory results have been achieved by the above authors, inoculation with soilborne pathogens in the field is problematic. It is prone to failure if the environmental conditions subsequent to inoculum are unsuitable for disease development. More importantly, most agricultural soils are biologically

buffered and soil microorganisms are scattered in their distribution, thus it is difficult to establish inoculum, and also there is a tendency of disease escape.

2.5.3 Microplot method

The term "microplot" is commonly used to describe containerised, miniature plots of the type used to study the effects of nematode on plant yield (Barker, 1985). Recently, this method has been used to study the interaction of soilborne fungi and nematodes, fungal root diseases, and some other aspects relevant to soilborne pathogens.

Most use of microplots has been made in studies on field crop problems. The only report involving forage legumes was a study of effects of northern root knot nematode (Meloidogyne hapla Chitwood) on lucerne, birdsfoot trefoil, and red and white clovers (Townshend and Potter, 1978).

Field soil has been used as the plant growth substratum in all experiments except one in which potting mix was used (Sippell and Hall, 1982). Preparation of microplots involves the following steps: - Fumigation of the soil, embedding barriers in the ground, infestation and planting.

Experimental areas are usually fumigated with methyl bromide or other appropriate fumigants before establishment of the microplots, but fumigation after establishment has also been reported (Kotcon et al., 1985). Fumigated soil is collected for inoculation with the test organisms.

Barriers of microplots are embedded into the ground. Open-ended, unglazed, clay drain tiles have been used as barriers in many experiments (Martin et al., 1982; Chun and Lockwood, 1985; Riedel et al., 1985; Rowe et al., 1985; Francl et al., 1987; Starr et al., 1989). However, polythene sheets (Townshend and Potter, 1978; Kotcon et al., 1985), fibreglass (Tooley and Grau, 1986; van Bruggen et al., 1986; Sidebottom and Beute, 1989; Sumner and Littrell, 1989), aluminum cans (Sippell and Hall, 1982), and plastic buckets (Pinkerton et al., 1989), all with drainage holes on the bottom, have also been used.

After the microplots have been set up, soil inoculated with the pathogen, as described in glasshouse studies, is placed in the appropriate microplots and

seeds sown.

The microplot method can offer many of the advantages of field plot studies while overcoming much of the variability associated with the field environment. It usually entails an enormous amount of work.

2.6 EFFECTS OF ROOT DISEASES ON GROWTH OF FORAGE LEGUMES

2.6.1 Effects of diseases on establishment

The effects of some common soilborne pathogens on seedling establishment under glasshouse conditions are summarised in Table 2-6-1. Pathogens which have been reported to reduce percent seedling survival of forage legumes 2-6 weeks after sowing were Phytophthora megasperma Drechs., Gliocladium roseum Bainier, Mycoleptodiscus terrestris (Gerd.) Ostazeski, and species of Pythium, Cylindrocladium, Fusarium, and Rhizoctonia (Table 2-6-1). The remaining fungi listed in the table had a slight effect on seedling establishment.

The influence of pathogenic fungi on establishment of forage legumes has also been demonstrated in experiments in which fungicides have been applied to seed or the soil. Improved emergence and survival of seedlings has been obtained in glasshouse studies (Vlitos and Preston, 1949; Kreitlow et al., 1950; Fulkerson, 1953; Jacks, 1956; Bretag and Kollmorgan, 1986; Skipp et al., 1986; Skipp and Watson, 1987) and field experiments (Sheaffer et al., 1982; Falloon and Skipp, 1982).

Table 2-6-1. Pathogens adversely affecting seedling establishment of forage legumes in pot trials.

Fungi	Hosts and references
Phytophthora clandestina	SC 12[1]
P. megasperma	AF 7"[2], 11^ SA 11^
Pythium acanthicum	SC 3
P.debaryanum	AF 7", 14^ RC 13^, 16^ SW[3] 14^ WC 14^
P.irregulare	AF 14^ RC 13" SC 3, 4", 12 SW 14^ WC 14^
P.paroecandrum	AF 14" RC 13 SW 14" WC 14"
P.splendens	AF 14^ RC 13" SW 14^ WC 14^
P.ultimum	AF 7", 14^, 15 RC 13" SW 14 WC 14^
Pythium sp.	AF 5 RC 5, 16" SW 5 AC 5 SC 17"
Rhizopus.sp	RC 9
Chaetomium cochliodes	AF 2
C.diliochotrichum	AF 2
Cladosporium sp.	AF 2
Cylindrocladium clavatum	AF 18
C.crotalariae	AF 18"
Fusarium acuminatum	AF 15
F.avenaceum	AC 1" AF 1" SC 12, 17 WC 1
F.culmorum	AC 1 AF 1, 15 WC 1
F.moniliforme	AF 8" RC 8^
F.oxysporum	AC 1 AF 1, 7, 8" RC 6, 8, 9, 16" SC 4
F.roseum	AF 8^ RC 6, 8^, 16
F.solani	AF 8^ 7 RC 6, 8^, 9, 16
Gliocladium roseum	AF 16 RC 9^
Mycoleptodiscus terrestris	AF 8^ RC 8^, 10^
Phoma medicaginis	SC 4
Phoma sp.	RC 16
Rhizoctonia solani	AC 5" AF 2, 5", 7", 15" RC 5" SC 7", 19 SW 5"
Rhizoctonia spp.	RC 9", 16
Trichoderma viride	AF 2 RC 16
Trichoderma sp.	RC 9

[1]. 1. Aubé, 1966. 2. Aubé and Gagnon, 1970. 3. Barbetti and MacNish, 1978. 4. Barbetti et al., 1986 b. 5. Benedict, 1954. 6. Chi, 1965. 7. Chi and Childers, 1966. 8. Elliott, et al., 1969. 9. Fulton and Hanson, 1960. 10. Gerdemann, 1954. 11. Gray and Wofford, 1987. 12. Greenhalgh and Taylor, 1985. 13. Halpin et al., 1952. 14. Halpin et al., 1954. 15. Hancock, 1983. 16. Kilpatrick et al., 1954. 17. McGee and Kellock, 1974. 18.Ooka and Uchida,1982. 19. Wong et al., 1985 b.

[2]: The symbols ^ and " indicate that percent survival of seedlings had been reported as less than 10%, and 11-50%, respectively; percentages survival in remainder were 51-90%.

[3]: SW, sweet clover (Meliotus officinalis (L.) Desr.); Others see Table 2-4-1.

2.6.2 Effects of diseases on yield

Although there is relatively little quantitative data to indicate the effects of diseases on forage legumes, estimates of yield losses of forage crops due to disease have been estimated in some countries, and effects of some individual pathogens have been demonstrated experimentally.

In Ireland, an estimated loss of 5% of the annual forage yield due to diseases represents a financial loss of over 20 million pounds annually (O'Rourke, 1982). In Canada the estimated annual yield loss of forage crops due to foliar diseases of about 6% was, in 1973, equivalent to a loss of $9.4 million (Berkenkamp,1974). Losses from root disease may approach or surpass those caused by foliar diseases (Berkenkamp, 1971). In the USA the losses of hay and seed yield of lucerne due to diseases have been estimated about $400 million in 1972 (Leath et al., 1988).

Ten fungal species have been reported to cause more than 50% of reduction in shoot weight in glasshouse experiments. They were T.basicola, Cy. scoparium, M. terrestris, Trichoderma viride Pers., and species of Pythium, Fusarium, and Rhizoctonia (Table 2-6-2 A), while the other fungi have given less than 50% of reduction .

Effects of diseases on root weight have been studied to less an extent than those on shoots. Of 12 fungi tested, only R. solani was found to reduce root weight more than 50% (Table 2-6-2 B).

Field inoculation study also indicated that yield can be decreased by root disease (Faris and Sabo, 1981). Two years of continuous study showed that yield of lucerne cultivars inoculated with Phytophthora megasperma were 42% (15 - 69%) less than those of uninoculated ones.

Fungicidal treatments have been employed in most field studies. Benomyl and metalaxyl have been used most frequently either singly or in combination (Leath et al., 1973; Willis and Thompson, 1975; James et al., 1980; Sheaffer et al., 1982; Greenhalgh and Clarke, 1985; Taylor et al., 1985 b; Rufelt, 1986). Captafol and chloropicrin have also been used (Irwin and Jones, 1977; Leach et al., 1963). Increased weights of shoot and root were achieved from most reported work but occasionally no increase resulted from the treatment.

Table 2-6-2. Pathogens reducing the weights of shoots (A) and roots (B) of forage legumes in pot trials.

A. Shoots

Fungi	Hosts and references
Phytophthora megasperma	AF[1] 13[2]
Pythium irregulare	BM 4^[3] SC 2, 3, 15
P.ultimum	BM 4^
Pythium sp.	RC 7
Rhizopus sp.	RC 7
Codinaea fertilis	WC 5
Cylindrocladium scoparium	RC 11^
Fusarium avenaceum	BM 4^ SC 15
F.equiseti	BM 4
F.oxysporum	AF 6^ 8 BM 4 BT 10^ RC 7 SC 15^
F.solani	BT 10^ RC 7, 11
Fusarium spp.	RC 12
Gliocladium roseum	AF 1
Mycoleptodiscus terrestris	BT 10^
Phoma medicaginis	BM 4 SC 15
Pyrenochaeta terrestris	AF 1
Rhizoctonia cerealis	SC 14
R. solani	AF 1 BM 4^ SC 3, 15
Rhizoctonia sp	RC 7^ SC 14
Trichocladium basicola	RC 11^
Trichoderma viride	AF 1^
Trichoderma sp.	RC 7
Verticillium dahliae	RC 11

B. Roots

Fungi	Hosts and references
Phytophthora megasperma	AF 13
Pythium irregulare	SC 2, 3
Codinaea fertilis	WC 5
Fusarium avenaceum	SC 3
F.oxysporum	AF 8
Fusarium spp.	RC 9, 12
Gliocladium roseum	AF 1
Pyrenochaeta terrestris	AF 1
Rhizoctonia cerealis	SC 14
R. solani	AF 1^ SC 3, 14^
Rhizoctonia sp	RC 14
Trichoderma viride	AF 1

[1]: See Table 2-4-1.

[2]: 1. Aubé and Gagnon, 1970. 2. Barbetti and MacNish, 1978. 3. Barbetti et al., 1986 b. 4. Bretag, 1985. 5. Campbell and Moyer, 1983. 6. Emberger and Welty, 1983. 7. Fulton and Hanson, 1960. 8. Mauza and Webster, 1982. 9. Newton and Graham, 1963.

10. Pettit et al., 1969. **11**. Skipp et al., 1986. **12**. Thompson and Willis, 1968. **13**. Welty et al., 1980. **14**. Wong et al., 1985 b. **15**. Wong et al., 1986.

[3]: The symbol ^ indicated that weights had been reduced more than 50%, reduction in remainder were 51-90%, as compared with control.

2.6.3 Effects of diseases on nitrogen fixation and nodulation

Biological nitrogen fixation is second only to photosynthesis as the most important biochemical process on earth. About 85% of nitrogen fixed in agricultural soil is derived from pulse and pasture legumes (Vance and Johnson, 1981). Any pathological factor that affects the health of the plant will influence the symbiotic balance (Tu and Ford, 1984). Although many plant pathologists have drawn attention to the effects of diseases, mainly virus diseases, on nitrogen fixation and nodulation, and considerable information has accumulated, there are only a few studies on forage legumes.

Guy et al. (1980) found that the number of nodules per plant of white clover seedlings was reduced by infection with white clover mosaic virus (WClMV). Khadhair et al. (1984) demonstrated that WClMV reduced nodulation (number and weight of nodules), rhizobial population, nitrogenase activity, and leghemoglobin concentration in red clover. Tu and Holmes (1980) reported that alfalfa mosaic virus (AMV) reduced nodulation of lucerne. Sawada (1983) reported that lucerne grown in soil naturally infested with F. oxysporum had fewer nodules than plants grown in sterilised soil. Skipp and Gaynor (1987) reviewed information on the effects of root invasion by endoparasitic nematodes on the nitrogen fixing ability of clover roots. In addition, reduced white clover nodulation by mycoplasma-like organisms (MLO) has also been reported (Joshi et al., 1967).

2.6.4 Effects of diseases on persistence

Poor persistence is a major limitation in the use of perennial species of forage legumes, especially red clover. Causes of poor persistence have been summarised and well documented recently by scientists from Australia, New Zealand, and USA, (Marten, 1989). Many studies have shown that root diseases

are an important factor in the depletion of stands. Root diseases can either kill plants directly during early stages of growth (section 2.6.1.) or cause poor vigour which results in plants having lower yields than healthy plants (section 2.6.2.), and greater susceptibility to death under stress conditions.

Some studies have shown that a single pathogen can cause poor persistence (Johnson et al., 1982; Faris and Sabo, 1981). However, it is generally recognised that root deterioration is the result of the interaction of many environmental and biological factors (Irwin, 1989; Leath, 1989; Watson et al., 1989). These have been partly summarised in section 2 and 3 of this chapter.

Field studies through both fungal inoculation and chemical manipulation have provided evidence of the role of a complex of several root-invading fungi as a cause of poor persistence. Irwin and Jones (1977) reported that total losses of lucerne stands at 3 experimental sites in Australia were between 22.8 and 84.15%, 2.5 years after establishment due to invasion of a number of fungal species. Stephen et al. (1982) concluded from a trial conducted in northern Canterbury, New Zealand over a 4 year period, that crown rot caused by Fusarium spp. was one of the most important factors in lucerne deterioration. Kilpatrick et al. (1954) reported that only 1.2% of the red clover plants were still alive one year after sowing in a field containing a complex of root-invading fungi (see Table 2-3-1) at Madison, Wisconsin.

Substantial increases in persistence resulting from application of chemicals have been reported for alsike clover (Leach et al., 1963), red clover (Leath et al., 1973), white clover (James et al., 1980), and subterranean clover (Greenhalgh and Clarke, 1985).

2.7 LIGHT AND ELECTRON MICROSCOPY STUDIES OF ROOT INVASION BY TRICHOCLADIUM BASICOLA AND OF EFFECTS OF FUNGICIDES ON FUNGAL GROWTH IN VITRO AND INVASION OF HOST TISSUE

2.7.1 Ultrastructure of T. basicola

A morphological description of the fungus has been given by Subramanian (1968), and Nag Raj and Kendrick (1975). According to DelVecchio et al. (1969), the hyphal cytoplasm of the fungus contains mitochondria, nuclei, and a well

developed endoplasmic reticulum, similar to those described for other fungi (Bracker, 1967; Beckett et al., 1974). However, the hyphal cell wall of T. basicola was composed of two regions: an outer electron-dense layer and an inner electron-transparent layer (DelVecchio et al., 1969).

Mature endoconidia contain vacuoles and clusters of vesicles at both ends of the cell as well as the other organelles described for vegetative hyphae. The vesicles contain storage products (DelVecchio et al., 1969; Hawes and Beckett, 1977). The cell walls of endoconidia have a two-layered structure similar to those of vegetative hyphae (Tsao and Tsao, 1970).

The endoconidiophores were always devoid of cytoplasmic components. The walls of these cells were identical to those of vegetative hyphae (DelVecchio et al., 1969).

Chlamydospores when grouped together in a chain are enveloped by a wall consisting of two layers: an electron-dense outer layer and an electron-transparent inner layer (DelVecchio et al., 1969; Christias and Baker, 1970; Tsao and Tsao, 1970). The outer surface of the envelope is rough and pitted (Christias and Baker, 1970). Within the chain envelope, each one-celled spore has its own thick wall, also consisting of two layers (DelVecchio et al., 1969; Christias and Baker, 970; Tsao and Tsao, 1970), but usually 2 - 3 times as thick as that of the chain envelope (Tsao and Tsao, 1970). A wedge-shaped structure at the junction of lateral and end walls was seen in one study (Tsao and Tsao, 1970). Septal pores exist between cells in a chain of chlamydospores (DelVecchio et al., 1969; Tsao and Tsao, 1970). A prominent feature of the "aged" chlamydospore cell (2 weeks or older) is a high concentration of spherical or ellipsoidal globules of reserve nutrient material, which completely fill the cell and largely obscure the contents (Christias and Baker, 1970; Tsao and Tsao, 1970). The size of these globules is fairly uniform. The cytoplasm occurs as a thin film between the globules (Christias and Baker, 1970). However, nuclei, endoplasmic reticulum, and numerous mitochondria are readily visible in chlamydospore cells of young (4-day-old) cultures (DelVecchio et al., 1969).

2.7.2 Root invasion by T. basicola

Light microscopy studies of root diseases incited by T. basicola have been made on bean (Phaseolus vulgaris L.) (Christou, 1962; Pierre and Wilkinson,

1970), cotton (Gossypium hirsutum L.) (Mathre et al., 1966), citrus (Citrus sp.) (Tsao and Van Gundy, 1962), strawberry (Fragaria X ananassa Duchesne) (Hildebrand and Koch, 1936), tobacco (Nicotiana tabacum L.) (Conant, 1927; Koch, 1935; Hildebrand and Koch, 1936; Stover, 1950), and white clover (Lim and Cole, 1984). Light and scanning electron microscopy studies have been made on Japanese holly (Ilex crenata Thunb.) (Wick and Moore, 1983) and cotton (G. hirsutum and G. barbadense L.) (Mauk and Hine, 1988).

Hyphae directly penetrate the epidermal cells of the hosts, with (Mauk and Hine, 1988), or without (Christou, 1962; Pierre and Wilknson, 1970; Wick and Moore, 1983; Lim and Cole, 1984) the formation of appressoria. Wounds do not appear to be necessary for penetration although Conant (1927) reported that the fungus entered tobacco roots through wounds.

Hyphae within penetrated cells develop in a characteristic beaded shape due to formation of a constriction just behind the hyphal tip. Continued growth is in a digitate form and eventually fills the cells. This type of hypha is of diagnostic value for the fungus. It has been found in all studies of root diseases incited by the pathogen. Accounts vary as to the subsequent development of the fungus. Slender nonconstricted intercellular hyphae were reported to form from beaded hyphae in bean (Christou, 1962). A similar type of hypha was seen in cotton but was only found intracellularly (Mathre et al., 1966; Mauk and Hine,1988). Thick walled pseudoparenchymatous hyphae were found in holly (Wick and Moore, 1983). The fourth type of hypha, termed "reproductive hypha", develops both inter- and intracellularly in bean (Christou, 1962; Pierre and Wilkinson,1970), but only intracellularly in white clover (Lim and Cole, 1984), cotton (Mauk and Hine, 1988), holly (Wick and Moore, 1983), and tobacco (Stover, 1950). Chlamydospores are produced intracellularly in bean (Christou,1962), white clover (Lim and Cole, 1984) and cotton (Mauk and Hine, 1988), but both inter- and intracellularly in citrus (Tsao and Van Gundy, 1962). At an advanced stage of infection, endoconidia and chlamydospores are produced abundantly on the root surfaces of all kinds of host plants; and the outer epidermal walls usually slough off.

The fungus invades all tissues including tracheary elements of bean (Christou,1962) and cotton (Mauk and Hine, 1988), but is confined largely to cortical regions of citrus (Tsao and Van Gundy, 1962).

Host cells show little sign of disturbance during early stages of infection.

Nuclei examined by light microscopy were intact and stained normally in infected cells of tobacco (Stover, 1950), bean (Christou, 1962; Pierre and Wilkinson, 1970),citrus (Tsao and Van Gundy, 1962), cotton (Mathre et al., 1966), holly (Wick and Moore, 1983), and white clover (Lim and Cole, 1984). As infection advances, abundant yellow-brown granules develop in the infected cells of bean (Christou,1962) and white clover (Lim and Cole, 1984). Lignituber-like structures were seen occasionally in roots of white clover (Lim and Cole, 1984). Barrier structures were found in holly (Wick and Moore, 1983).

2.7.3 Effects of benomyl on ultrastructure of fungi grown in culture and in hosts

Little information is available on cytological changes induced in fungi by benomyl treatment in culture apart from a paper by Richmond and Pring (1979) on Botrytis fabae Sard.. In benomyl-treated cells the orientation of organelles towards the hyphal tip is disorganised. The endoplasmic reticulum becomes reduced to short broken fragments, or sometimes, a branched reticulated network. Lomasome formation seems to be stimulated, which results in the surface of the plasmalemma becoming far more convoluted than in of normal conidia. Nuclei appear normal in thin section, but deeply lobed and convoluted when examined in material processed by freeze etching. In general, mitochondria and lipid bodies appear unaffected by the fungicide although some mitochondria have looped cristae membranes.

Studies on the effect of benomyl on the fine structure of fungi within their hosts have been reported for Puccinia coronata Corda on oat seedlings (Avena sativa L.) (Simons, 1975), and Venturia inaequalis (Cke.) Wint. on leaves of apple (Malus sylvestris Mill.) (Hoch and Szkolnik, 1979).

Application of benomyl at post-infection did not eradicate P.coronata or V.inaequalis from their hosts, but disease development was greatly reduced (Simons, 1975; Hoch and Szkolnik, 1979). Mitochondria were swollen in the treated cells of both fungi. The cristae of mitochondria in V. inaequalis remained platelike (Hoch and Szkolnik, 1979), but often formed peculiar, looped configurations in P. coronata (Simons, 1975). The endoplasmic reticulum cisternae were dilated in V. inaequalis (Hoch and Szkolnik,1979). Nuclear membranes were missing and the plasmalemma was fragmented in haustoria of

P. coronata (Simons, 1975).

Benomyl did not cause protoplasmic abnormalities in the cells of apple leaves (Hoch and Szkolnik, 1979).

Benomyl does not directly interfere with energy metabolism, but acts on microtubule assembly resulting in disruption of mitosis and meiosis in plant cells (Davidse, 1986). The abnormal mitochondria, nuclei and other organelles of the fungi treated with benomyl could possibly then be interpreted as secondary effects in cells which had been unable to divide normally (Davidse, 1986; Simons, 1975).

2.7.4 EFFECTS OF ERGOSTEROL BIOSYNTHESIS INHIBITING FUNGICIDES ON ULTRASTRUCTURE OF FUNGI GROWN IN CULTURE AND IN HOSTS

Electron microscopy studies on the effects of ergosterol biosynthesis inhibiting (EBI) fungicides on fungal ultrastructure of plant pathogenic fungi have been reported for the fungicides triadimefon, numarimol, and imazalil nitrate on sporidia of Ustilago avenae (Persoon) Rostrup (Hippe, 1984 a and b; Hippe and Grossmann, 1982), triadimefon on Sclerotinia sclerotiorum (Libert) de Bary (Stiers et al., 1980) and on Botrytis allii Munn (B. aclada Fresenius) (Richmond, 1984), and propiconazole on Taphrina deformans (Berkeley) L. R. Tulasne (Sancholle et al., 1988), and on Puccinia graminis Persoon f.sp.tritici (Dahmen et al., 1988).

Fungicide treatments induced uneven thickening of peripheral cell walls; and accumulation of vesicles in the walls of U. avenae, T. deformans, and B. allii (Hippe 1984 a and b; Sancholle et al., 1988; Richmond, 1984). The plasma membrane was invaginated at random locations and separated from the inner face of the cell wall of U. avenae (Hippe, 1984 a and b), and T. deformans (Sancholle et al., 1988). The cytoplasm became granulated and electron opaque materials were deposited on the plasma membrane of P. graminis f.sp. tritici (Dahmen et al., 1988). In addition, mitochondria were increased in number in U. avenae (Hippe 1984 a and b), and S. sclerotiorum (Stiers et al., 1980), became enlarged or irregular in shape in cells of U. avenae (Hippe, 1984 a,b), P. graminis f.sp. tritici (Dahmen et al., 1988) and B. allii (Richmond, 1984). Numbers of endoplasmic reticulum (ER) figures were increased in the cells of S. sclerotiorum and they occurred both as randomly oriented material and as membrane

complexes in stacks (Stiers et al., 1980). As incubation continued the integrity of cells of the treated fungi was destroyed.

Studies of the effects of EBI fungicides on the fine structure of fungi within treated hosts have been reported for triadimefon on Puccinia recondita Rob ex Dorm. infecting wheat leaves, Uromyces vicia-fabae (Pers.) Schroet. infecting broad bean leaves (Vicia fabae L.) (Pring, 1984), and triadimenol on Erysiphe graminis f.sp.hordei deCan. on barley seedlings (Smolka et al., 1988).

Lower concentrations of triadimefon reduced rust development in wheat leaves, but did not prevent the formation of sporulating pustules. The urediniospores thus produced were able to reinfect normally (Pring, 1984). Incomplete and multiperforate septa occurred, and extensive wall thickening was found in intercellular mycelia of both rust fungi within triadimefon-treated plants (Pring, 1984). Haustoria of the powdery mildew fungus in triadimenol-treated seedlings developed vacuolised cytoplasm with extracytoplasmic vesicles accumulating between the plasma membrane and cell wall. They became completely encapsulated by deposition of a thick amorphous layer at the haustorium - plant cytoplasm interface, after which their cytoplasm degenerated (Smolka et al., 1988).

The primary mode of action of EBI fungicides is the inhibition of C 14 demethylation (DMI) during sterol biosynthesis. It is presumed that the depletion of functional sterols and the accumulation of sterol intermediates lead to a disruption of membrane functions and to growth inhibition (Siegel, 1981). Therefore, the various observations of effects of the fungicides on cell ultrastructure of different fungi may be an indirect consequence of sterol inhibition instead of direct effects (Hippe 1984, a, b; Dahmen et al., 1988).

CHAPTER 3

GENERAL MATERIALS AND METHODS

3.1 SEED

The red clover cultivar 'Grasslands Hamua', which had been used in previous studies at the experimental site (Skipp et al., 1986), was used throughout this study. Seed (F2056) was obtained from the New Zealand Forage Crop Germplasm Centre, Grasslands Division, DSIR, Palmerston North. The seed was harvested in the 1979/1980 growing season and stored in the Germplasm Centre at 30% R.H. and 0°C until 1986, then at 4°C in a laboratory refrigerator. Germination on filter paper in laboratory tests was 74%.

All seed used for field and glasshouse trials conducted in soil from the plant breeder's evaluation block (details below) was treated with metalaxyl (Ridomil 25% WP; Giba-Geigy Ltd) at a rate of 3 g a.i./kg seeds to ensure adequate seedling emergence by control of damping-off. Ten g of seed were mixed with 0.12 g Ridomil in a 50 ml flask and 0.25 ml water was added. The mixture was shaken on a Cyclo-Mixer until the walls of the flask became free of fungicide. This indicated that all added fungicide had been deposited on the seed. Germination of treated seeds (71%) was not significantly lower than that of untreated seeds ($P > 0.05$).

3.2 EXPERIMENTAL SITES

The field experiments were conducted at DSIR Grasslands Division, Palmerston North. The plots were situated at two sites.

3.2.1 Plant breeder's evaluation block

The block chosen for these studies had previously been used for evaluation of overseas and New Zealand cultivars of red clover (Claydon and Rumball, 1982).

It had been maintained as a red clover monoculture since 1981 (R.B. Claydon, pers. comm.) and red clover plants generally did not persist well in the block. Previous work (Skipp et al., 1986; R.A. Skipp and M.J. Christensen, pers. comm.) has demonstrated that the soil of this block contains several pathogenic root-invading fungi which appeared to cause destruction of the tap root and thus hasten the death of plants.

The soil has been classified as Manawatu mottled silt loam plus Manawatu silt loam (T.G. Shepherd, pers. comm.). The natural nutrient status of the soil is given in Table 3-2-1. The soil had a reasonable ability to hold cations in a form that was not easily soluble in water, but was available to plants, as indicated by the cation-exchange capacity (CEC) and the levels of the individual elements (Blakemore, 1978). The soil drained readily in winter, yet retained sufficient soil moisture for satisfactory plant growth during most of the summer (Cowie, 1978).

3.2.2 Pasture block

This block had also been previously used by plant breeders of Grasslands Division. White clover had been grown before 1983, then ryegrass from 1983 to 1985. After 1985 it became a volunteer ryegrass-white clover pasture. It has had no history of growing red clover during the last 10 years (R.B. Claydon, pers. comm.).

The soil has been classified as Karapoti black silt loam (T.G. Shepherd, pers.comm.). It is regarded as slightly less fertile than the Manawatu soils. It has more acid (Table 3-2-1), thus to maintain good permanent pasture, lime and phosphorus topdressing is needed. It is a well-drained soil, but the plants dry out slightly in summer (Cowie, 1978).

Table 3-2-1 Chemical analyses of soils of the two sites (From Blakemore, 1978)

Soil type	Depth (cm)	PH	Truog P mg/100g	C %	N %	CEC me%	Ca me%	Mg me%	K me%
Manawatu	0-18	6.2	5	4.1	0.41	22.0	15.3	3.4	0.64
Manawatu	18-25	5.8		1.8	0.22	15.1	8.5	2.5	0.43
Karapoti	0-8	5.4	5	5.3	0.45	23.1	13.3	2.7	0.64
Karapoti	10-18	5.3		2.7	0.24	17.4	6.7	2.1	0.40

3.3 SOIL PROCESSING FOR POT EXPERIMENTS

Field soil from the experimental blocks was used for the pot experiments. The soil samples were taken at different positions throughout the block. Surface vegetation was removed, blocks of soil 20 x 20 x 20-25 (depth) cm in size were dug out with a spade and placed in plastic bins (50 x 32 x 22 (depth) cm) for transfer to the laboratory. Combined samples of soil were spread over the surface of metal trays in the glasshouse and air-dried for 2-3 days (11 - 25°C). The soil was further mixed, crushed, and then freed from coarse roots and plant debris.

3.4 DETERMINATION OF SOIL WATER HOLDING CAPACITY

The term "soil water holding capacity" (WHC) is defined here as the amount of water required to saturate a unit weight of soil. The matric potential is approximately equivalent to zero at soil water holding capacity. In some papers, this is called "saturated soil" (Griffin, 1966).

WHC = Weight of saturated soil - weight of oven dried soil.

Weight of oven-dried soil: 100 g crushed soil was put in a weighed glass petri dish, dried in an oven at 105°C overnight, then re-weighed. The mean of 5 replicates was calculated.

Weight of saturated soil: the bottoms of plastic pots (65 mm diam. x 95 mm) were perforated with 5 holes each about 3.5 mm in diameter and approximately 15 mm apart. A piece of filter paper cut to fit the pot bottom was placed in each pot and 100 g crushed soil was put in each of 5 of these weighed pots. One hundred ml of tap water was added to each pot to saturate the soil. Thirty min later the soil was re-saturated. Pots were weighed when no surplus water was present on the soil surface, and the mean weight of the saturated soil was calculated.

The amount of water needed to adjust the water content of soil to x% of WHC = (x% x WHC / 100) x weight of soil placed in pot.

The total weight of each pot (soil + pot) required to maintain a given % of WHC = the amount of water needed to adjust the water content of soil to x% of WHC + dry weight of soil in pot + pot weight.

CHAPTER 4
QUANTITATIVE ASSESSMENT OF ROOT INVASION BY SOILBORNE FUNGI UNDER CONTROLLED ENVIRONMENT AND FIELD CONDITIONS

4.1 INTRODUCTION

Studies on mechanisms of disease resistance, effects of environment or fungicides on disease development, or the response of crop yield to fungal invasion, can often require a quantitative assessment of the amount of fungal biomass in plant tissue. Techniques used for quantitative studies have included plating of fragments or macerates, and recently, enzyme linked immunosorbent assay (ELISA). In addition, some special techniques have been developed for quantifying microsclerotia of Vertcillium dahliae within plant tissues. These have included photographing stripped pieces of infected tissue (Evans et al., 1966), blending fresh (Tsai and Erwin, 1975), or dried diseased tissue (Isaac et al., 1971), and collecting microsclerotia, grinding air-dried stems and plating on media (Davis et al., 1983; Davis and Everson, 1986).

Although ELISA has been used to detect fungal pathogens in root tissue (Leach and Swinburne, 1984; El-Nashaar, et al., 1986; Hawthorne, pers .comm.), a lack of highly specific antisera has restricted the widespread use of this technique. Those special techniques listed above have achieved desirable results in the V. dahliae studies, but none seemed suitable for general purposes. Tissue maceration followed by dilution and plating appeared to be the most useful technique presently available to provide a quantification of fungal invasion of roots.

A previous glasshouse experiment (Skipp et al., 1986) indicated that the soil in the plant breeder's evaluation block at Grasslands Division, DSIR, Palmerston North, contains several fungi pathogenic to red clover.

Environmental factors such as soil temperature and moisture may markedly affect the development of root disease (Chapter 2), but their effects on root invasion of red clover by pathogenic fungi have not been investigated. Experiments reported in this chapter were: -

1. Assessment of a root maceration method for studying invasion of red clover roots by soilborne fungi.

2. Quantitative appraisal of the effects of soil temperature and moisture on invasion of red clover roots by fungi from soil of the plant breeder's evaluation block.

4.2 EXPERIMENT ONE: ASSESSMENT OF A ROOT MACERATION METHOD FOR STUDYING INVASION OF RED CLOVER ROOTS BY SOILBORNE FUNGI

4.2.1 Introduction

The root maceration technique has been satisfactorily used to isolate fungi from a wide range of field and horticultural crops. However, most studies did not examine more than one fungal species (Matta and Dimond, 1963; Lacy and Horner, 1966; Busch and Schooley, 1970; Hall and Busch, 1971; Busch and Hall, 1971; Evans and Gleeson, 1973; Brandt et al., 1984; Kellam and Coffey, 1985;) or genus (Stover and Waite, 1953; Pegg, 1978). Watson and Guthrie (1964) used the technique to isolate Fusarium spp. and Tetracoccosporium paxianum Szabo from roots of inoculated red clover plants. Clarke and Parkinson (1960) examined total numbers of colonies but not those of individual species.

A few studies have tested the efficiency of the method. Clarke and Parkinson (1960) concluded that a maceration technique is more suitable than a tissue segment plating method for the assessment of fungal colonisation, and it is applicable to many plant species unless fungitoxic substances are released during the disruption of root tissue. Singh (1965) found that the root maceration method was superior to a washing and plating technique for studying the mycoflora of the root surface of Vaccinium myrtillus L. Pegg (1978) indicated the danger of

drawing general conclusions on the suitability of the maceration method based on the findings for one host plant species because the germination of V. dahliae conidia was stimulated by macerated stems of chrysanthemum, and the germination of V. albo-atrum conidia was inhibited by those of tomato. The validity of using the method to study the mycoflora of soilborne, root-invading fungi of red clover has not previously been assessed.

Although tissue maceration has been often used since it was developed (Stover and Waite, 1953), the exact procedure used has varied. Blending times have ranged from 30 sec to 2 min (most were 1 min). The amount of tissue blended has also varied between studies and has been expressed in different ways eg. as length (2 mm to 100 cm), or weight of tissue (0.5 to 2 g), or the number of plant organs (leaves). Correspondingly, results have been expressed either as number of propagules per gram, per unit length (mm, 100 cm), or as a percentage of the maximum number of colonies obtained. A broadly applicable method for the routine isolation of root-invading fungi by tissue maceration has not been described.

The objectives of the present study were to:

1. compare the suitability of root maceration and root segment plating methods for studying invasion of red clover roots by fungi;

2. evaluate factors which influence estimates of the number of fungal propagules in macerates of root tissue;

3. use this information to develop a standard isolation procedure for detailed quantitative studies.

4.2.2 Materials and methods

Red clover plants were grown in a field plot in the plant breeder's evaluation block, and in pots of soil from the field plot in the glasshouse. The field plot was sown in November 1986 and plants were maintained under local field management conditions until used.

Two separate glasshouse trials were carried out. One hundred and fifty grams of processed soil (details see Chapter 3) was placed in each of 140

unperforated plastic pots (65 mm diameter x 95 mm). Twenty seeds were sown on the soil surface of each pot and covered to 1-2 cm depth with another 50 g of soil. The pots were watered immediately after sowing to adjust the soil water content to 60% of water holding capacity (WHC) and then were placed in a waterbath at 20°C in a glasshouse (air temperature 11 - 25°C). The pots were weighed and watered daily to 60% of WHC until used.

Root tissues used for fungal isolation were washed free of soil under a jet of tap water, surface sterilised in 0.3% of sodium hypochlorite solution for 1 min, rinsed in sterile tap water (3 changes), and blotted dry on sterilised filter paper.

4.2.2.1 Comparison of root segment and root maceration methods

Fungi were isolated by both the root segment and root maceration methods from tap roots of plants growing in the pots 8 weeks after sowing. The lateral roots were trimmed off and eight, 1 g samples of tap roots were taken at random. Each sample was treated as one replicate. Four replicates were used for each isolation method.

In the root segment method, the roots were cut into 1 - 2 mm long segments with a sterile scalpel. All of the 365 segments were placed in total of 37 plastic petri dishes containing antibiotic PDA (ABPDA) (10 μg oxytetracycline (Sigma) /ml).

In the root maceration method, the roots were cut into 3 - 4 mm long segments. All segments from each 1 g sample were added to 50 ml of sterile tap water in a 1000 ml sterilised Waring blender cup (Watson Victor Ltd) and macerated at high speed for 60 sec. The cup was rinsed with another 50 ml sterile tap water and the macerate suspension made up to 100 ml. A 0.25 ml aliquot of the macerate was pipetted onto each of 3 ABPDA plates and spread over the surface of the medium using a sterilised bent glass rod. After each maceration, the blending cup was thoroughly washed with a stream of tap water for about 30 sec, disinfected with 95% ethanol for another 30 sec, and rinsed with sterile tap water (4 changes). A preliminary test showed that this procedure was adequate to prevent contamination of subsequent samples with the residue from macerated

roots.

4.2.2.2 Effects of the quantity of roots blended and blending time on the number of fungal colonies isolated

Two experiments were carried out to determine the amount of root tissue and blending times to be used to obtain optimum recovery of propagules. In the first experiment the blending time was fixed at 60 sec and the amount of roots to be blended was 2, 4, or 8 g. In the second, the amount of roots was fixed at 2 g and the period of blending was 30, 60, 120, or 240 sec. Plants of two different ages were used for each experiment (12-week-old pot grown plants and 1-year-old field grown plants). Five replicates were used for each treatment.

The roots (both tap roots and lateral roots) of the 12-week-old plants were cut into 5 mm long segments. All the segments were mixed thoroughly. Random samples were made up to the appropriate treatment weight. The cortices of tap roots of the 1-year-old plants were removed with a scalpel. Each of the stele cylinders was cut longitudinally into four quadrants, each being used for a different treatment. Steles from different roots were cut, mixed and sampled as described for 12-week-old plants. The root tissues were blended as described above except that macerates were diluted 2-fold and 4-fold when 4 and 8 g samples of roots were blended, respectively.

All the plates were incubated at 20°C in the dark until fungi could be identified or subcultured on PDA. Colonies of individual fungal species were counted from 2 or 3 days. The results were expressed as number of colonies per g fresh roots. With root segments, these were the total number of colonies from all segments from each 1 g root sample. With root maceration, the number of colonies per g fresh roots was calculated as : (number of colonies on 3 plates x 100 / 0.75) / weight of roots blended.

4.2.2.3 Effects of the quantity of roots blended and blending time on percentage of root tissue macerated

A preliminary test showed that after maceration only finely macerated root tissue would pass through the aperture of the pipette used for plating. The fine material was approximately equivalent to that which would pass through a 600 μm aperture sieve. One-year-old plants were used in an experiment to determine the effects of the quantity of roots blended and the blending time on the proportion of finely macerated root tissue. The macerate was poured into a sieve (600μm diam. aperture) and washed under a stream of tap water for about 2 min. The residue was collected, blotted dry with filter paper and weighed. Because the weight of residue included the water which it absorbed, it was necessary to determine the amount of water absorbed by the roots during the blending. This was done by fragmenting three, 2 g samples of unblended root segments, soaking in water for 2 or 4 min, and then collecting, blotting dry and weighing. The percentage of finely macerated root tissue was calculated as: (weight of root sample - weight of residue - weight of water absorbed by the residue after 2 or 4 min soak) x 100 / weight of root sample.

4.2.2.4 Calculation of maximum number of colonies

The maximum number of fungal colonies per gram fresh roots was calculated as: number of colonies per gram roots / percentage of root tissue macerated

4.2.2.5 Statistical analyses

Coefficients of variance (C.V.) were calculated for data from the experiment which compared root segment and root maceration methods. Analyses of variance (LSD test) were carried out for transformed (sq.rt.) colony number data from experiments on blending times and amounts of root blended.

Similar tests were performed for the percentage data.

4.2.3 Results

4.2.3.1 Comparison of the root segment and root maceration methods

A similar range of fungal species was isolated from the roots of 8-week-old red clover plants by the root segment and the root maceration methods (Table 4-2-1). However, far more colonies were obtained by root maceration. As a whole, there was less variation among replicates of root macerate plates than of root segment plates except for species of Acremonium and Penicillium as indicated by the lower coefficients of variance (Table 4-2-1).

Fusarium solani was the predominant species found on both root segment and root macerate plates. Colonies of T. basicola, F. oxysporum, C. destructans, Gliocladium roseum and Acremonium spp. were also common on plates from both isolation methods.

4.2.3.2 Effects of the quantity of roots blended and blending time on the number of colonies isolated

Fusarium solani was the predominant fungal species (Fig.4-2-1 A and Fig.4-2-2 A) in the roots of 12-week-old plants from pots. F. oxysporum and Cylindrocladium scoparium were also common. Other fungi present included G. roseum, Cladosporium herbarum, V. dahliae, C. destructans, T. basicola, and species of Acremonium, Penicillium, Trichoderma, and Periconia. A few colonies of zygomycetes and sterile mycelium were also found.

Verticillium dahliae was most common (Fig.4-2-1 B and Fig.4-2-2 B) in the root stele of one-year-old field grown plants. Fusarium solani, and a range of other fungi including F. oxysporum, G. roseum, Phoma medicaginis, C. destructans, and species of Acremonium, Paecilomyces, Penicillium and Alternaria were also present.

Two grams samples of roots gave the greatest number of colonies per

gram root in total, and of the predominant fungal species from 12-week-old (F. solani Fig.4-2-1 A) and 1-year-old- (V. dahliae Fig.4-2-1 B) plants. Blending for 60 (and 120) sec gave the greatest number of colonies in total, and of F. solani, from 12-week-old plants (Fig.4-2-2 A). Blending for 120 sec gave the greatest number of colonies in total, and of V. dahliae, from stele tissue of 1-year-old plants (Fig.4-2-2 B). Differences between 60, 120 and 240 sec treatments were not significant, however.

4.2.3.3 Effects of the quantity of roots blended and blending time on the percentage of root tissue macerated

The proportions of 2, 4, or 8 g root samples which passed through a 600 μm aperture sieve after blending for 60 sec were greatest at 2 g (62%) and decreased significantly with increments in the weight of samples macerated (Fig.4-2-3 A). The proportions of 2 g root samples blended for 30, 60, 120, or 240 sec which passed through the sieve increased with increasing blending time to reach a maximum of 74% at 240 sec (Fig.4-2-3 B).

4.2.3.4 Maximum number of colonies per gram fresh roots

There were no significant differences among root sample weights (Fig.4-2-4 A) and blending times (Fig.4-2-4 B) in the range 2 to 8 g and 30 to 240 sec, respectively. However, 2 g root samples blended for 240 sec gave significantly lower number of colonies in total fungi and for V. dahliae (Fig. 4-2-4 B).

Table 4-2-1 Mean numbers of colonies isolated from one gram of fresh tap roots of 8-week-old red clover plants by a root segment or a root maceration method.

Fungal species	Root segment	C.V	Root maceration	C.V
Acremonium sp.	4.75	26.32	1475	69.15
Cladosporum herbarium	0.50	58.00	2500	53.60
Cylindrocarpon destructans	5.50	21.64	1400	15.00
Fusarium oxysporum	12.50	14.80	3475	13.81
F. solani	13.50	21.04	17950	13.70
Fusarium spp.	1.50	79.33	300	60.00
Gliocladium roseum	3.75	22.67	4450	24.27
Penicillium sp.	2.25	28.00	125	100.00
Trichocladium basicola	7.25	20.55	6530	15.01
Sterile mycelium	1.25	38.40	1325	18.11
Zygomycetes	0.25	100.00	1325	46.79
Total no. colonies	53.00		40855	

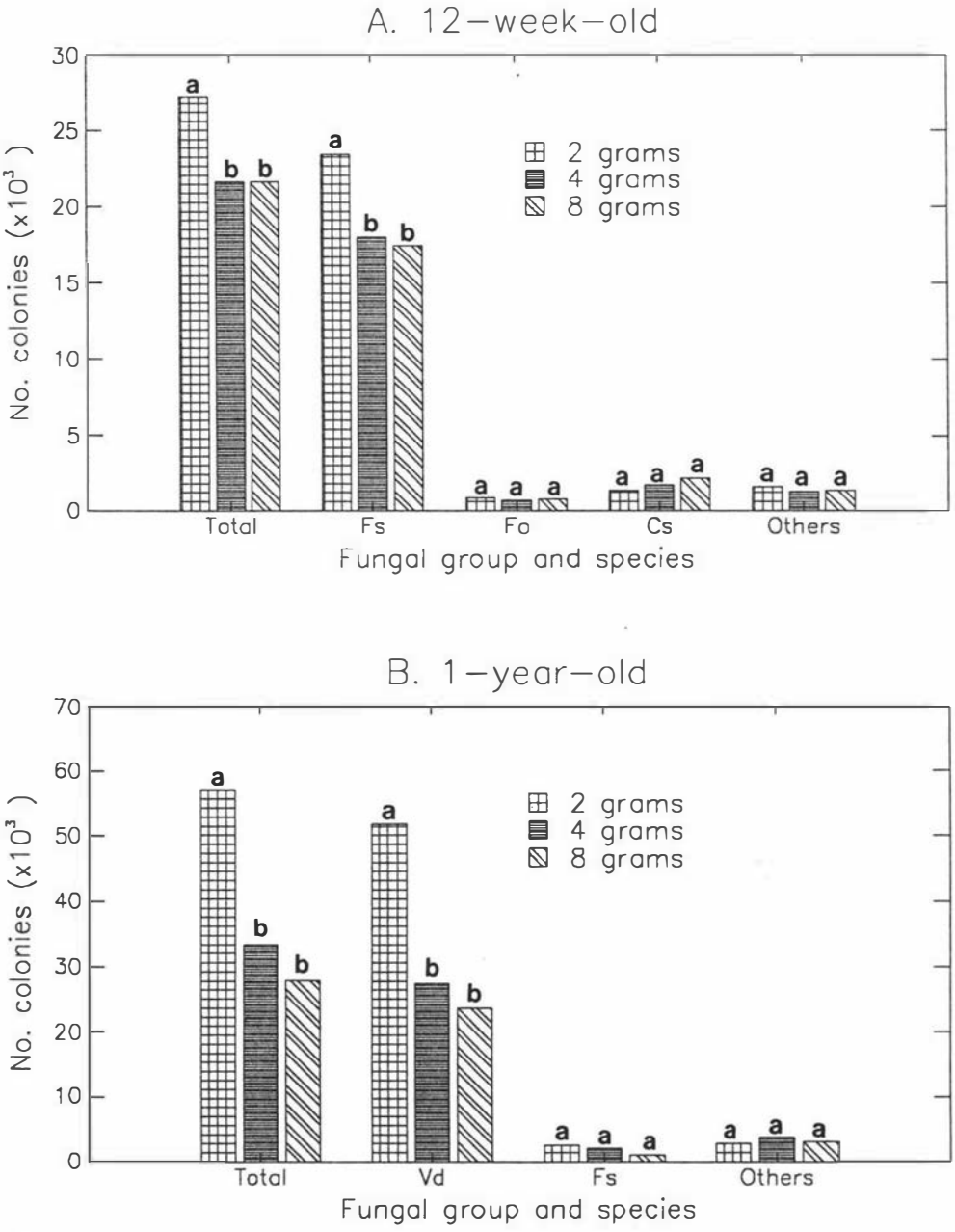


Figure 4-2-1. Mean numbers of fungal colonies in total, and of the major fungal species, per gram fresh roots of 12-week- (A) and 1- year- (B) old red clover plants after adding various amounts of root and blending for 60 seconds. Columns followed by the same letters were not significantly different at 5% probability level in an analysis of transformed values (sq.rt). Cs, Cylindrocladium scoparium; Fo, Fusarium oxysporum; Fs, F. solani; Vd, Verticillium dahliae.

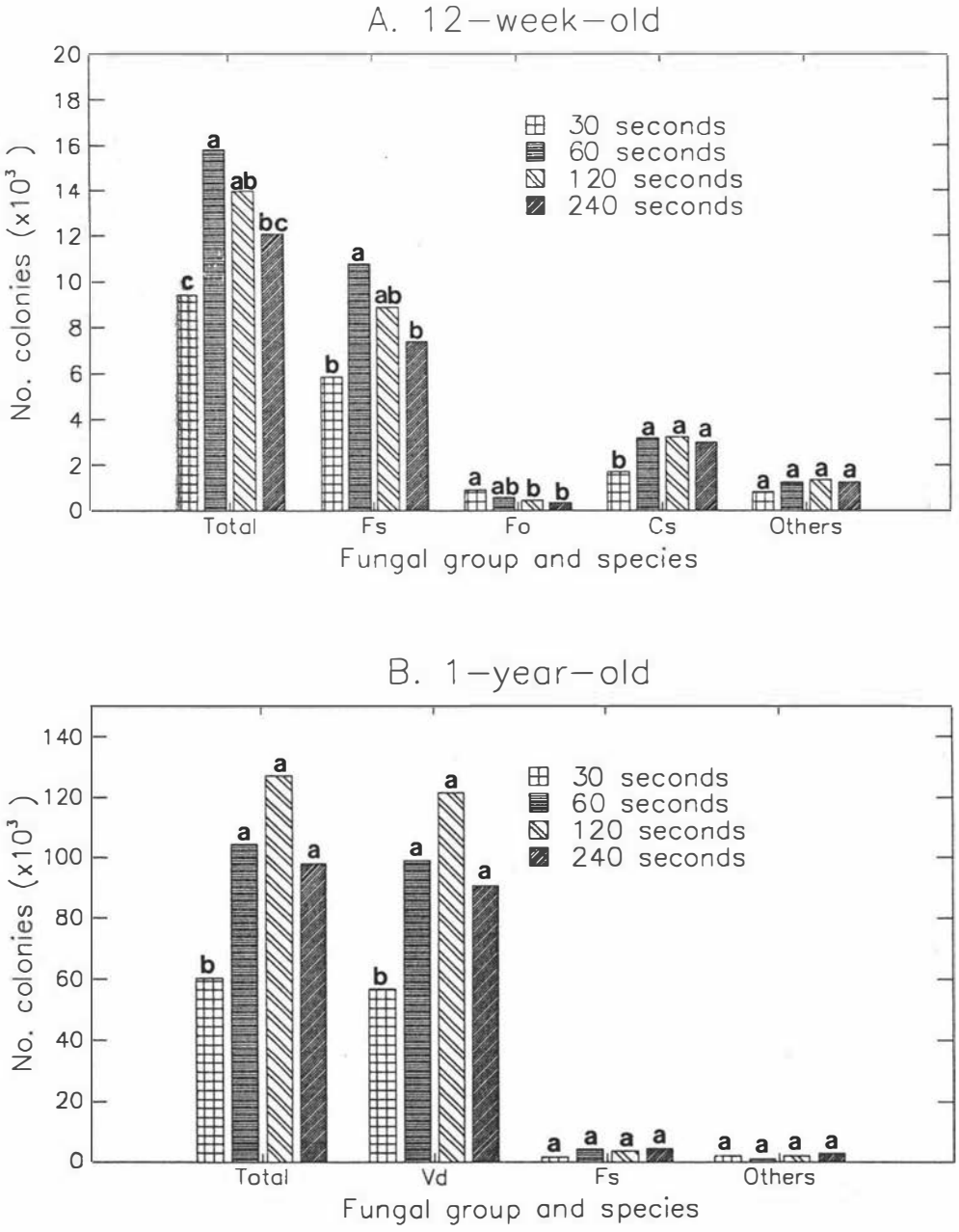


Figure 4−2−2. Mean numbers of fungal colonies in total, and of the major fungal species, per gram fresh roots of 12−week− (A) and 1−year− (B) old red clover plants after adding 2 g of root and blending for various times. Columns followed by the same letters were not significantly different at 5% probability level in an analysis of transformed values (sq.rt). For abbrevations see Fig. 4−2−1.

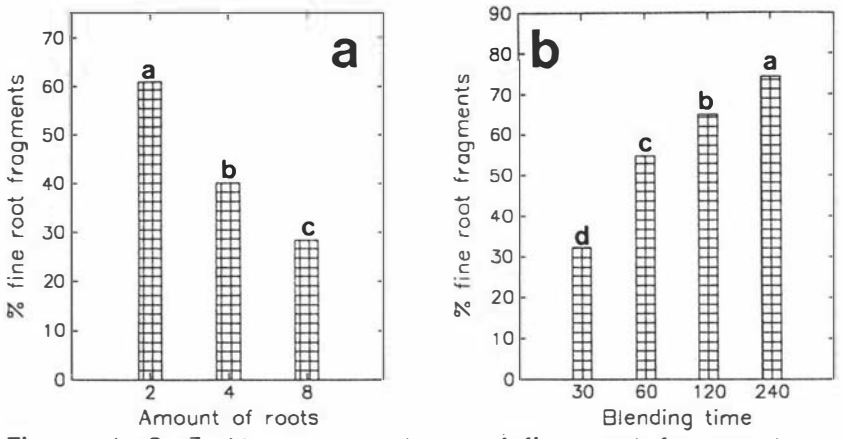


Figure 4–2–3. Mean percentage of fine root fragments in macerates of root steles of 1–year–old red clover plants using various amount of tissue (A) and different blending times (B). Columns followed by the same letter were not significantly different at 5% probability level.

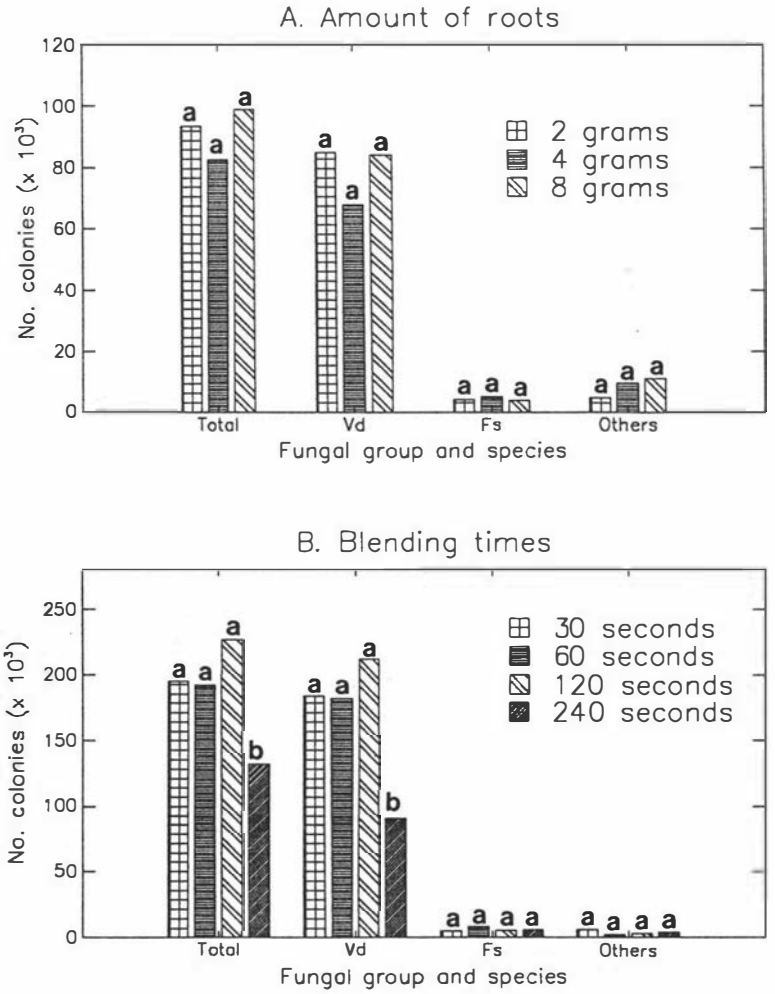


Figure 4–2–4. Mean numbers of fungal colonies in total, and of major fungal species, corrected for the proportion coarse (unplated) fragments in the macerate using different blending times (A) and various amount of tissue (B). Columns followed by the same letters were not significantly different at 5% probabilty level in an analysis of transformed values (sq.rt.).

4.2.4 Discussion

Maceration methods can underestimate fungal colonisation of roots if antifungal chemicals are released from the roots. Comparison of results obtained using the root maceration and the root segment methods would indicate whether fungitoxic compounds have been released during maceration. Clarke and Parkinson (1960) found that roots of leek yielded fewer colonies from macerates than from segments. Maceration of healthy tomato tissue with V. albo-atrum was also found to inhibit fungal germination (Pegg, 1978). Antifungal chemicals have been reported from roots of red clover (McMurchy and Higgins, 1984). However, many more colonies were obtained from root macerate than from root segments (Table 4-2-1), thus there was no evidence of suppression of fungal growth by materials in the macerate. Similar results were obtained by Clarke and Parkinson (1960) for broad bean and Singh (1965) for V. myrtillus.

Plant tissue macerates can also stimulate fungal growth. Pegg (1978) found that maceration of chrysanthemum stem tissue with mycelium of V. dahliae increased colony numbers compared with maceration of agar discs bearing the mycelia alone. It is possible that the host stem acted as a specific stimulant for fungal germination. Alternatively, the presence of host tissue may have reduced the killing of fungal cells and propagules during the maceration process. In the present experiment, no stimulation was observed, but it was found that when 2 g of roots were blended for 240 sec, the treatment which gave the greatest proportion of finely macerated tissue (Fig.4-2-3 B), colony numbers were lower than those with macerate from shorter blending times (Fig.4-2-4 B). It is likely that fungal propagules are killed during prolonged maceration through continuous exposure to shearing forces.

Presentation of data as number of colonies per g fresh roots rather than per unit length (Stover and Waite, 1953; Brandt et al., 1984) seemed most appropriate for red clover roots, and can more accurately reflect the concentration of fungi in the plant since there was often considerable variation in diameter for a given length of root. Fresh weight was preferred to dry weight

as it allowed measurement of the tissue actually being blended, rather than a subsample.

It is concluded that using standardised amounts of tissue and blending times, root maceration provides a useful method for comparison of fungal populations in roots subjected to different treatments. The method detected a similar range of fungi to that found by plating segments but yielded more colonies and showed less variation. Even using a narrow aperture pipette, at least half the potential viable fungal colony forming units (Fig 4-2-3) will be recovered from diluted macerate. Furthermore results can be corrected easily to allow for the loss of tissue fragments too large to pass through the pipette (Fig. 4-2-4).

4.3. EXPERIMENT TWO: QUANTITATIVE APPRAISAL OF THE EFFECTS OF SOIL TEMPERATURE AND MOISTURE ON INVASION OF RED CLOVER ROOTS BY SOILBORNE FUNGI

4.3.1 Introduction

Soil temperature and moisture are two major factors affecting root rot development of red clover (Kreitlow and Hanson, 1950; Chi and Hanson, 1959; Fulton and Hanson, 1960; Fezer, 1961; McCarter and Halpin, 1962; Ylimäki, 1967; Siddiqui and Halisky, 1968; Elliott et al., 1969), and other forage legumes (Leath et al., 1971; Wong et al., 1984). Studies cited above have mainly examined relationships between soil temperature and/or moisture, and the effects of fungi inoculated into sterilised plant growth media. Little work has been done on the effects of soil environmental factors on invasion of roots from natural soil. O'Rourke and Millar (1966) found that the recovery of root-invading fungi (mainly E. oxysporum) from field grown lucerne was highest during the summer, but effects of soil temperature and moisture on these fungi were not the major concern in their studies.

Most investigations on forage legumes have centred on rots of seedling roots or tap roots with less attention being paid to lateral ("feeder") roots. The identity and effect of fungi invading lateral roots has been studied for lucerne (Jones, 1943; O'Rourke and Millar, 1966; Hancock, 1985), subterranean clover (Wong et al., 1985 a; Barbetti et al., 1986 b; 1987), and alsike clover (Hardison, 1952; Leach et al., 1963). Aubé and Deschenes (1967) isolated fungi from lateral roots of red clover, but quantitative data were not given.

Results of Experiment 1 showed that root maceration is a useful method for comparison of fungal populations in roots of red clover subjected to different treatments.

The objectives of the present experiments were, using the root maceration method to:

1. study the effects of soil temperature and moisture on root invasion of

red clover plants grown in soil from the plant breeder's evaluation block in the glasshouse;

2. assess the fungal populations in tap and lateral roots of those plants;

3. monitor fungal invasion of roots of red clover plants grown in field plots in the plant breeder's evaluation block.

4.3.2 Materials and methods

4.3.2.1 Glasshouse trial

The experiment was carried out as, soil temperature x soil moisture (4 x 3), a 2 factor-factorial design. Three replicate unperforated plastic pots (95 mm diam. x 110 mm), and a spare pot were used for each treatment. Field soil was obtained from the field trial plot at the time of sowing (see below) and processed as described in Chapter 3. Soil (450 g) was placed into each of 144 pots. Previous work (Skipp et al., 1986) suggested that the soil contains a high level of inoculum of Pythium spp. causing severe damping-off of red clover. All seeds used in the glasshouse and field (see below) trials were treated with metalaxyl as described in Chapter 3 to ensure that sufficient seedlings emerged. Thirty fungicide-treated seeds were sown in each pot and covered to a depth of 10 mm with another 50 g soil. The pots were watered to 40, 60, or 80% water holding capacity (WHC) from an initial 27% WHC. This was equivalent to 22.41, 33.61, 44.82, and 15.04 g water/100 g dry soil, respectively.

Soil moisture was controlled by weighing the pots daily during the first 6 weeks, and twice a day for the remaining period, and adding water as required to adjust soil moisture to the designated pot WHC. After 8 weeks, the weight of 8-week-old plants in pots was added to the weight of pot plus soil for calculation of amount of water required at each watering. The pots were placed into temperature controlled waterbaths (Plate 4-1) at 10, 15, 20, or 25°C (± 0.2°C). Measurement of plant growth and isolation of root-invading fungi were undertaken 4, 8, and 12 weeks after sowing for each combination of temperature and moisture level.

Plate 4-1 General view of the waterbaths showing the effects of different temperatures on red clover growth. R - L 10 - 25°C at 5°C intervals.

4.3.2.2 Field trial

The experimental plot was situated in the plant breeder's evaluation block. It was 2 x 2 m, divided into 20 x 20 cm small plots. The seed bed was prepared by hand removal of the top vegetation and digging to a 25 cm depth, followed by mechanical rotovation to produce a fine tilth. The plot was sown on the 19th November 1986 by placing 30 metalaxyl-treated seeds in the central 100 cm^2 area of each small plot. Three small plots were randomly selected (using random number tables) 4, 8, and 12 weeks after sowing, and plants and soil removed to a depth of 25 cm for laboratory assessment.

4.3.2.3 Numbers of emerged seedlings

Numbers of emerged seedlings were counted at 7, 9, and 20 days after sowing for the pot experiment and at 20 days for the field experiment.

4.3.2.4 Shoot and root fresh weight

Plants were harvested from both field and glasshouse experiments 4, 8, and 12 weeks after sowing and their roots were washed free of soil with a jet of tap water. Numbers of plants per pot (or small plot) were counted. Shoots and roots were separated and fresh weights were determined. The roots of plants grown in pots were further divided into tap roots and lateral roots before weighing separately at 8 and 12 weeks.

4.3.2.5 Isolation of root-invading fungi

The weighed whole roots, or the separated tap roots and lateral roots, were surface-sterilised, then macerated following the procedure described in Experiment 1. The blending time was 60 sec at 4 and 8 weeks, and 90 sec at 12 weeks. The suspension of macerated root pieces was pipetted onto the surface of ABPDA, spread, and incubated as described in Experiment 1.

Before each complete isolation was undertaken, a preliminary test was done to determine the concentrations of the macerated suspension which would give a suitable number of colonies per plate for counting. These tests were done using plants from the spare pots prepared for each treatment. No dilutions were made for macerates obtained at 4 and 8 weeks, and 5-fold dilutions were made at 12 weeks. Identification and colony counts of individual fungal species growing on ABPDA were achieved by frequent inspection with a stereomicroscope for characteristic reproductive and/or vegetative structures. This started 2 or 3 days after plating by examination for chains of endoconidia of Trichocladium basicola. By 10 days, most of fungi had been identified. A few unidentified fungi were subcultured onto PDA for further examination and identification. The colonies growing on the surface of ABPDA plates were then removed by swabbing with cotton wool so that colonies of Verticillium dahliae, which possess dark microsclerotia, could be identified under a stereomicroscope, and counted.

4.3.2.6 Calculation of parameters of fungal invasion

Numbers of colonies per gram fresh roots = Total numbers of colonies on 3 plates x 100 x dilution factor / 0.75 / weight of roots macerated

Numbers of colonies per root = Numbers of colonies per gram x mean weight per root.

When the isolations were made separately from tap roots and lateral roots, the number of colonies per gram of whole roots was calculated as: % of whole root weight as tap root x No. of colonies per gram tap root + % of whole root weight as lateral roots x No. of colonies per gram lateral roots.

The total number of colonies per gram fresh root, or per root, was the sum of the no. of colonies of all individual fungal species per g or per root.

Ratios of mean numbers of colonies per gram tap roots to lateral roots were the quotients of mean numbers of colonies per gram tap roots divided by mean numbers of colonies per gram lateral roots.

4.3.2.7 Field soil moisture and temperature

Soil moisture at 0 - 10 cm depth was measured at 10 day intervals during the experiment. Soil cores were taken from five locations in the plot and soil moisture content in each was determined by the oven-drying method. Daily soil temperatures at 10 cm depth and daily rainfall data were obtained from the meterological station, DSIR, Palmerston North, which is about 500 m from the field plot. Total rainfall and mean daily soil temperature at 10cm depth were calculated for the same 10 day intervals used for soil moisture determinations.

4.3.2.8 Relationship between soil water content and matric potential

Soil samples (1 kg) were taken from the field plot just before sowing, and from pot soil just before the pots were filled. The drying boundary of both field and pot soils was determined by Haine's Apparatus for -0.5 and -0.05 bars and by the Pressure Plate Apparatus for -1, -3, and -15 bars. Two replicates of each soil per matric potential were used. After equilibration, (about 4 h for the Haine's Apparatus and 7 days for the Pressure Plate Apparatus), the soil samples were dried at 105°C for 24 h. Mean percentage of soil water was calculated and plotted versus the corresponding matric potentials. Water percentage at 0 bar was determined by oven drying five replicate samples of saturated soil.

4.3.2.9 Statistical analysis

Statistical analyses were performed after arcsine (seedling emergence data) or square root (fungal colony data) transformation except for fresh weight data which were not transformed.

A two way analysis was carried out on the data obtained from the glasshouse trial at each harvest (counting) as soil temperatures x moisture. Data from the field trial were subjected to one way analysis of variance (LSD test).

4.3.3 Results

4.3.3.1 Glasshouse trial

A. Seedling emergence

Numbers of seedlings from soil in pots held at 10°C were fewer than those at 15, 20, and 25°C, 7 and 9 days after sowing, but the total numbers of seedlings which emerged did not differ significantly at 20 days (Table 4-3-1).

In contrast, the numbers of emerged seedlings did not differ among the different soil moisture contents until 20 days after sowing when there were fewer emerged seedlings at 80% WHC than at 40 or 60% WHC (Table 4-3-1).

Table 4-3-1. Mean numbers of emerged seedlings 7, 9, or 20 days after sowing 30 seeds treated with metalaxyl into each of 12 pots at different soil temperatures (°C) and moistures (% WHC).

Factors	Levels	Days after sowing 7	9	20
Temperature	10	0.39 a[1]	5.28 a	12.97 a
	15	10.14 b	12.00 b	13.25 a
	20	12.89 c	13.11 b	13.58 a
	25	12.36 c	12.92 b	13.36 a
Moisture	40	9.25 a	11.33 a	14.44 b
	60	9.67 a	11.48 a	13.79 b
	80	7.92 a	9.67 a	11.65 a

[1]: Numbers followed by the same letters were not significantly different at 5% probability level in an analysis of transformed values (arcsine).

B. Shoot and root fresh weight

The greatest shoot weights initially were recorded from plants grown in pots held at 25°C (Table 4-3-2 A). However, by 12 weeks shoot weights were

similar at temperatures of 15, 20, and 25°C, but remained much lower at 10°C (Table 4-3-2 A).

The highest weights of whole roots at 4 and 8 weeks were obtained from plants grown at 20 and 25°C, but at 12 weeks greatest root weights were from plants grown at 15°C. The lowest weights at all harvests were from pots kept at 10°C (Table 4-3-2 A).

The mean weights of shoots, whole roots, tap roots and lateral roots per plant were significantly higher at 60% WHC than at 40 or 80% WHC, except at 4 weeks where root weight was similar at 40 and 60% WHC (Table 4-3-2 B).

C. Numbers of fungal colonies per gram fresh roots

The fungi most frequently isolated from roots of red clover were (listed in order of decreasing numbers) V. dahliae, Fusarium solani, F. oxysporum, Cylindrocladium scoparium, and T. basicola. Other less frequently isolated fungi were Cylindrocarpon destructans, Gliocladium roseum, Phoma medicaginis, species of Acremonium, Alternaria, Aspergillus, Chrysosporium, Cladosporium, Fusarium, Humicola, Paecilomyces, Penicillium, Phoma, Rhizopus, Trichoderma, Volutella, and sterile grey and sterile white mycelium. A few colonies of species of Colletotrichum, Epicoccum, Geotrichium, Myrothecium, Morteriella, Pythium, Rhizoctonia (binuclate type), Stilbum, and Ulocladium were also obtained from different ages of plants.

Higher temperatures (20 and 25°C) favoured most fungal species, but the greatest number of colonies of Cy . scoparium was consistently obtained at 20°C at all harvests (Table 4-3-3 A). Moreover, the effects of temperature on root invasion by V. dahliae and F. solani varied with plant age. Greatest numbers of colonies of these fungi at 4 weeks were obtained from plants grown at 10°C and at 12 weeks, 25°C.

Numbers of colonies per gram whole roots increased as moisture content increased. Most plants grown at 80% WHC were stunted, had some rotted roots, and chlamydospores of T. basicola were found on some dark lesions examined under a stereomicroscope. The influence of soil moisture on root invasion by

F.solani differed for different-aged plants. Most colonies were recovered from plants grown in soil at 40% WHC after 4 and 8 weeks, and at 80% WHC after 12 weeks. In contrast, most colonies of V. dahliae were isolated from roots of plants grown at 80% WHC at 4 weeks, but no significant differences were detected among all levels of WHC at 8 and 12 weeks (Table 4-3-3 B).

Generally, numbers of colonies of the major fungal species increased as plant age increased with the exception of V. dahliae, for which more colonies were isolated at 4 weeks than at 8 or 12 weeks (Table 4-3-3)

D. Numbers of colonies per root

Numbers of colonies in total, and of the major fungal species occurred to the greatest extent at higher temperatures (20 and 25°C) at 8 and 12 weeks (Table 4-3-4 A). However, temperature did not affect the numbers of colonies per root, in total at 4 weeks, or of V.dahliae at 4 and 12 weeks, where similar numbers of colonies were isolated at each temperature.

Moisture content showed a similar effect on numbers of colonies in total, and of F. oxysporum, T. basicola, and Cy. scoparium, as when results were expressed as number per gram roots (Table 4-3-4 B). However, the effect of moisture content on numbers of colonies of F. solani recovered was only evident at 12 weeks (Table 4-3-4 B). Furthermore, most colonies of V. dahliae were obtained at 60% WHC, for all ages of plant.

Numbers of colonies gradually increased during the experimental period to reach the highest at 12 weeks with exception of V. dahliae (Table 4-3-4). More V. dahliae colonies were isolated at 4 weeks than at 8 or 12 weeks, but the magnitude of the decline was much less apparent than when results were expressed on a per gram basis.

Table 4-3-2. Mean weights (mg) of shoots and roots per red clover plant grown in pots as affected by soil temperatures (A) and moistures (B) 4, 8, and 12 weeks after sowing.

A. Temperatures.

Ages (weeks)	Temp. (°C)	Shoot	Whole root	Tap root	Lateral root
4	10	33.0 d[1]	20.8 c		
	15	72.8 c	50.7 b		
	20	124.6 b	69.2 a		
	25	153.9 a	68.1 a		
8	10	87.2 d	87.0 c	14.4 d	72.6 c
	15	473.4 c	328.5 b	52.7 c	275.7 b
	20	1133.7 b	541.3 a	84.0 b	457.3 a
	25	1421.3 a	601.9 a	98.6 a	502.5 a
12	10	220.2 b	270.8 c	46.3 b	224.5 c
	15	1624.3 a	1297.1 a	197.1 a	1100.0 a
	20	1671.2 a	856.7 b	196.2 a	660.5 b
	25	1882.4 a	771.9 b	169.3 a	602.7 b

B. Moistures.

Ages (weeks)	Mois. (% WHC)	Shoot	Whole root	Tap root	Lateral root
4	40	76.6 c	51.5 a		
	60	124.1 a	59.8 a		
	80	87.5 b	45.3 b		
8	40	425.1 c	322.6 b	39.6 c	283.0 b
	60	1178.7 a	530.5 a	88.0 a	442.5 a
	80	733.0 b	315.4 b	59.8 b	255.6 b
12	40	912.8 b	685.1 b	121.0 b	564.1 b
	60	2156.8 a	1130.8 a	216.7 a	914.1 a
	80	979.0 b	581.6 b	119.0 b	462.5 b

[1]: Means for one factor were averaged over all levels of the other factor. Weights followed by the same letters for the each harvest date were not significantly different at 5% probability level.

Table 4-3-3. Mean numbers of colonies ($x10^3$) per gram fresh roots in total, and of the major fungal species isolated from whole roots of red clover plants grown in pots, as affected by soil temperatures (A) and moistures (B) 4, 8, and 12 week after sowing.

A. Temperatures (°C)

Temp.	Total	Vd[2]	Fs	Fo	TB	Cs	Other
			4 weeks				
10	64.40 a[1]	54.97 a	7.70 a	0.09 b	0.00 c	0.07 b	1.58 a
15	14.54 b	12.37 b	0.24 b	0.57 a	0.57 b	0.04 b	0.75 a
20	18.91 b	12.99 b	1.25 b	1.07 a	1.78 a	1.14 a	0.68 a
25	12.99 b	8.34 b	1.92 b	0.73 a	1.32 ab	0.10 b	0.57 a
			8 weeks				
10	11.30 a	0.13 a	4.45 a	0.64 a	0.00 b	0.02 c	6.06 a
15	3.54 a	0.07 a	0.37 a	0.37 a	0.88 a	0.51 b	1.34 b
20	6.73 a	0.22 a	2.60 a	0.52 a	0.96 a	1.43 a	1.00 b
25	6.46 a	0.39 a	2.87 a	0.77 a	1.37 a	0.53 b	0.53 b
			12 weeks				
10	9.43 b	0.02 a	6.52 b	0.09 b	0.07 b	0.00 c	2.74 b
15	3.03 b	0.05 a	0.93 b	0.14 b	0.22 b	0.23 c	1.47 b
20	43.77 a	0.50 a	21.77 a	3.15 a	1.05 b	8.94 a	8.39 a
25	51.88 a	0.40 a	34.74 a	3.19 a	3.53 a	1.80 b	8.22 a

B. Moistures (% WHC)

Mois.	Total	Vd	Fs	Fo	Tb	Cs	Other
			4 weeks				
40	24.66 b	17.63 b	5.04 a	0.33 a	0.79 a	0.04 b	0.83 a
60	20.33 b	16.76 b	1.12 b	0.84 a	0.61 a	0.22 ab	0.78 a
80	38.14 a	32.12 a	2.17 b	0.67 a	1.36 a	0.75 a	1.07 a
			8 weeks				
40	8.19 a	0.17 a	4.84 a	0.87 a	0.86 a	0.32 b	1.13 a
60	5.90 a	0.34 a	1.19 b	0.28 a	0.31 a	0.22 b	3.55 a
80	6.93 a	0.01 a	1.69 b	0.57 a	1.24 a	1.32 a	2.03 a
			12 weeks				
40	12.83 b	0.22 a	5.72 b	1.25 a	1.04 a	0.92 b	3.68 a
60	9.58 b	0.41 a	5.17 b	1.04 a	0.61 a	0.59 b	1.77 b
80	58.67 a	0.07 a	37.08 a	2.63 a	2.00 a	6.72 a	10.17 a

[1]: Data for each factor were averaged over all levels of the other factor. Means followed by the same letters for each fungus at each harvest date were not significantly different at 5% probability level in an analysis of transformed values (sq.rt.).

[2]: Vd: V. dahliae, Fs: F. solani, Fo: F. oxysporum, Cs: Cy. scoparium, TB: T. basicola

Table 4-3-4. Mean numbers of colonies ($x10^3$) **per root** in total, and of the major fungal species isolated from whole roots of red clover plants grown in pots, as affected by soil temperatures (A) and moistures (B) 4, 8, and 12 weeks after sowing.

A. Temperatures (°C)

Temp.	Total	Vd[2]	Fs	Fo	Tb	Cs	Other
				4 weeks			
10	1.28 a[1]	1.09 a	0.16 a	0.01 b	0.00 b	0.01 b	0.04 a
15	0.74 a	0.63 a	0.01 b	0.03 ab	0.03 b	0.01 b	0.04 a
20	1.18 a	0.79 a	0.08 ab	0.08 a	0.12 a	0.07 a	0.04 a
25	0.89 a	0.60 a	0.12 a	0.05 ab	0.08 ab	0.01 b	0.04 a
				8 weeks			
10	0.89 b	0.01 c	0.52 b	0.04 b	0.00 a	0.01 b	0.31 a
15	1.02 b	0.03 bc	0.11 b	0.10 b	0.23 a	0.11 b	0.43 a
20	3.28 a	0.14 ab	1.28 a	0.27 a	0.51 a	0.59 a	0.62 a
25	3.48 a	0.24 a	1.46 a	0.38 a	0.80 a	0.31 a	0.30 a
				12 weeks			
10	2.08 b	0.01 a	1.27 b	0.02 b	0.02 b	0.00 c	1.05 a
15	3.22 b	0.06 a	0.89 b	0.17 b	0.23 b	0.29 c	1.59 a
20	21.74 a	0.46 a	10.43 a	1.81 a	0.74 b	4.27 a	4.03 a
25	23.18 a	0.35 a	14.86 a	1.92 a	1.96 a	0.71 b	3.39 a

B. Moistures (% WHC)

Mois.	Total	Vd	Fs	Fo	Tb	Cs	Other
				4 weeks			
40	0.74 b	0.52 b	0.11 a	0.02 a	0.05 a	0.01 b	0.04 a
60	0.99 ab	0.78 ab	0.06 a	0.06 a	0.05 a	0.02 ab	0.05 a
80	1.34 a	1.04 a	0.10 a	0.04 a	0.08 a	0.04 a	0.05 a
				8 weeks			
40	2.04 a	0.06 b	1.02 a	0.20 a	0.33 a	0.13 b	0.30 a
60	2.10 a	0.24 a	0.77 a	0.20 a	0.23 a	0.17 b	0.47 a
80	2.36 a	0.02 b	0.74 a	0.20 a	0.60 a	0.46 a	0.38 a
				12 weeks			
40	8.74 b	0.15 b	3.81 b	0.85 a	0.74 a	0.73 b	2.42 a
60	11.14 b	0.48 a	6.17 b	1.22 a	0.86 a	0.77 b	1.81 a
80	17.78 a	0.02 b	10.60 a	0.86 a	0.61 a	2.44 a	3.24 a

[1], [2]: See Table 4-3-3 for explanation.

E. Comparison of fungal colonies in tap roots and lateral roots

Tap roots and lateral roots were invaded by a similar range of fungi, but the numbers of colonies differed considerably between the two sorts of roots (Appendix 4-3-1; 4-3-2), especially when the results were expressed on per g basis.

The ratio of fungal colonies recovered (tap roots : lateral roots; Table 4-3-5) varied with age of plant, levels of soil temperature and moisture, and particularly on whether results were expressed on a per g root or per root basis. Generally, more colonies in total, and of the major fungi were obtained from tap roots than from lateral roots of the same-aged plants. The highest ratios were obtained for F. solani (155.7; 10°C) and V. dahliae (67.7; 25°C).

4.3.3.2 Field trial

A. Seedling emergence and shoot and root fresh weights

The mean number of seedlings emerged per small plot at 20 days was 9.6. Mean weights of shoots per plant at 4, 8, and 12 weeks were 44.3, 220.2, and 5081.5 mg, respectively. Corresponding mean weights of roots per plant were 22.0, 86.0, and 1127.7 mg.

B. Numbers of fungal colonies per gram fresh roots

The major fungal species isolated from roots of plants growing in the field (Table 4-3-6 A) were the same as those from plants in pots. Other fungi isolated were species of Acremonium, Alternaria, Chrysosporium, Cladosporium, Fusarium, Humicola, Paeciliomyces, Penicillium, Phoma, Rhizopus, and those with sterile mycelium.

More colonies in total, and of V. dahliae, were recovered at 4 weeks than at 8 and 12 weeks. Whereas fewer colonies of F. solani were obtained at 12 weeks than at 4 or 8 weeks. The other fungi listed in Table 4-3-6 remained at a relatively constant level.

Table 4-3-5. Ratios of mean numbers of colonies (x 10^3) per gram tap root to lateral roots (A), and per tap root to lateral roots per pot plant (B) as affected by plant age (weeks), soil temperature (°C), and moisture (%WHC).

A. Per gram fresh weight.

Factors	Levels	Total	Vd[2]	Fs	Fo	Tb	Cs	Other
Ages	8	13.5[1]	24.0	40.5	3.3	20.1	11.5	5.5
	12	2.5	54.0	3.0	1.1	11.3	1.4	1.9
Temp.	10	19.0	6.3	155.7	0.9	0.3	5.0	6.1
	15	6.5	14.0	22.1	1.1	12.8	14.6	3.6
	20	2.0	39.5	2.0	1.1	13.7	1.9	1.6
	25	3.2	67.7	3.2	2.4	18.6	2.0	1.6
Mois.	40	6.6	19.4	8.6	1.8	8.8	2.0	1.6
	60	4.4	63.7	3.7	1.9	12.3	2.2	4.8
	80	2.8	17.0	3.6	1.3	33.4	2.0	1.3

B. Per root.

Factors	Levels	Total	Vd	Fs	Fo	Tb	Cs	Other
Ages	8	1.6	4.5	4.6	1.0	3.9	2.1	1.6
	12	1.1	10.0	1.2	0.5	4.3	1.0	0.8
Temp.	10	4.9	5.0	21.3	0.2	1.0	1.0	2.1
	15	1.0	1.5	3.2	0.3	1.6	1.5	0.6
	20	1.1	14.0	1.0	0.6	4.3	1.2	0.9
	25	1.3	14.0	1.3	0.6	5.3	0.8	0.8
Mois.	40	0.8	4.0	0.7	0.4	1.9	0.5	0.8
	60	1.1	17.0	1.0	0.4	4.0	0.5	1.2
	80	1.7	1.0	2.0	1.2	14.3	1.8	0.7

[1]: Ratios were calculated after three way analyses for tap and lateral roots.

[2]: See Table 4-3-3 for explanation.

C. Numbers of fungal colonies per root

More colonies in total, and of V.dahliae, and F.oxysporum were obtained at 12 weeks than at 4 and 8 weeks (Table 4-3-6). However, no differences were detected on the numbers of colonies of F. solani, Cy. scoparium, and T. basicola between the different sampling dates.

Table 4-3-6. Mean numbers of colonies (x 10^3) per gram fresh roots (A), and per root (B) in total, and of the major fungal species isolated from roots of red clover plants grown in field 4, 8, and 12 weeks after sowing.

A. Per gram fresh weight.

Fungi isolated	4 weeks	8 weeks	12 weeks
Total	56.00 a[1]	19.22 b	8.48 b
Verticillium dahliae	41.70 a	0.80 b	3.76 b
Fusarium solani	10.29 a	6.14 a	0.59 b
F. oxysporum	1.01 a	6.33 a	2.77 a
Cylindrocladium scoparum	0.00 a	0.00 a	0.03 a
Trichocladium basicola	0.00 a	1.66 a	0.33 a
Other	3.00 a	4.29 a	1.01 b

B. Per root.

Fungi isolated	4 weeks	8 weeks	12 weeks
Total	1.15 b	1.58 b	8.84 a
Verticillium dahliae	0.78 b	0.05 b	4.40 a
Fusarium solani	0.28 a	0.55 a	0.77 a
F. oxysporum	0.03 b	0.51 ab	1.88 a
Cylindrocladium scoparum	0.00 a	0.00 a	0.05 a
Trichocladium basicola	0.00 a	0.15 a	0.46 a
Other	0.07 b	0.33 b	1.28 a

[1]: Means followed by the same letters for each fungal species were not significantly different at 5% probability level in an analysis of transformed values (sq.rt.).

4.3.3.3 Field soil temperature and moisture

Mean soil temperatures at 0 - 10 cm depth in the field plot taken at 10 day intervals during the experiment ranged from 14.5 to 21°C. Mean soil moisture contents ranged from 10.19 to 23.65 g water/100 g dry soil, and corresponding rainfall from 0.5 to 52.3 mm (Fig.4-3-1).

4.3.3.4 Relationships between soil water content and matric potential

The soil moisture character curves were similar for the two soils (Fig. 4-3-2). At 0 bar, water contents were 63.76 and 56.02%, and at -15 bars were 15.4 and 15.1%, for field and pot soil, respectively (Fig. 4-3-2).

4.3.4 Discussion

Temperature appeared to affect the speed of emergence of red clover seedlings from field soil rather than the total numbers of emerging seedlings (Table 4-3-1). Similar results were reported by Hampton et al (1987) for laboratory germination tests. Slow emergence at lower temperatures may favour attack by seedling pathogens such as Pythium spp. (Chi and Hanson, 1962; Wong et al., 1984), although Skipp (1986) has reported damping-off of red clover to be greatest at high temperatures. In the present study, seed was treated with metalaxyl before sowing in pots and field plots to minimise plant loss due to damping-off caused by oomycete fungi during establishment. Post-emergence damping-off was not observed in the experiments suggesting that the treatment had probably been effective. The reduced emergence of seedlings from wet soil (80% WHC Table 4-3-1) after 20 days may reflect a lack of oxygen, or the action of pathogens other than oomycetes.

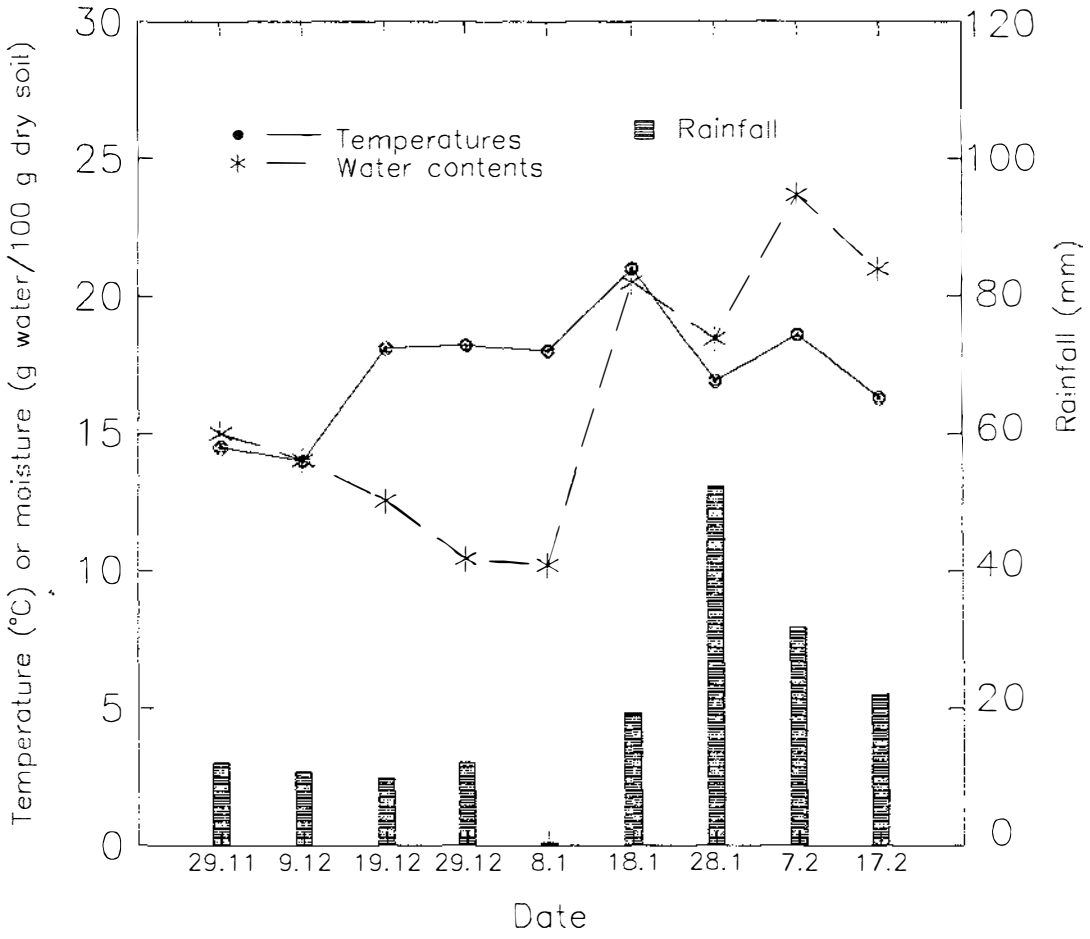


Figure 4–3–1. Mean soil temperatures, soil water contents at 0 ––10 cm depth, and rainfall at 10 days intervals from 19 Nov. 1986 to 17 Feb. 1987.

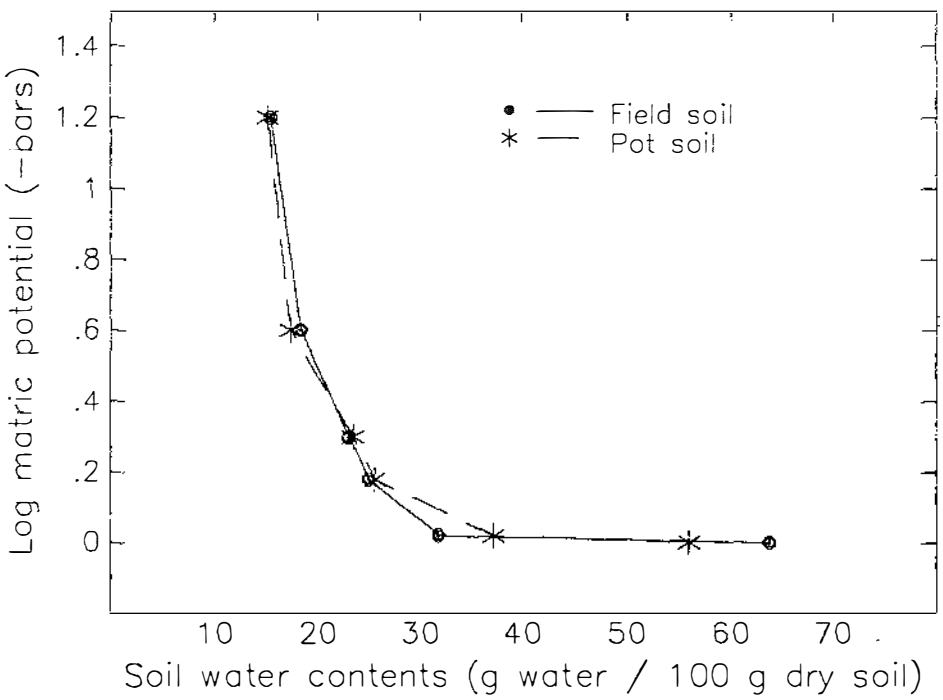


Figure 4–3–2. Relationship between water content and matric potential for field and pot soils. Matric potential data presented after Log transformation.

Red clover plants grew better at the higher temperatures used in the water bath experiments (Table 4-3-2, see also Kendall, 1958; Fergus and Hollowell, 1960; Bowley et al., 1984). Similarly, shoot and root weight have generally been found to be positively related to available soil moisture (Gist and Matt, 1957; Kilmer et al., 1960). However, in the present experiments, shoots and roots grew less well at 80% WHC than at lower water contents (Table 4-3-2). This was possibly due to effects on subsequent growth of the factors discussed above which affected emergence in wet soil.

Fungal invasion of red clover roots appeared to occur progressively from the seedling stage onwards (Tables 4-3-3; 4-3-4; Skipp et al., 1986), so that by 12 weeks the numbers of colonies of most of the major fungal species were much higher than at 4 weeks. Garber and Houston (1966) showed that V. albo-atrum could infect cotton (Gossypium hirsutum L.) roots within 24 h after inoculation. Although the period needed for V. dahliae to invade red clover roots in field soil is unknown, it had become the predominant component of the internal root mycoflora of pot, and field grown plants by 4 weeks after sowing (Tables 4-3-3; 4-3-6).

The reason for the marked decline in the numbers of colonies of V. dahliae recovered from roots of pot plants, and of V. dahliae and F. solani from field grown plants, from 4 to 8 weeks is not very clear (Table 4-3-3, 4-3-6). It is unlikely that the relatively high numbers of colonies of V. dahliae on the plates at 4 weeks were contaminants from the blender since the washing/disinfection process was consistently effective in removing viable propagules from the blender cup. A similar decline in fungal colonisation of roots has been reported for F. oxysporum f.sp. lycopersici in stems of tomatoes (Matta and Dimond, 1963), and Gaeumannomyces graminis (Saccardo) Arx & Olivier var. tritici J. Walker in roots of wheat (El-Nashaar et al., 1986), using maceration and ELISA techniques, respectively. The most likely explanation is that the fungal biomass in the roots was rapidly diluted by the development of new, uninfected root tissue from 4 weeks after sowing (Table 4-3-2). However, once the organisms were well established, the relative biomass of the fungi was able to increase rapidly (Table 4-3-3). On the other hand, it is also possible, as seen in the other studies cited

above, that many hyphae which had penetrated root tissue during early stages of seedling development (thus being available to form colonies on plates inoculated with root macerate) failed to reach the vascular region (Garber and Houston, 1966), or died within the root tissue (Matta and Dimond, 1963).

More fungal colonies were isolated from plants grown at higher temperatures (20 and 25°C) than at low temperatures (10 and 15°C) (Table 4-2-3, 4-2-4). Root rot of red clover has often been reported to be enhanced by high soil temperatures (Kreitlow and Hanson, 1950; Chi and Hanson, 1959; Fulton and Hanson,1960; McCarter and Halpin, 1962; Ylimäki, 1967; Siddiqui and Halisky, 1968; Elliott et al., 1969). However, more colonies of the two predominant fungal species, V. dahliae and F. solani, were isolated from plants grown at 10°C than at higher temperatures at 4 weeks (Table 4-3-3). Wong et al (1984) reported that the most severe root rot of subterranean clover caused by species of Fusarium and other soilborne fungi occurred at 10°C. As mentioned above, low soil temperatures are less favourable for red clover growth and may increase vulnerability to disease. Although low temperature also affects fungal growth (See Chapter 2 and Table 2-4-1), fungi may have a pathogenic advantage if plant development and vigour are reduced more dramatically. Another possibility is that there is less root growth (therefore less dilution by new roots) at low temperatures.

The soil temperatures at which the highest numbers of colonies were obtained at 12 weeks fell within the range of optimum temperatures for mycelial growth of the same species in culture (see Chapter 2). Thus by 12 weeks the higher temperatures (20 and 25°C) may be facilitating growth within host tissues.

Higher soil moisture contents have been considered most favourable for colonisation by the major fungal species encountered in this study (See Chapter 2). More fungal colonies were isolated from the plants grown at 80% WHC than at lower soil moisture contents (Table 4-2-3, 4-2-4), and correspondingly, root rot was most severe on plants grown under these conditions. Previous work has shown that Cy. scoparium and T. basicola were highly pathogenic to red clover plants grown in potting mix in a glasshouse at high soil moisture levels (Skipp et al., 1986). The lower yield of shoots and roots of plants grown in field soil at 80%

WHC may have been mainly due to fungal infection.

However, the highest numbers of colonies of F. solani were isolated from the roots of plants grown at 40% WHC at 4 and 8 weeks (especially at 10°C) (Table 4-3-3). This agreed with other work which has shown that root rot of red clover incited by species of Fusarium was more severe in dry soil than in wet soil (Chi and Hanson, 1959; Fezer, 1961; Ylimäki, 1967). As discussed previously (Chapter 2), the minimum moisture requirements of the fungi are well below those of their hosts, thus they will be less adversely affected than their hosts by decreased soil moisture. Furthermore, species of Fusarium required a lower water content for minimum growth than V. dahliae, Cy. scoparium, and T. basicola (Table 2-4-1). Thus, F. solani may take advantage of lower vigour of plants grown under low temperature and low moisture conditions.

In the present study, a similar range of fungal species was isolated from tap roots and lateral roots. However, lateral roots contained far fewer fungal colonies per gram of tissue than did tap roots (Table 4-3-5). One reason may be the short life cycle of the lateral roots. The presence of dark lateral root scars on tap roots indicated that diseased lateral roots had sloughed off. These would therefore have been lost prior to processing for fungal isolation. Presumably, the loss of fungi in the infected lateral roots may have prevented continued invasion into the tap root. The relatively high level of fungal infection in tap roots compared with lateral roots and the importance of the tap roots as the main channel for transfer of water and nutrients, suggests that the tap root should be given most attention in the study of root invasion.

On the other hand, as root systems develop, a greater proportion (Table 4-3-2) is made up of lateral roots. The effects on pasture production of fungal infection of lateral roots should not be ignored. Jones (1943) noticed that yield of lucerne was affected by the health of lateral roots. Hancock (1985) found that shoot growth of lucerne was reduced markedly by fungal invasion of rootlets. Hardison (1952) reported that poor persistence of alsike clover was mainly due to rootlet rot incited by soilborne fungi. The present research appears to be the first detailed study on fungal infection of lateral roots of red clover.

Fungal infection of young lateral roots and rootlets of plants is an area

which needs more scientific input. The present results showed that root maceration is a useful technique for this type of study.

Numbers of colonies per gram fresh roots and per root were both used as quantitative parameters of fungal invasion in the present experiments. Number of colonies per gram fresh roots was more suitable for expressing fungal invasion of plants of a similar age, whereas number of colonies per root was more useful to show the progressive development of fungi in roots. The consideration of both parameters for the 12-week experimental period gave a better indication of the process of root invasion by fungi.

The range of soil temperature and moisture content during most periods of the year in the major pastoral areas of New Zealand (Grasslands Division, 1984) is within the range used in the pot experiment. Consideration of the results of the field and pot trials together provides a comprehensive basis for understanding invasion of red clover by fungi during the year.

CHAPTER 5
A STUDY OF EFFECTS OF DISEASES ON THE GROWTH OF RED CLOVER BY USE OF FUNGICIDES DRENCHES

5.1 INTRODUCTION

Fungicides are not widely used to control root diseases of forage legumes other than as seed treatments, because they are perceived to be uneconomic, and because of concerns about exposure of animals and humans to toxic residues (Leath, 1981). However, they have been used in glasshouse and field studies to assess the effects of disease on plant growth and development, and to estimate yield reductions that can be attributed to the infection of different fungal groups (Chapter 2). Few reports have been made of this type study on red clover (Leath et al., 1973; Skipp et al., 1986;). Work with some fungicides has shown that effects are not confined to the target fungi, but also non-target micro-organisms, which results a marked change in the microbial equilibrium of the soil (Bollen, 1961; Wainwright and Pugh, 1974; Atlas et al., 1978; Ingham and Coleman, 1984; Ingham, 1985). EBI fungicides have been little studied in this respect apart from a paper by Elmholt and Smedegaard-Petersen (1988). Thus there appeared to be a need to establish experimental methods for the present investigation which would take account of these factors. Experiments reported in this chapter examined : -

1. Effects of fungicides on growth of root-invading fungi, Rhizobium trifolii, and red clover seedlings in the laboratory.

2. Effects of prochloraz drenches on populations of soil micro-organisms and red clover growth in the glasshouse.

3. Effects of fungicidal drenches on root-invading fungi and plant yield in the plant breeder's evaluation block.

5.2 EXPERIMENT THREE : EFFECTS OF FUNGICIDES ON GROWTH OF ROOT-INVADING FUNGI, RHIZOBIUM TRIFOLII, AND RED CLOVER SEEDLINGS IN THE LABORATORY

5.2.1 Introduction

Since organic fungicides first became available in 1934 (Spencer, 1977) numerous reports have been published on the use of fungicides to control soilborne fungal pathogens. The fungicides used included protectants, systemics, and recently, ergosterol biosynthesis inhibitors (EBIs). According to their chemical structures, the protectants most frequently used were disulphides and cylic imides; the systemics were benzimidazoles, dicarboximides, and acyl alanines; the EBIs were triazoles, imidazoles, and pyrimidines. Although many fungicides appear to have the potential to control root diseases of red clover, none has been reported to inhibit growth of all the major fungal species isolated from red clover roots during previous experiments.

It has been reported that some fungicides used on forage legumes adversely affect the growth of Rhizobium (Fisher et al., 1979; Fisher and Hayes, 1981; 1982; Sirois et al., 1981; Tu, 1981; 1982; Heinonen-Tanski, et al., 1982; Antoun et al., 1984; Ruiz-Sainz et al., 1984). Some were known to decrease (Fisher and Hayes, 1981; 1982; Tu, 1981; Sirois et al., 1981; Antoun et al., 1984), or increase (Sirois et al., 1981; Tu, 1981; Antoun et al., 1984) the growth of white clover or lucerne. No information is available for red clover. Therefore, it was necessary to assess the effects of fungicides on the growth of Rhizobium and red clover plants.

The objectives of the present experiment were to assess the effects of some candidate fungicides on the growth of:

1. the major root-invading fungi isolated from red clover;
2. red clover seedlings;
3. Rhizobium trifolii.

5.2.3 Materials and methods

5.2.3.1 Fungicides

Eleven fungicides were used in these experiments. They were six EBIs: fenarimol (Rubigan 12% WP. May and Baker N.Z.Ltd), flutriafol (Impact 12.5% EC. ICI Ltd), prochloraz (Sportak 45% EC. FBC Ltd), propiconazol (Tilt 25% EC. CIBA-GEIGY Ltd), triadimefon (Bayleton 25% EC. Bayer Ltd.), and triadimenol (Baytan 25% EC.Bayer Ltd.), three other systemics: benomyl (Benlate 50% WP. Du Pont Ltd.), iprodione (Rovral 25% EC. May and Baker N.Z.Ltd.), and metalaxyl (Ridomil 25% WP. GIBA GEIGY Ltd.), and two protectants: captafol (Difolatan 80% WP. N.Z.Farmer's Fertilizer Company Ltd.), and thiram (Thiram 40% EC.).

Each fungicide was tested at concentrations of 0.1, 1, 10, and 100 μg active ingredient (a.i.) per ml of culture medium for effects on mycelial growth and fungal spore germination, and 1, 10, 100, and 1000 μg a.i. per ml of distilled water for effects on growth of rhizobium, and on germination and seedling growth of red clover. Stock solutions were prepared by suspending the appropriate amount of fungicide in 20 ml sterile distilled water, and performing serial dilutions to give the required concentrations.

5.2.3.2 Fungi and rhizobia

Seven major root-invading fungi of red clover isolated during the previous experiments were tested. They were Cylindrocarpon destructans, Cylindrocladium scoparium, Fusarium oxysporum, F. solani, Gliocladium roseum, Trichocladium basicola, and Verticillium dahliae. The cultures were maintained on PDA medium and kept at 20°C in the dark until used.

A stock culture of Rhizobium trifolii Dangeard 1926 (Biotechnology Division Culture Collection NZP578) was kindly provided by Dr.P.J.Patel, Biotechnology Division, DSIR. Broth cultures were grown in yeast extract

mannitol broth (YMB) (K_2HPO_4 0.5 g, $MgSO_4.7H_2O$ 0.2 g, NaCl 0.1 g, mannitol (Difco) 10.0 g, yeast extract 1.0 g, in 1000 ml of distilled water) and incubated at 25°C for 48 h. Cultures were maintained in the YMB medium. For solid media, 15 g agar per L was added (YMA).

5.2.3.3 Effects on mycelial growth and spore germination

The ability of the fungicides to inhibit radial growth of the fungi was evaluated using an agar plate method (Torgeson, 1967). Ten ml of each fungicide suspension, made as described above was added to 190 ml molten corn meal agar (CMA Difco) cooled to 50°C in a water bath. The medium and fungicide were mixed together and 20 ml was pipetted into each of 10 petri plates. In the controls, sterile distilled water was used instead of fungicide. One plate was prepared for each combination of fungicide, concentration and fungal species. Plates were inoculated with three 4 mm diam. agar plugs taken from the margin of 10-day-old fungal colonies on PDA medium. The plates were incubated in the dark at 20°C. Colony radius was measured after 8 days.

At certain concentrations some fungicides completely stopped fungal growth. To determine whether the chemicals had had a fungistatic or a fungicidal effect, the plugs of inoculum from plates showing complete inhibition of fungal growth were transferred to corn meal agar without added fungicide, and the plates incubated for 8 days under the conditions as described above.

To assess the effects of the fungicides on spore germination, duplicate plugs (8 mm diam. x 4mm deep) were cut from CMA medium containing the same range of concentrations of the fungicides as above. The plugs were put on glass slides and one drop (25 μl) of spore suspension was placed onto each of the plugs. The spore suspensions were prepared by placing 10 mm diameter plugs from colonies of 15-day-old cultures (Cy.scoparium 2-month-old) in 10 ml distilled water, shaking for 1 min. and filtering through 4 layers of muslin to remove the agar particles and mycelium. The concentration of spore suspension was measured using a haemocytometer. The numbers of spores per drop ranged 10,000 to 20,000 for all fungal species tested except Cy. scoparium which only

contained about 725 spores per drop. After incubation at 20°C for 20 h, spores were killed by adding one drop of 0.05% Trypan blue in alcoholic lactophenol per plug. Fifty spores per field, 3 fields per plug were assessed for germination for each treatment. Spores were considered to have germinated when a germ tube had been produced.

5.2.2.4 Effects on growth of rhizobia

The level of tolerance of R. trifolii to the fungicides was evaluated using an agar cup method. Seeded medium was prepared as follows: 4 drops of diluted rhizobium culture (about 10^8 cells per ml) were added to a screw top bottle containing 3 ml molten soft water agar (0.6%) cooled to 50°C. The mixture of the inoculum and water agar was poured into each of 33 petri plates already containing 20 ml of solidified YMA medium. The top layer was allowed to set and four cups, 10 mm diameter, per plate were cut through to the bottom of the plate with a sterilised cork borer. The cups were filled with fungicide solution (150 μl) by a micropipette. Each of the four cups within the same plate contained different concentrations of the same fungicide. Three replicate plates were used for each of the fungicides. Plates were maintained at room temperature (10 - 20°C) overnight to allow the fungicide to diffuse through the agar then they were incubated at 25°C for 3 days. The size of the inhibition zone around each cup was recorded.

5.2.2.5 Effects on germination and seedling growth of red clover

The effects of fungicides on germination and seedling growth of red clover were studied in petri dishes containing two layers of filter paper. Ten seeds were sown per plate and the filter papers moistened with 3 ml of fungicide suspension. Control plates received the same amount of distilled water. Three replicate plates of each treatment were sealed in a plastic bag with a rubber band. A separate series of plates was incubated at 15 and 25°C. Seeds were considered to have germinated when a radicle had emerged. The numbers of germinated seeds

were counted after 7 days, and lengths of each whole seedling and primary root measured. Lengths of each primary shoot were calculated. Roots of three randomly selected seedlings per plate were separated and transferred to glass slides. Lengths of root hairs were measured under a compound microscope (31.25 magnification) fitted with an eyepiece micrometer.

5.2.2.6 Statistical analysis

Analysis of variance (LSD test) was performed for percentage mycelial growth inhibition data after arcsine square root transformation. A similar analysis was carried out for lengths of shoot, root, and root hair data except that these were not transformed.

5.2.3 Results

5.2.3.1 Effects on mycelial growth and spore germination

The EBI prochloraz was the most effective of the 11 fungicides tested in control of the seven fungal species (Table 5-2-1). More than 50% growth inhibition was obtained at 0.1 μg/ml, and 90 - 100% inhibition at 1 μg/ml, for all fungi except Cy. scoparium which needed higher concentrations (10 and 100 μg/ml, respectively) to achieve the similar results. Propiconazol also showed a strong inhibitory effect to most of the fungi tested. Although other EBI fungicides were highly inhibitory to some fungi at relatively low concentrations, generally, they were not as effective as prochloraz.

Benomyl showed a similar spectrum of antifungal activity as prochloraz, but it did not completely stop the growth of F. solani or G. roseum. Metalaxyl had no effect on any fungal species at 0.1 - 10 μg/ml but was slightly inhibitory occurred at 100 μg/ml.

The protectant fungicides did not affect fungal growth at 0.1 and 1 μg/ml, but some strong inhibition occurred at higher concentrations.

The number of fungal species inhibited in mycelial growth by 90% or more

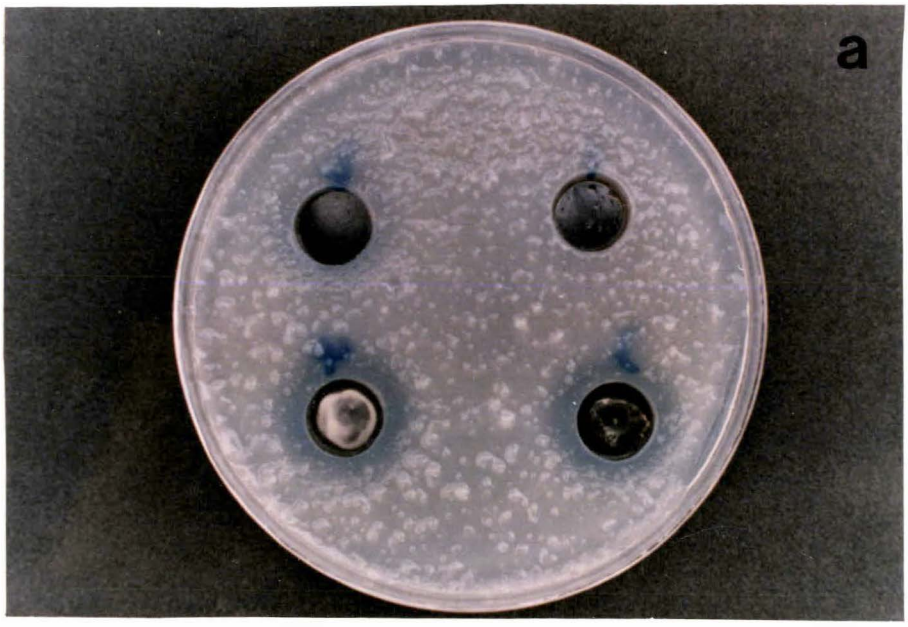
by each fungicide at concentrations of 100 μg/ml or less were: prochloraz 7, propiconazol and benomyl 6, thiram 4, flutriafol 3, fenarimol and triadimenol 2, iprodione, triadimefon, and captafol 1, and metalaxyl 0.

Vigorous fungal regrowth was noted from the plugs treated with benomyl, thiram, or captafol after transfer of plugs of inoculum from plates showing 100% inhibition to medium lacking fungicide. However, only limited regrowth (only on the plug) occurred from plugs from the lower concentration (0.1 μg/ml) of prochloraz (Table 5-2-1). No regrowth was observed from plugs from the higher concentrations of the fungicide even after 50 days incubation.

The EBIs and other systemics, generally, did not affect fungal spore germination at the concentrations tested (Appendix 5-2-1) although slight inhibition occurred for some fungal species. However, germ mycelia from the conidia treated with these fungicides were shortened, thickened, and sometimes swollen compared with those of conidia germinated on non-fungicide medium. Prochloraz was the only EBI fungicide which showed strong inhibitory effects on spore germination of any fungal species (G. roseum and V. dahliae). On the other hand, the protectants showed strong inhibition of spore germination at 1 and 10 μg/ml (Appendix 5-2-1).

5.2.3.2 Effects on Rhizobium growth

None of the fungicides inhibited the growth of Rhizobium trifolii at the range of concentrations tested except triadimefon, captafol, and thiram. The minimum concentrations for triadimefon, captafol, and thiram to inhibit rhizobial growth were 1000, 10, and 100 μg/ml, inhibition zones at 1000 μg/ml were 3, 10, and 15 mm, respectively (Plate 5-1).



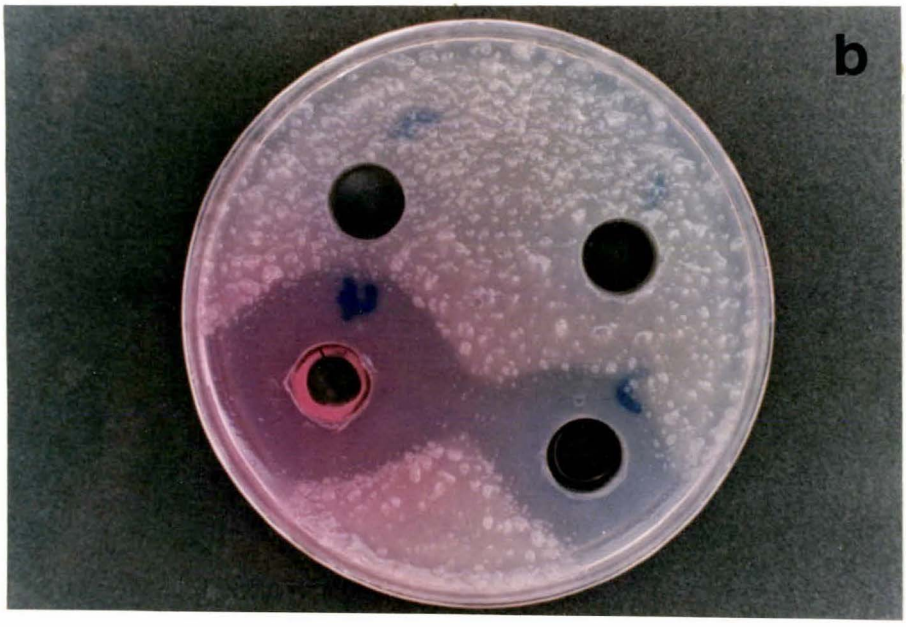


Plate 5-1 a-b Effects of captafol (a) and thiram (b) on Rhizobium growth after 3 days incubation at 25°C. Each of the 4 cups in each plate contained fungicide at concentrations of 1, 10 μg a.i./ml (Top R - L) and 100, 1000 μg a.i./ml (Bottom R-L).

5.2.3.3 Effects on germination and seedling growth of red clover

None of the fungicides tested affected germination of red clover at 1 and 10 μg/ml. Propiconazol, flutriafol, triadimefon, and metalaxyl reduced germination at concentrations of 100 μg/ml or greater at one or both incubation temperatures (15 or 25°C) (Appendix 5-2-2).

The EBI fungicides had some inhibitory effects on seedling growth of red clover (Fig. 5-2-1). Propiconazol was toxic to shoots at the lowest concentration. It reduced the length of primary shoots at 1 μg/ml at 15 and 25°C. Other EBIs significantly decreased shoot growth at 10 μg/ml or greater at both temperatures with the exception of prochloraz, which showed inhibitory effects only at 100 and 1000 μg/ml at 25°C, and at 10 μg/ml and above at 15°C.

Other systemics and protectants stimulated shoot elongation at concentrations up to and including 100 μg/ml at 25°C, but not at 15°C. Most fungicides reduced root elongation at concentrations of 10 μg/ml or greater (Fig. 5-2-1), thiram and iprodione reduced root length at 1 μg/ml. Prochloraz reduced root length at 10 μg/ml at 15°C, but it was the only fungicide which did not reduce root growth at any concentration tested at 25°C (Fig. 5-2-1).

Root hair growth was also affected by fungicides (Table 5-2-2). Lengths of root hairs were greatest at low concentrations and lowest at higher concentrations of some fungicides. Again, prochloraz was the only fungicide which did not affect the root hair growth at any concentration tested .

Table 5-2-1. Mean percentage inhibition of mycelial growth 8 days after inoculation of seven fungal species on corn meal agar containing four concentrations of different fungicides.

Fungicides	Tb	Cd	Cs	Fo	Fs	Gr	Vd[1]
				0.1 µg/ml			
EBIs							
Fenarimol	64 b[2]	0 e	0 d	7 c	12 d	2 ef	11 c
Flutriafol	91 a	0 e	21 a	0 d	0 f	0 f	0 d
Prochloraz	91 a	80 a	4 bc	71 a	59 a	95 a	56 a
Propiconazol	95 a	18 b	6 b	6 c	18 b	47 b	32 b
Triadimefon	5 d	1 d	0 d	0 d	0 f	6 d	1 d
Triadimenol	95 a	10 c	1 dc	1 d	8 e	5 de	7 c
Other systemics							
Benomyl	95 a	0 e	0 d	0 d	0 f	1 f	2 d
Iprodione	38 c	0 e	24 a	0 d	0 f	0 f	0 d
Metalaxyl	0 e	0 e	0 d	0 d	0 f	2 ef	0 d
Protecants							
Captafol	64 b	0 e	3 bc	26 b	16 c	17 c	18 bc
Thiram	2 de	0 e	24 a	0 d	0 f	0 f	0 d
				1 µg/ml			
EBIs							
Fenarimol	95 b	0 f	1 e	43 d	37 c	8 e	50 cd
Flutriafol	100=[3]a	0 f	30 bc	11 fg	20 d	0 f	64 c
Prochloraz	96 b	100+a	39 b	100+a	94 a	100=a	94 b
Propiconazol	100=a	75 c	9 d	41 d	41 c	87 b	89 b
Triadimefon	61 c	8 e	0 e	3 h	1 f	4 e	2 e
Triadimenol	100=a	10 e	2 e	7 g	12 de	3 ef	58 c
Other systemics							
Benomyl	100=a	97 b	100+a	89 b	76 b	84 b	100+a
Iprodione	44 d	8 e	31 bc	27 e	10 e	25 d	6 e
Metalaxyl	1 e	0 f	0 e	1 h	0 f	3 ef	0 e
Protecants							
Captafol	96 b	22 d	8 d	55 c	44 c	41 c	34 d
Thiram	41 d	0 f	23 c	15 f	3 f	0 f	7 e

Table 5-2-1 Continued.

Fungicides	Tb	Cd	Cs	Fo	Fs	Gr	Vd
				10 µg/ml			
EBIs							
Fenarimol	100=a	11 f	24 f	67 e	62 d	30 e	86 c
Flutriafol	100=a	0 h	36 e	58 f	51 e	13 g	94 b
Prochlora	100-a	100=a	67 b	100-a	100-a	100=a	100-a
Propiconazol	100-a	98 b	58 c	75 d	77 c	100=a	100=a
Triadimefon	100+a	22 e	10 g	44 h	25 g	25 ef	64 d
Triadimenol	100=a	4 g	6 h	37 i	38 f	21 f	89 bc
Other systemics							
Benomyl	100-a	100+a	100+a	100+a	88 b	81 b	100+a
Iprodione	66 b	64 d	63 b	79 c	65 d	61 d	21 e
Metalaxyl	7 c	0 h	0 i	0 j	2 h	9 g	6 f
Protecants							
Captafol	100+ a	58 d	41 d	84 b	69 d	62 d	21 e
Thiram	100+ a	87 c	37 de	54 g	31 fg	72 c	83 c
				100 µg/ml			
EBI							
Fenarimol	100=a	27 c	24 g	89 d	81 c	45 g	94 b
Flutriafol	100-a	60 b	62 d	98 b	83 c	48 fg	100+a
Prochloraz	100-a	100-a	100=a	100-a	100-a	100=a	100-a
Propiconazol	100-a	100+a	78 b	92 c	92 b	100=a	100-a
Triadimefon	100=a	63 b	31 f	70 e	67 e	51 f	85 c
Triadimenol	100-a	36 c	19 h	62 f	62 f	36 h	100=a
Other systemics							
Benomyl	100-a	100+a	100=a	100+a	92 b	81 c	100+a
Iprodione	92 b	58 b	71 c	58 g	55 g	72 d	26 e
Metalaxyl	22 c	9 d	24 g	45 h	20 h	50 f	28 e
Protecants							
Captafol	100+a	67 b	41 e	89 d	79 c	62 e	78 d
Thiram	100+a	100+a	72 c	100+a	72 d	88 b	94 b

[1]. Tb: T. basicola, Cd: C. destructans, Cs: Cy. scoparium, Fo: F. oxysporum, Fs: F. solani, Gr: G. roseum, Vd: V. dahliae.

[2]. Analysis of variance was performed after arcsine square root transformation. Data followed by the same letters were not significantly different within the each column at each concentration at 5% probability level.

[3]. Eight days after the agar plugs were transfered to non-fungicide CMA, the fungus showed no visible growth under microscope (-); growth on the agar plug only (=); vigorous growth on the medium (+).

Table 5-2-2. Mean root hair lengths (μm) of 7-day-old red clover seedlings in petri dishes[1] on filter papers soaked with different fungicides at four concentrations.

Treatments	1 μg/ml		10 μg/ml		100 μg/ml		1000 μg/ml	
	25°C	15°C	25°C	15°C	25°C	15°C	25°C	15°C
Control	345	381	345	381	345	381	345	381
EBIs								
Fenarimol	325	325	320	299	294	254-[2]	401	304
Flutriafol	467	502	452+	375	441+	411	355	167-
Prochloraz	360	411	299	315	325	330	310	310
Propiconazol	421	360	315	259-	162-	335	142-	66-
Triadimefon	244-	360	264	264	162-	173-	208-	218-
Triadimenol	315	315	335	345	238-	249-	107-	208-
Other systemics								
Benomyl	330	411	289	284	122-	198-	46-	76-
Iprodione	370	396	345	345	274	320	157-	198-
Metalaxyl	436	360	396	441	315	386	96-	102-
Protecants								
Captafol	406	401	594+	355	436	355	441+	310
Thiram	497+	325	411	320	315	330	228-	279-
LSD (0.05)	92	79	89	106	94	94	94	93

[1]. The petri dishes were incubated in the dark at 25°C or 15°C.

[2]. The symbols + or - indicated that the mean lengths of root hairs were significantly greater or less than the control.

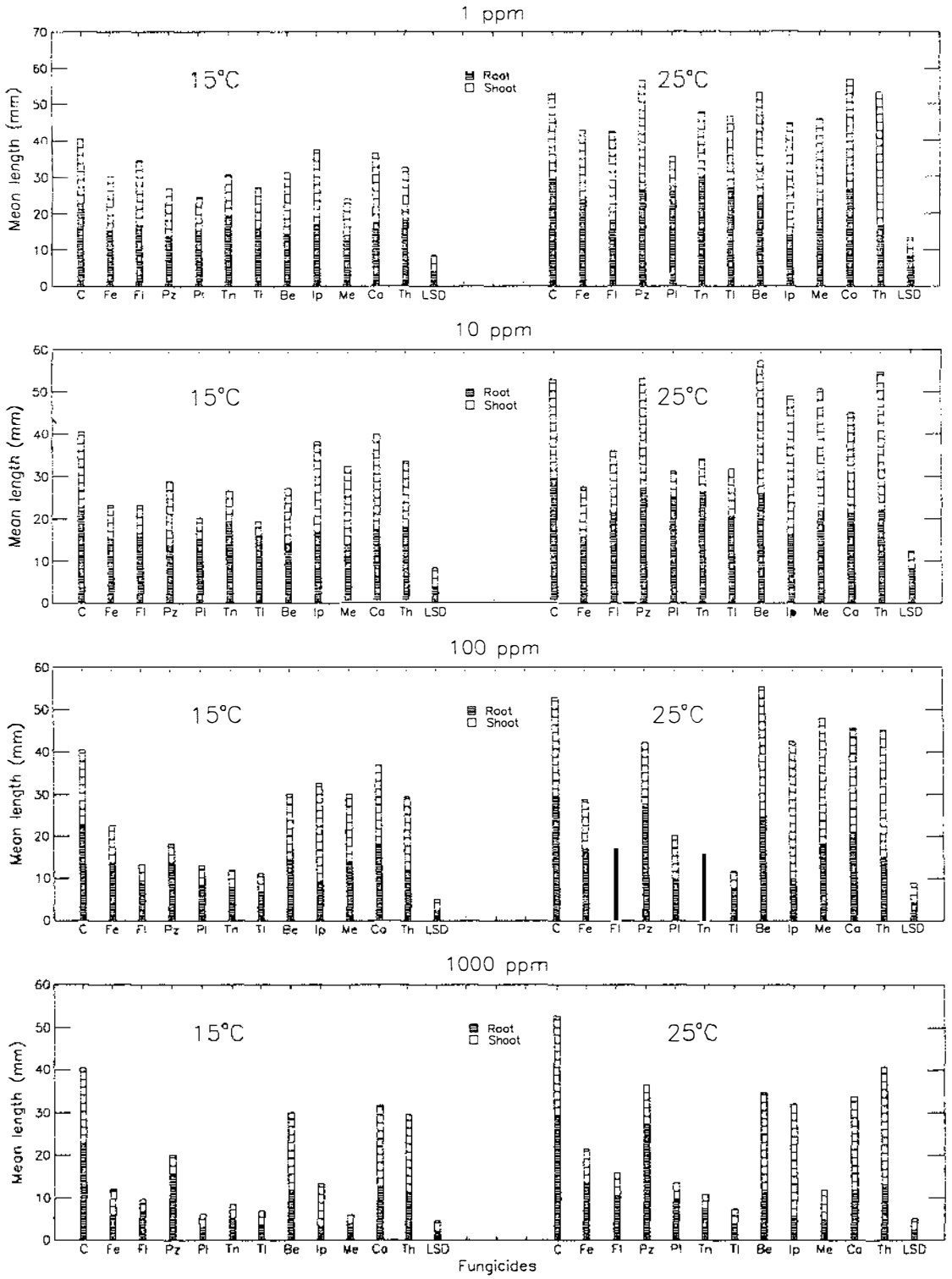


Figure 5-2-1. Mean lengths of primary shoot and root per seedling of 7-day-old red clover growing on two layers of filter paper in petri plates at 25 or 15°C. The filter papers were moistened with different concentrations of 11 fungicides. Statistical analyses were performed at each level of concentration and temperature. C: Control; Fe: Fenarimol; Fl: Flutriafol; Pz: prochloraz; Pl: Propiconazol; Tn: Triadimefon; Tl: Triadimenol; Be: Benomyl; Me: Metalaxyl; Ca: Captafol; Th: Thiram; LSD: Least significant difference.

5.2.4 Discussion

Although the intervals in the range of concentrations used in the mycelial growth bioassay were not narrow enough to give accurate EC50 or EC90 values for each fungicide against each of the potential pathogens of red clover roots, concentrations of some fungicides which inhibited mycelial growth of T.basicola, F.oxysporum, F.solani, and V. dahliae by about 50% were similar to those reported as EC50 values by others (Table 5-2-1, and Birchmore et al., 1977; Antoun et al., 1984; Gisi et al., 1986; Roberston et al., 1987 a). No information is available in the literature on the effects of the fungicides on C.destructans, Cy.scoparium, and G.roseum.

Prochloraz demonstrated the greatest inhibition of all fungal species tested among the 11 fungicides tested (Table 5-2-1). The fungicide was also found the most effective of 24 fungicides tested against the pea pathogens, F.solani and Phoma medicaginis (Roberston et al., 1987 a). Benomyl also has a broad spectrum of activity (Table 5-2-1 and Birchmore et al., 1977). Its antifungal effects were mainly fungistatic at the concentrations tested, whereas prochloraz was fungicidal at high concentrations (Table 5-2-1).

Metalaxyl has high selective toxicity for oomycetes (Urech et al., 1977; Kerkenaar and Sijpesteijn, 1981), and showed little toxicity to the deuteromycete fungi tested (Table 5-2-1). It can be said therefore that the very small amounts of metalaxyl introduced as seed treatments in the experiments described in Chapter 4 were unlikely to have affected fungi other than oomycetes.

The EBI and other systemic fungicides inhibited mycelial growth but had little effect on spore germination of any fungus except G. roseum and V. dahliae, even at high concentrations (Appendix 5-2-1; see also Siegel 1981 and Siegel et al., 1977). Characteristically, for protectant fungicides (Sijpesteijn, 1984), captafol and thiram were strongly inhibitory to spore germination.

Captafol and thiram were very toxic to R. trifolii (Plate 5-1) and triadimefon was slightly inhibitory at high concentrations. This agreed with other reports (Fisher et al., 1979; Sirois et al., 1981; Antoun et al., 1981; Tu, 1981; 1982; Ruiz-Sainz, 1984). Fungicide sensitivity may differ among strains of Rhizobium (Heinonen-Taski et al., 1982). Rhizobium trifolii used in the current trial was the

most effective and predominant strain in New Zealand pastures (Dr P. J. Patel per. com.). Therefore, the result would have indicated the likehood of deleterious effects of fungicides under local conditions.

The EBI fungicides reduced the length of shoots and roots of red clover seedlings at 10 μg/ml or greater (Fig.5-2-1). Similar effects have been recorded for seedlings of wheat (Buchenauer, 1977), barley, tomato, and cotton (Buchenauer and Rohner, 1981) treated with triadimefon, triadimenol, fenarimol, or imazilil. Reduced shoot weight of white clover growing in the soil treated with diclobutrazol or tridemorph as compared with control has also been reported (Fisher et al., 1979; Fisher and Hayes, 1981). EBI fungicides have plant growth regulating properties, blocking gibberellin- and sterol-biosynthesis in plants (Buchenauer and Rohner, 1981), which results in treated plants being shorter (Fig.5-2-1) and having darker leaves (as also observed in the present work). Prochloraz was the only fungicide which did not affect root length at the concentrations tested (Fig. 5-2-1).

Benomyl, thiram, and captafol stimulated growth of red clover seedlings (Fig. 5-2-1). Benzimidazole compounds such as benomyl have cytokinin-like activity and are well known to stimulate growth (Gayed, 1970; Skene, 1972). Stimulatory effects of thiram and captan (structurally similar to captafol) have also been reported (Sirois et al., 1981; Tu, 1981).

Many factors are known to affect root hair growth (Cormack, 1949; 1962), however, effects of fungicides have not been reported previously. The present results demonstrated that some fungicides affected the growth of root hairs substantially at higher concentrations (Table 5-2-2). Root hairs play an important role in nutrient uptake (Bouldin, 1961) and subsequently affect dry matter production by plants (Caradus, 1981). Again, prochloraz was the only fungicide tested which did not affect the growth of root hairs.

Although ultimately the successful use of a fungicide depends on field performance, data obtained in the laboratory are valuable to help determine the type of fungicide which might be effective, and the rate of application that may provide maximum pathogen suppression and minimum phytotoxicity. It was concluded that prochloraz has a broad spectrum of activity, was not toxic to R. trifolii, and had the least retardant effect on the growth of plant. Prochloraz, followed by benomyl seemed to be the most suitable chemicals for use in field trials.

5.3 EXPERIMENT FOUR : EFFECTS OF PROCHLORAZ DRENCHES ON POPULATIONS OF SOIL MICRO-ORGANISMS, INVASION OF ROOTS BY FUNGI, AND GROWTH OF RED CLOVER SEEDLINGS UNDER GLASSHOUSE CONDITIONS.

5.3.1 Introduction

As described in Experiment 3, prochloraz was the most promising fungicide of those tested in vitro for use in control of root-invading fungi of red clover and it showed the least adverse effects on seedling growth. However, there are a myriad biological, chemical, and physical interactions in soil which do not exist under laboratory conditions (Kaufman, 1977), and which can affect the ultimate behaviour and effectiveness of fungicides. Therefore, it was necessary to test the fungicide further under more natural conditions before using it in field experiments.

An experiment was set up in pots in constant temperature waterbaths in the glasshouse to assess the effects of prochloraz on:

1. microbial populations in soil;
2. fungal invasion of the roots;
3. plant growth.

5.3.2 Materials and methods

Field soil was obtained from the plant breeder's evaluation block. Samples of soil were taken, mixed and processed as described in Chapter 3. The bulk soil was then divided into two equal parts: (a): "Field soil" and (b): "microwave-treated-soil" (MW soil). Field soil was put into pots directly (details below). Microwave-treated-soil was processed as follows: Polyethylene bags were filled with about 1200 g soil, placed open in a microwave oven (National, Model NE-8070), and full power (650 W heating power, 2450 MHz) applied for 5 min. In a preliminary test in which 1200 g soil was treated for 3, 5, 10, or 15 min,

fungal colonies failed to grow on soil dilution plates from soil sterilised for 5 min or longer. After treatment, the bags were closed and the soil was allowed to cool to room temperature before it was placed into the pots.

The experiment was carried out as, soil temperature x prochloraz concentration (3 x 5), a two factor factorial design for both Field and MW soil. Five replicate unperforated plastic pots (65 mm diam. x 95 mm) were used for each treatment. Soil (175 g) was placed into each of 75 pots. Twenty seeds were sown onto the surface of the soil in each pot. Another 25 g soil was added to each pot to cover seeds to a depth of 10 mm. The soil in the pots was drenched immediately after sowing, with prochloraz emulsions diluted with water to give concentrations in the soil of 80, 240, 730, and 2200 mg a.i./kg dry soil, and a moisture content of 80% of WHC. The concentrations were equivalent to 1.15, 3.46, 10.52, and 31.72 g a.i. per square metre of soil surface, respectively. The control pots received the same amount of water. The pots were placed into temperature controlled waterbaths at 15, 20, or 25°C in a glasshouse, weighed daily, and soil moisture content adjusted to 80% WHC by adding appropriate amounts of water.

Measurements of plant growth, and assessment of populations of soil micro-organisms and root-invading fungi were undertaken 4 weeks after sowing.

5.3.2.1 Microbial population of the soil

Soil dilution plates with different selective media were used to assess populations of bacteria and fungi in the soil. Bacto-tryptic soy broth (Difco) at 0.3%, solidified with 1.5% agar, was used for total bacteria (Martin, 1975), and Martin's rose bengal medium (Martin, 1950) containing oxytetracycline at 10 μg/ml instead of streptomycin, for fungi. A preliminary experiment showed that the latter medium was superior to several other media tested in the total number of colonies obtained, and numbers of colonies of species of Gliocladium, Fusarium, Cylindrocarpon, and zygomycetes isolated from soil. It was also best in preventing overgrowth of the plate by zygomycetes.

At the end of experiment (4 weeks after sowing) two 10 g samples of fresh

soil were taken from each pot with a sterilised cork borer (10 mm inner diam.) through the whole depth of the soil. One core sample was used for measuring soil water content. The other was comminuted with 100 ml sterile distilled water in a Waring blender for 1 min at high speed. A series of 10-fold dilutions for each soil sample suspension was prepared. Maximum dilutions used were 10,000-fold, for bacteria in both types of soil, and 100- and 10-fold, for fungi from "Field soil" and "MW soil", respectively. A 0.25 ml of aliquot of an appropriate dilution was pipetted onto duplicate plates of the bacterial and the fungal media, and spread evenly over the surface using a bent glass rod.

The plates were incubated at 20°C in the dark. Colony counts were made at intervals from 2 days after inoculation (until colony numbers reached a maximum). For bacteria, only the total number of colonies was counted. For fungi, the numbers of colonies in total, and of the major fungal species previously isolated from roots of red clover (Chapter 4) were counted. The data were expressed as numbers of colonies per gram dry soil.

5.3.2.2 Seed germination and seedling growth of red clover

Numbers of seedlings per pot were counted 12 days after sowing. At the end of the experiment, seedlings were washed free of soil with a jet of tap water, the lengths of each whole seedling and its root were measured, and the length of its shoot was calculated. Shoots and roots were then separated and fresh weights were determined.

5.3.2.3 Root-invading fungi

Fungi were isolated from roots as described in Experiment 1 using all the weighed whole roots from each pot. The blending time was 60 sec, and no dilution was made.

5.3.2.4 Statistical analysis

Statistical analyses were performed after square root (colony data) or arcsine (seedling emergence data) transformation except for the length and weight data which were not transformed.

A two way analysis was carried out on the data obtained from each type of soil as soil temperature x prochloraz concentration. When the interaction of the two factors was significant, the data are present for all combinations of temperature and concentration, otherwise these were averaged over one or another factor.

5.3.3 Results

5.3.3.1 Populations of bacteria and fungi in the soil

Generally bacterial populations increased as concentrations of prochloraz applied to the soil increased (Table 5-3-1). However, there was a strong temperature x concentration interaction ($P < 0.05$) and the effect of temperature differed in Field soil and MW soil. In Field soil at 15 and 20°C, bacterial colony forming units (CFU) were significantly increased, compared with untreated soil, by treatment with 730 mg prochloraz/kg soil or more, and at 25°C with 2200 mg/kg. In MW soil, however, concentrations required to increase bacteria populations decreased as temperature increased (730 mg/kg at 15°C, 240 mg/kg at 20°C, and 80 mg/kg at 25°C).

The fungi most frequently isolated from Field soil were Chrysosporium spp., C. destructans, Fusarium spp., G. roseum, Penicillium spp., and some species of zygomycetes. Fungal colonies of Acremonium, Chaetomium, Cladosporium, Geotrichum, Paecilomyces, Phoma, Trichoderma species, and of some sterile mycelial forms were also found.

From MW soil the fungi most frequently isolated were Humicola spp., and Penicillium spp.. Other fungi isolated occasionally were species of Acremonium,

Chrysosporium, Cladosporium, Phoma, and some sterile mycelium. Three of the major fungal species (genus) isolated from field soil, C. destructans, Fusarium spp., and G. roseum, were rarely isolated from undrenched MW soil; no isolates were obtained from prochloraz drenched MW soil.

As with the bacteria, temperature x concentration interactions were also found for total numbers of fungal CFU in the soils. At 15°C a lower concentration of prochloraz (80 mg/kg) was required to reduce the total fungal CFU per gram of dry Field soil below the level in untreated soil than at 20 and 25°C (240 mg/kg) (Table 5-3-1).

The total number of fungal CFU obtained from MW soil was less than 10% of that obtained from Field soil. Numbers of CFU were further lowered significantly in soil treated with the fungicide at 80 mg/kg and above (20 and 25°C), or 240 mg/kg and above (15°C), compared with those from undrenched soil (Table 5-3-1).

The individual fungal species isolated from Field and MW soils showed different responses to the treatments (Table 5-3-2). Species of Chrysosporium and Fusarium (mainly F. solani, F. oxysporum, and F. culmorum) in Field soil were markedly affected by the prochloraz treatments. Numbers of CFU were significantly lower in soil receiving any of the prochloraz treatments than in untreated soil. Numbers of G. roseum were reduced at 730 mg/kg and above. Others were not affected dramatically by any concentration (Table 5-3-2 A). In MW soil, the number of CFU of Humicola spp. was reduced significantly by fungicidal drenches at 730 mg/kg and 2200 mg/kg (Table 5-3-2 A). Numbers of CFU of Penicillium spp. were lower in prochloraz drenched soils, compared with those in untreated soil, but the differences were not statistically different.

Temperature did not affect the numbers of fungal CFU in either soil except of G. roseum in Field soil and Humicola spp. in MW soil, which were significantly higher at 20°C and 25°C, respectively than at 15°C (Table 5-3-2 B).

Table 5-3-1. Mean numbers of colony forming units of bacteria and fungi per gram dry Field and microwave-oven-treated soil (MW) 4 weeks after pots[1] were drenched with different concentrations of prochloraz.

Temp. (°C)	Conc. (mg/kg)	Bacteria ($x10^8$) Field	Bacteria ($x10^8$) MW	Fungi ($x10^3$) Field	Fungi ($x10^3$) MW
15	0	2.61 e[2]	8.20 bcd	437.33 a	11.06 bcd
	80	3.46 de	8.16 cd	230.20 c	4.60 cde
	240	3.74 de	7.79 cd	193.00 cd	0.27 e
	730	8.09 bc	17.37 a	182.40 cd	0.27 e
	2200	16.81 a	22.19 a	157.60 de	0.33 e
20	0	3.31 de	7.87 cd	367.40 ab	20.07 ab
	80	3.83 de	6.83 de	315.17 b	1.83 de
	240	6.16 cd	11.77 b	197.40 cd	11.34 abc
	730	7.72 bc	11.00 bc	154.93 de	1.23 de
	2200	10.82 bc	19.30 a	123.93 e	0.07 e
25	0	2.66 e	4.44 e	335.53 b	21.87 a
	80	2.30 e	7.21 d	316.07 b	8.77 bcd
	240	4.03 de	9.06 bcd	194.77 cd	7.70 bcd
	730	4.10 de	9.17 bcd	151.40 de	4.60 cde
	2200	11.11 b	17.84 a	120.40 e	0.10 e

[1]. The pots were kept at 15, 20, or 25°C in waterbaths.

[2]. Means in each column followed by the same letters were not significantly different at 5% probability level in an analysis of transformed values (sq.rt.)

Table 5-3-2. Mean numbers of colony forming units ($x10^3$) of some fungal species per gram dry Field and microwave-oven-treated soil (MW) as affected by prochloraz concentrations (A) and temperatures (B).

A. Concentrations

Soil	Fungi	Concentration (mg/kg) 0	80	240	730	2200
Field	Chrysosporium.sp	14.17 a[1]	5.90 b	5.32 b	5.61 b	3.87 b
	C. destructans	1.49 a	0.90 a	2.39 a	2.39 a	2.37 a
	Fusarium spp.	23.31 a	6.51 b	5.91 b	5.90 b	6.21 b
	G. roseum	41.91 a	52.23 a	49.27 a	29.80 b	28.02 b
	Penicillium spp.	17.11 a	18.01 a	19.17 a	15.64 a	17.12 a
	zygomycetes	17.69 a	18.28 a	19.78 a	20.64 a	18.00 a
MW	Humicola sp.	6.88 a	2.42 ab	2.80 ab	1.07 bc	0.00 c
	Penicillium spp.	2.61 a	0.54 a	0.14 a	0.39 a	0.18 a

B. Temperatures

Soil	Fungi	Temperature (°C) 15	20	25
Field	Chrysosporium.sp	8.87 a	7.09 a	4.97 a
	C. destructans	2.32 a	1.97 a	1.43 a
	Fusarium spp.	8.86 a	9.93 a	9.91 a
	G. roseum	33.81 b	46.75 a	40.19 ab
	Penicillium spp.	16.82 a	17.18 a	18.23 a
	zygomycetes	18.59 a	20.17 a	17.88 a
MW	Humicola sp.	0.94 b	2.26 ab	4.70 a
	Penicillium spp.	0.21 b	0.23 b	1.89 a

[1]. Data for each factor were averaged over all levels of the other factor. Means followed by the same letters in each line were not significantly different at 5% probability level in an analysis of transformed values (sq.rt.).

5.3.3.2. Seedling emergence and growth of red clover

Generally, seedling emergence was greater from MW soil than from Field soil (Table 5-3-3). Treatment of either soil with prochloraz at a rate of 730 mg/kg or more resulted in reduced seedling emergence compared with other treatments (Table 5-3-3 A).

Soil temperature affected seedling emergence in Field soil, but not in MW

soil (Table 5-3-3 B). Significantly greater numbers of seedlings were obtained from Field soil in pots kept at 25°C than in those at 15 and 20°C.

Table 5-3-3. Mean emergence percentage of red clover seedlings 12 days after sowing 20 seeds into each pot containing Field or microwave-oven-treated soil (MW) as affected by prochloraz concentrations (A) and soil temperatures (B).

A. Concentration.

Soil	Concentration (mg/kg) 0	80	240	730	2200
Field	43.3 a[1]	43.0 a	49.3 a	24.0 b	13.0 c
MW	61.3 a	49.7 bc	55.7 ab	43.7 c	31.7 d

B. Temperature.

Soil	Temperature (°C) 15	20	25
Field	32.4 b	31.4 b	39.8 a
MW	48.4 a	44.4 a	52.4 a

[1]. Data for each factor were averaged over all levels of the other factor. Means followed by the same letters in each line were not significantly different at 5% probability level in an analysis of transformed values (arcsine).

Prochloraz concentrations, soil temperatures, and interactions of the two factors, all affected red clover growth (Fig. 5-3-1). At 15°C, treatment of either soil with prochloraz at concentrations of 80 mg/kg or more resulted in shorter shoots compared with those of plants from untreated soil, while at 20 and 25°C significant reductions in shoot growth were detected at 730 and 2200 mg/kg for Field soil , and 240 mg/kg and above for MW soil. Shoot weight showed similar responses except that in Field soil significant reductions in growth were only detected at concentrations of 730 mg/kg and above, at all temperatures.

Lengths of roots of plants grown in Field soil treated with prochloraz at 2200mg/kg were significantly lower than those of plants from untreated soil at all temperature levels in Field soil. In MW soil, however, while root length was only significantly lower at the highest concentration (2200 mg/kg) at 15°C, at 20 and 25°C inhibition was evident at concentrations of 240 mg/kg and above . Results were somewhat different when weight was used as the parameter of root growth. In Field soil at 15 and 25°C root weight was significantly lower with treatments of

730 and 2200 mg/kg, while at 20°C, it was reduced by 80 mg/kg and above. In MW soil it was reduced at concentrations of 80 mg/kg and above at 15°C, 730 mg/kg and above at 20°C, and 240 mg/kg and above at 25°C.

5.3.3.3 Root-invading fungi

In Field soil, fewer colonies were recovered from root tissue of plants from soil drenched with prochloraz (Table 5-3-4). The lowest concentration of prochloraz treatment which resulted in significantly lower colony counts than untreated controls varied with temperature. They were 80 mg/kg at 15°C, 240 mg/kg at 20°C, and 80 mg/kg at 25°C. Concentrations above these gave significant inhibition except 730 mg/kg at 15 and 20°C. Numbers of colonies of Fusarium spp. (mainly F. solani, and less frequently F. oxysporum and F. culmorum) were reduced in all treatments receiving prochloraz. Trichocladium basicola, C. destructans, and G. roseum were isolated from the plants growing in the pots without prochloraz, but no isolates were obtained from the roots in drenched soil. Numbers of colonies of Cy. scoparium and a group of other fungi (including species of Acremonium, Chrysosporium, Humicola, Penicillium, and some with sterile mycelium) did not show a marked or consistent response to the fungicide (Table 5-3-4 A).

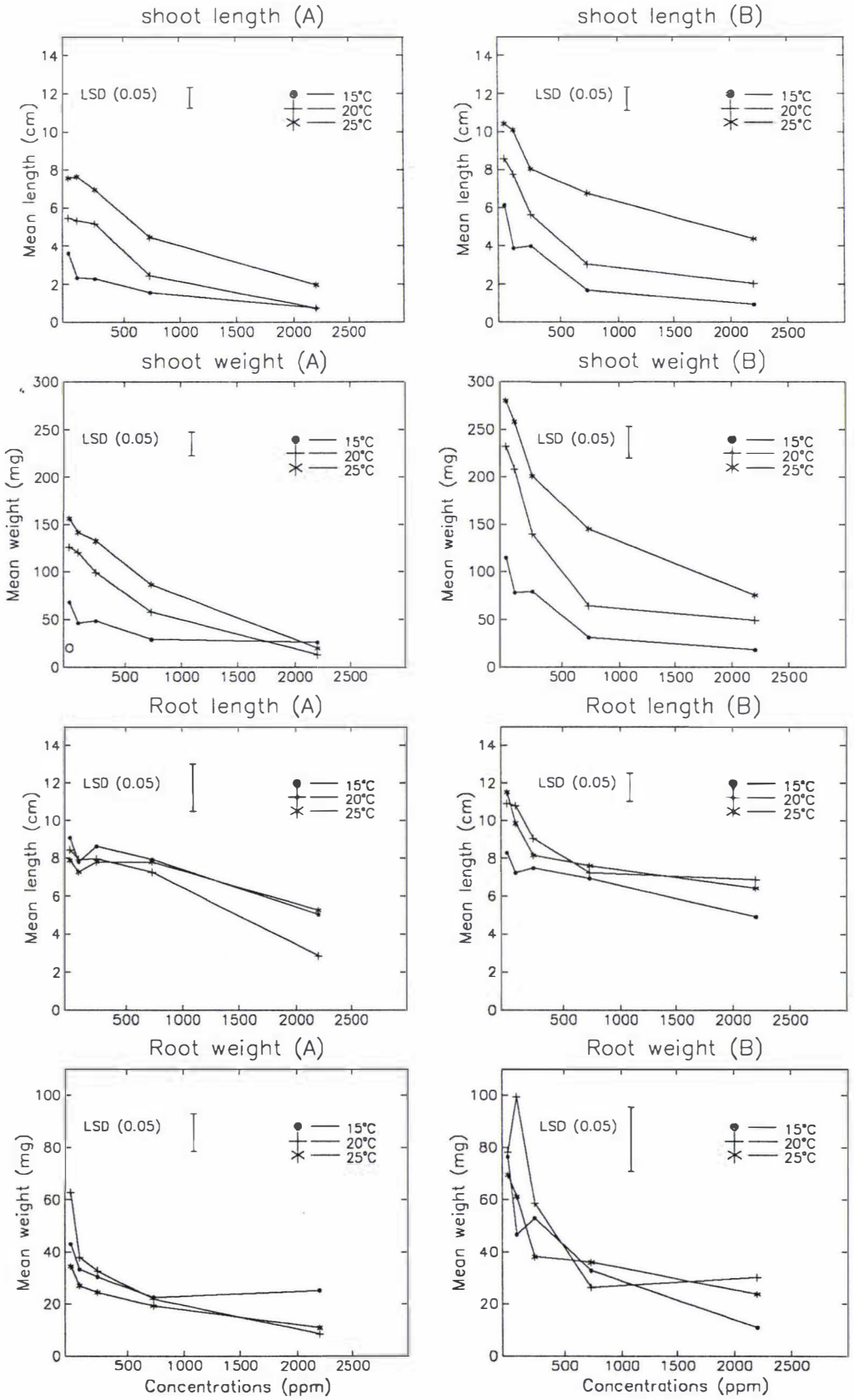


Figure 5-3-1. Mean lengths and weights of shoot and root per plant of 4-week-old red clover growing in pots containing field soil (A) or microwave-oven-treated soil (B). The pots were drenched with different concentrations of prochloraz and kept in water baths at 15, 20, or 25°C.

Table 5-3-4. Mean numbers of colonies per g fresh roots (x10²) in total, and of some major fungal species isolated from roots of 4-week-old red clover plants growing in pots[1] containing Field (A) or microwave-oven-treated (MW) soil (B).

A. Field soil Temp. (°C)	Conc. (mg/kg)	Total	Fspp[2]	Cs	Others
15	0	116.33 bc[3]	30.47 bc	4.30 a	81.56 ab
	80	15.50 defg	4.33 cd	0.00 a	11.17 cd
	240	28.67 defg	0.00 d	0.00 a	28.67 bcd
	730	135.57 b	4.43 cd	0.00 a	131.14 a
	2200	0.00 g	0.00 d	0.00 a	0.00 d
20	0	66.93 bcd	39.60 b	1.90 a	25.43 bcd
	80	26.23 defg	0.00 d	11.37 a	20.23 bcd
	240	4.23 fg	0.00 d	0.00 a	4.23 cd
	730	38.10 defg	0.00 d	4.71 a	33.33 bcd
	2200	22.23 efg	0.00 d	0.00 a	22.23 bcd
25	0	442.47 a	352.33 a	8.73 a	81.41 ab
	80	52.50 bcde	7.13 cd	0.00 a	45.37 bc
	240	37.10 defg	25.60 bc	3.17 a	8.33 cd
	730	0.00 g	0.00 d	0.00 a	0.00 d
	2200	0.00 g	0.00 d	0.00 a	0.00 d

B. MW soil Temp. (°C)	Conc. (mg/kg)	Total	Acre.	Humi.	Others
15	0	3.03 bcde	0.00 d	0.00 e	3.03 b
	80	8.17 abcd	5.00 b	0.00 e	3.17 b
	240	12.17 abcd	12.17 a	0.00 e	0.00 c
	730	0.00 e	0.00 d	0.00 e	0.00 c
	2200	0.00 e	0.00 d	0.00 e	0.00 c
20	0	15.97 ab	2.10 c	3.00 c	10.87 a
	80	1.43 e	0.00 d	1.43 d	0.00 c
	240	12.23 abc	1.67 cd	7.77 b	2.79 b
	730	0.00 e	0.00 d	0.00 e	0.00 c
	2200	0.00 e	0.00 d	0.00 e	0.00 c
25	0	22.77 a	0.00 d	16.67 a	6.10 b
	80	2.90 cde	0.00 d	0.00 e	2.90 b
	240	1.17 de	0.00 d	0.00 e	1.17 b
	730	0.00 e	0.00 d	0.00 e	0.00 c
	2200	0.00 e	0.00 d	0.00 e	0.00 c

[1]. The pots were drenched with different concentrations of prochloraz and kept at 15, 20, or 25°C in water baths.

[2]. Fspp, Fusarium spp. Cs, Cy. scoparium Acre, Acremonium spp. Humi, Humicola sp.

[3]. Means in each column followed by the same letters were not significantly different at 5% probability level in an analysis of transformed values (sq.rt.).

Far fewer colonies were recovered from plants grown in MW soil than in Field soil (Table 5-3-4 B). High concentrations of prochloraz (730 and 2200 mg/kg) completely eliminated fungal invasion of roots, and the numbers of colonies were reduced significantly by 80 and 240 mg/kg at 25°C. Results with lower concentrations of prochloraz were inconsistent at lower temperatures. Species of Acremonium and Humicola were isolated from a few treatments but never at concentrations above 240 mg/kg. Numbers of colonies of others fungi (species of Cladosporium, Chrysosporium, Penicillium, and some sterile mycelium) were reduced significantly at concentrations above 240 mg/kg at all temperatures (Table 5-3-4 B).

5.3.4 Discussion

Most previous investigations on effects of fungicides on soil micro-organisms have been carried out with non-EBI fungicides. One recent report on the effects of the EBI fungicide propiconazol found no significant differences in fungal populations in the soil 30 days after the last of a series of treatments at 10 day intervals at a rate of 0.125 g/m^2 as compared with untreated soil (Elmholt and Smedegaard-Petersen, 1988). In the present experiment, marked differences between treatments were detected 4 weeks after a single application of prochloraz (Table 5-3-1, 5-3-2). Although the two studies are not really comparable, effects would have been more likely to have shown up in the present trial since prochloraz has stronger antifungal activity than that of propiconazol (Experiment 3) and it was used at high concentrations.

The significant reduction in the total fungal population of the soils (Table 5-3-1) confirmed the broad spectrum of antifungal activity of prochloraz which has been reported in vitro (Buchenauer, 1977). Among the fungal species listed in Table 5-3-2 different reactions to prochloraz were found. Fungi, such as species of zygomycetes and Penicillium, known to be insensitive or slightly sensitive to prochloraz in vitro (Buchenauer, 1977), remained at relatively constant levels whereas others known to be sensitive to the chemical (Experiment 3) were, with the exception of C. destructans, reduced in the treated soil. These results showed that in vitro tests gave a reasonable indication of the likely effect of the fungicide

on fungi in soil. On the other hand, no major fungal group was eliminated from soil by prochloraz even at a concentration as high as 2200 mg/kg, in contrast to the results of the in vitro tests, where strong fungicidal effects were achieved at 100 ppm or lower. This supports the view that the bioactivity of fungicides can be reduced markedly by physical, chemical, and microbial factors in the soil (Helling et al., 1971).

Effects of temperature on the efficacy of prochloraz varied with the soils. In Field soil for instance, the percentage reduction in the total fungal population following prochloraz treatment with 80 mg/kg was greater at 15°C than that at 20 and 25°C, whereas in MW soil, the same concentration caused more reduction at 20 and 25°C than at 15°C (Table 5-3-1). The different responses may be due to the different mycofloras in the soils (Table 5-3-2), and may indicate that effects of temperature on antifungal activity of prochloraz varied with fungal group, or the individual fungal species present. The temperature effect seems more likely to have occurred through changes in the physiological state of test organisms, and hence their susceptibility to toxicants, rather than changes in the chemical itself.

Fungal invasion of roots was substantially reduced by prochloraz drenches (Table 5-3-4). This probably was because of great reduction of inoculum level in the soil (Table 5-3-1, and 5-3-2), and of further antifungal activity from chemicals accumulated on and in plants.

Following the application of the fungicide to the soil, the numbers of bacteria increased dramatically (Table 5-3-1; see also Farley and Lockwood, 1969; Wainwright and Pugh, 1973; 1974; 1975; Ferriss and Mitchell, 1981; Ingham and Coleman, 1984), and such increases may reflect changes in the fungal population which resulted in : (a) reduced competition with fungi for nutrients and/or (b) increased substrate in the form of dead hyphae (Bollen, 1961; Simon-Sylverstre and Fournier, 1979; Ingham, 1985).

Seedling emergence from both soils was reduced by the higher concentrations of prochloraz (Table 5-3-3). Similar results have been reported for peas drenched with the same fungicide (Robertson et al., 1987 c), and for prairie grass seeds treated with numarimol or propiconazol prior to sowing (Falloon, 1988). Although germination was not affected by prochloraz in Experiment 3 it did inhibit elongation of shoots and roots of red clover.

Therefore, it is likely that reduced emergence was the result of the growth retarding effects of the fungicide at higher concentrations. Similar effects have been reported for some other EBI fungicides (Buchenauer, 1977; Buchenauer and Rohner, 1981).

The adverse effects of prochloraz on shoot growth of red clover seedlings were more severe at lower (15°C) than at higher (20-25°C) temperatures (Fig. 5-3-1). This corresponded with previous observations in laboratory tests (Experiment 3). The optimum temperature for red clover growth is 20-25°C (Chapter 2). Plants growing at lower temperatures may be more susceptible to the growth retardant effect of prochloraz.

Microwave oven treatment was found to be an effective method to kill fungi in soil. Four weeks after treatment, the fungal populations in undrenched MW soil were still less than 10% those in Field soil receiving the highest concentration of prochloraz (Table 5-3-1). The major fungi in MW soil were species of Humicola and Penicillium rather than fungi known to invade red clover roots. They are ubiquitous soil inhabiting fungi. Some, like Penicillium, whose conidia are easily distributed through the atmosphere (Domsch et al., 1980), may have re-invaded the soil from this source. However, the fungal re-establishment of MW soil was successfully suppressed by the prochloraz treatments. Since MW treatment has little effect on soil (Ferriss, 1984) apart from killing fungi, it is suitable for use to detect direct effects of the fungicide on plant growth.

Although results obtained in the glasshouse may vary from those under field conditions owing to greater variability in the field environment and the more heterogeneous biotic populations encountered in field soil, they can give some indication of the effects that application of prochloraz in the field may have. It is concluded from the present results that since prochloraz was strongly antifungal towards a wide range of fungi in soil, and reduced fungal invasion of red clover roots, it was suitable for further use as an experiment tool for field studies on the effects of fungal invasion on red clover growth. However, it had a retardant effect on the plants, especially at lower temperatures, thus choice of suitable concentrations for field application should strike a balance between maximum pathogen suppression and minimum retardant effects.

5.4 EXPERIMENT FIVE : EFFECTS OF FUNGICIDAL DRENCHES ON ROOT-INVADING FUNGI AND GROWTH OF RED CLOVER UNDER FIELD CONDITIONS.

5.4.1 Introduction

As described in Chapter 2, the application of fungicidal drenches to soil in field plots can be a useful experimental tool to study aspects of root rot of forage legumes. Benomyl has been the fungicide used most in this kind of study. It has been reported to increase persistence and yield of red clover (Leath et al., 1973). Benomyl drenches have also increased yield (Greenhalgh and Clarke, 1985) and reduced root rot severity (Smiley et al., 1986) of subterranean clover under field and glasshouse conditions. Although in one of these studies root invasion by several soilborne fungi was monitored (Greenhalgh and Clarke, 1985), generally, there was no direct evidence that the positive effects on plant growth were due to control of pathogenic fungi by the fungicide. Furthermore, the results of Experiment 3 and 4, reported in the previous sections, showed that prochloraz was the fungicide most effective against root-invading fungi of red clover in vitro (Experiment 3), and could indeed be a suitable fungicide for experimental use in the field (Experiment 4). Prochloraz has been mainly used in foliage sprays (Gallimore et al., 1987; Harris et al., 1979; Mercer and McGimpsey, 1985), and seed or seed tuber treatments (Hide and Cayley, 1985; Hide et al., 1987; Roberston et al., 1987 b; Falloon, 1988). It has also been used as a spray to control diseases of mushrooms (Nair and Macauley, 1987). But no one has used the fungicide in soil drenches to study effects of root rot on red clover, or any other crops under field conditions.

Field experimental plots drenched with prochloraz or benomyl were set up in the plant breeder's evaluation block to assess effects on :

1. fungal invasion of the red clover roots;
2. red clover growth.

5.4.2 Materials and methods

The experimental area was situated in the plant breeder's evaluation block. Existing vegetation was killed by spraying with glyphosate (Roundup, Monsanto Ltd) at rate of 2.16 L a.i./ha on the 11th of Dec. 1987. Ten days later, the area was rotovated to produce a fine tilth.

The experiment was carried out as a randomized complete block design with 4 treatments and 6 replicates. The treatments were control, benomyl 3.46 g/m^2, prochloraz 1.15 g/m^2, and 3.46 g/m^2. The blocks were spaced 1 m apart and there was a 1 m wide path around the whole experimental area. Each block was divided into 1 x 3.5 m plots, 1 m apart. Each plot consisted of 6 rows, 1 m long and 70 cm apart.

Fifty metalaxyl-treated seeds as described in Chapter 3 were sown into each row on the 22nd of Dec. 1987. The appropriate fungicide suspensions were drenched into the soil of each plot at a rate of 14.3 L of water/m^2 immediately after sowing using a watering can. The control plots received the same amount of water. An examination of the soil profile of an area receiving the standard drench treatment showed that the fungicide suspensions penetrated to about 20 cm depth.

The plots were irrigated using a hose and a rose attachment when the soil was too dry during the first 2 weeks after sowing to ensure seedling establishment, and hand weeded thereafter as necessary.

Numbers of emerged seedlings were counted 6 weeks after sowing. One row of plants was randomly selected from the middle 4 rows of each plot 6, 12, 18, and 45 weeks after sowing, and plants and soil (to a depth of 25 cm) removed for laboratory assessment.

5.4.2.1 Assessment of plant growth

At each sampling date the number of plants in the row was counted and symptoms of leaf diseases were noted. The shoots of all plants per row were cut from the crown and fresh weights were determined. Roots were washed free of

soil with a jet of tap water, blotted dry, and cut transversely to examine stele symptoms. The percentage of roots with stele browning was calculated, and the fresh weights of roots were then determined.

5.4.2.2 Isolation of root-invading fungi

It had been found previously (Experiment 1) that more colonies were obtained by blending 2 g roots than 4 g and higher. The total roots harvested from each row at 6 weeks were macerated, since there was rarely more than 2 g tissue per row. The total roots harvested from each row at 12, 18, and 45 weeks were cut into 5-10 mm long segments, thoroughly mixed. Two, 2 g samples were then taken and used for maceration since the weights of roots per row harvested at these times greatly exceeded 2 g.

The roots of 6-, 12-, and 18-week-old roots were blended for 60 sec as described in Experiment 1, and no dilution was made. The roots of 45-week-old plants were blended for 120 sec and one dilution was made. The procedure was as follows: the roots were blended for 60 sec then the sides of blending cup were rinsed with 50 ml sterile distilled water to resuspend the macerate. This was blended for further 60 sec, and the cup rinsed with another 100 ml sterile distilled water. The suspension was pipetted onto the surface of ABPDA, spread, incubated, and fungi identified and counted, as described in Experiment 1.

Numbers of colonies per gram fresh roots, and per root, were calculated as described in Experiment 2.

5.4.2.3 Statistical analysis

Analysis of variance (LSD test) was performed after appropriate transformation i.e. arcsine for seedling emergence, Log for percentage, and square root for numbers of fungal colonies. A similar analysis was carried out for shoot and root data except that these were not transformed.

5.4.3 Results

5.4.3.1 Seedling emergence and plant growth

Mean numbers of emerged seedlings per row 6 weeks after sowing were 11.5, 17.2, 16.4, and 14.4 respectively from the control, benomyl and prochloraz (1.15 g/m^2 and 3.46 g/m^2) treatments. Number of seedlings were significantly increased, compared with the control, by the benomyl and prochloraz 1.15 g/m^2 treatments, but not by prochloraz 3.46 g/m^2 treatment.

Foliage diseases found during the experimental period were: rust (caused by Uromyces fallens (Arthur) Barth.), pseudopeziza leafspot (caused by Pseudopeziza trifolii (Biv.) Fckl.), sooty blotch (caused by Mycosphaerella killianii Petr.), and virus disease (indicated by leaf mottling and streaking), but none was predominant and there were no obvious differences in severity among the different treatments.

No obvious root rot symptoms were observed among the treatments until 12 weeks after sowing. Nearly 50% of the plants from the control plots had brown or dark brown lesions (0.5 - 1 cm long) at 12 weeks, whereas plants from plots receiving chemical treatments had a lower proportion (20 - 30%) of roots bearing lesions, and most lesions were smaller (most less than 0.5 cm) than those from control plots. At the end of experiment, no great differences were found in root rot symptoms on tap roots among the treatments, but there was still a high proportion of lateral roots which remained white in colour on the plants receiving the chemical treatments, especially on the roots from the plots receiving the high rate of prochloraz.

The percentages of roots with stele browning gradually increased from 0 at 6 weeks to 86 - 94% at 45 weeks (Table 5-4-1). Fewer roots showed stele browning at 12 weeks in plants from plots treated with the high rate of prochloraz (3.46 g/m^2), but no differences among the treatments were detected at 18 and 45 weeks.

Table 5-4-1. Mean percentages of roots exhibiting stele browning of 6-, 12-, 18-, and 45-week-old plants growing in a field plot drenched with benomyl or prochloraz (Proch.) immediately after sowing.

Weeks after sowing	Control	Benomyl 3.46g/m²	Proch. 1.15g/m²	Proch. 3.46g/m²
6	0.00	0.00	0.00	0.00
12	48.71 a[1]	38.53 ab	33.04 ab	27.22 b
18	78.29 a	90.47 a	81.49 a	78.28 a
45	89.61 a	94.50 a	86.37 a	91.45 a

[1]: Means followed by the same letters within each line were not significantly different at 5% probability level in an analysis of transformed values (Log).

Fresh weights of shoots per plant were consistently higher from plots treated with 3.46 g prochloraz/m² than from control plots throughout the experiment, although differences were not significant at 45 weeks (Fig. 5-4-1 A). Compared with control plots, significantly higher yields were also obtained from plots treated with 1.15 g prochloraz/m² at 6 weeks, and with benomyl at 12 weeks. However, shoot fresh weights were not significantly different among the chemical treatments.

Fresh weights of roots per plant showed no significant differences among treatments until 18 weeks after sowing (Fig.5-4-1 B). Plants from plots drenched with 3.45 g prochloraz/m² had produced greater root fresh weight than those from control plots at 18 and 45 weeks.

Results expressed as percentages of control values show the effects of fungicides on plant growth more clearly (Table 5-4-2). Treatment with prochloraz at 1.15 and 3.46 g/m² resulted in up to 72 and 95% increases in herbage production (6 weeks) and with benomyl, of 46% (12 weeks). Although the effect of treatments then gradually diminished, at 45 weeks yield was still 29% higher in plots drenched with prochloraz at 3.46 g/m² than in the control plots. However, effects of the low prochloraz treatment appeared to have been lost by 12 weeks, and of benomyl treatments by 18 weeks.

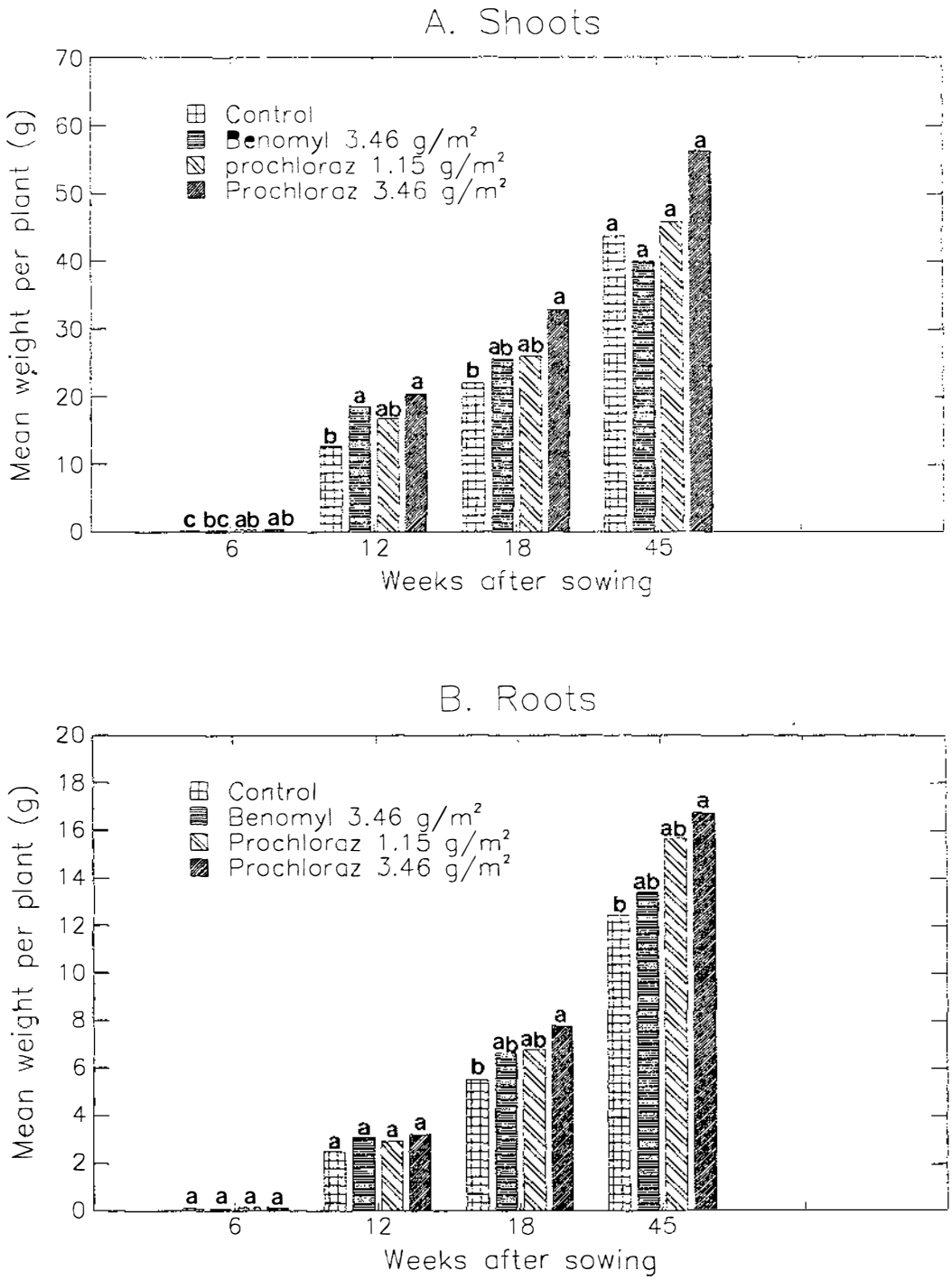


Figure 5-4-1. Mean weights of shoots (A) and roots (B) per plant of 6-, 12-, 18-, and 45-week-old red clover plants growing in a field plot drenched with benomyl or prochloraz immedately after sowing. Columns followed by the same letters were not significantly different at 5% probability level within each harvesting date.

Similarly with root weights, treatment with prochloraz at 1.15 and 3.46 g/m^2 resulted in increases of 50 and 40%, respectively at 6 weeks, and with benomyl, of 46% at 12 weeks. At 45 weeks, root weights were still 25% and 34% higher than controls in the respective prochloraz treatments; root weights from benomyl treated plots were similar to those from control plots (Table 5-4-2).

Table 5-4-2. Relative shoot and root weights (% of control) of 6-, 12-, 18-, and 45-week-old red clover plants growing in a field plot drenched with benomyl or prochloraz (Proch.) immediately after sowing.

A. Shoots Weeks after sowing	Benomyl 3.46g/m^2	Proch. 1.15g/m^2	Proch. 3.46g/m^2
6	118.18	172.33	195.45
12	146.04	104.76	160.08
18	116.02	118.07	149.06
45	91.53	104.81	128.67
B. Roots **Weeks after sowing**	**Benomyl 3.46g/m^2**	**Proch. 1.15g/m^2**	**Proch. 3.46g/m^2**
6	110.00	150.00	140.00
12	124.40	116.00	128.80
18	120.58	123.68	141.53
45	107.88	125.88	134.88

5.4.3.2 Root-invading fungi

Chemical treatments reduced, but did not eliminate, root invasion by fungi. A similar range of fungi was isolated from all treatments. The major fungal species are listed in Table 5-4-3. Other fungi isolated at any time from different treatments included species of Alternaria, Arthrinium, Aspergillus, Chaetomium, Cladosporium, Fusarium, Humicola, Leptographium, Mucor, Penicillium, Phoma, Pyrenochaeta, Pythium, Rhizoctonia, Trichoderma, and some with sterile mycelium.

Table 5-4-3. Mean numbers of colonies per gram fresh roots ($x10^3$) in total, and of the major fungal species isolated from 6-, 12-, 18-, and 45-week-old red clover plants growing in a field plot drenched with benomyl or prochloraz immediately after sowing.

Fungi	Control	Benomyl 3.46 g/m²	Prochloraz 1.15 g/m²	Prochloraz 3.46 g/m²
Six weeks				
Total	38.00 a[1]	11.33 b	8.60 b	7.28 b
Acremonium sp.	3.35 a	1.17 b	0.35 b	0.53 b
Cy. scoparium	0.07 a	0.00 a	0.14 a	0.05 a
C. destructans	0.00	0.00	0.00	0.00
F. oxysporum	8.40 a	1.87 a	1.38 a	1.75 a
F. solani	16.08 a	3.80 b	2.77 b	2.72 b
G. roseum	2.80 a	0.75 a	0.55 a	0.40 a
T. basicola	4.73 a	2.60 ab	2.17 ab	1.05 b
V. dahliae	0.00	0.00	0.00	0.00
Others	2.56 a	1.15 a	1.25 a	0.78 a
Twelve weeks				
Total	16.33 a	11.32 b	10.73 b	6.48 c
Acremonium sp.	0.51 a	0.43 a	0.58 a	0.21 a
Cy. scoparium	0.90 a	1.15 a	1.13 a	0.68 a
C. destructans	0.32 a	0.22 a	0.23 a	0.16 a
F. oxysporum	1.32 a	0.73 b	0.73 b	0.56 b
F. solani	4.93 a	4.12 a	5.02 a	2.08 b
G. roseum	1.44 a	1.03 ab	0.68 b	0.53 b
T. basicola	5.09 a	2.48 ab	1.65 b	1.38 b
V. dahliae	1.08 a	0.83 a	0.58 a	0.54 a
Others	0.78 a	0.35 ab	0.16 b	0.34 ab
Eighteen weeks				
Total	39.56 a	13.68 b	13.80 b	7.95 b
Acremonium sp.	3.38 a	0.70 b	0.65 b	0.68 b
Cy. scoparium	1.47 a	0.80 b	0.87 ab	0.61 b
C. destructans	0.67 a	0.32 a	0.35 a	0.50 a
F. oxysporum	2.38 a	0.72 b	0.73 b	0.59 b
F. solani	16.08 a	4.81 b	4.10 b	1.96 b
G. roseum	3.45 a	1.26 b	1.37 b	0.75 b
T. basicola	5.42 a	2.85 ab	2.55 ab	0.56 b
V. dahliae	1.72 a	0.70 a	2.07 a	1.25 a
Others	4.93 a	1.60 b	1.13 b	1.05 b
Forty five weeks				
Total	106.83 a	63.07 b	60.61 b	44.69 b
Acremonium sp.	5.97 a	2.59 ab	5.78 ab	2.66 b
Cy. scoparium	1.71 a	0.64 ab	0.15 b	0.13 b
C. destructans	13.00 a	8.37 a	10.28 a	8.37 a
F. oxysporum	2.07 a	1.08 a	1.33 a	0.79 a
F. solani	33.21 a	13.41 b	11.09 b	11.44 b
G. roseum	4.21 a	2.03 ab	1.21 b	0.96 b
T. basicola	18.65 a	14.11 ab	9.64 ab	5.68 b
V. dahliae	13.87 a	17.36 a	10.63 a	12.23 a
Others	14.14 a	3.48 ab	10.52 ab	2.43 b

[1]: Means in each line followed by the same letters were not significantly different at 5% probability level in an analysis of transformed values (sq.rt.).

Total numbers of fungal colonies per gram fresh roots remained significantly lower from plots treated with any fungicide than from control plots throughout the 45 weeks of the experiment (Table 5-4-3).

The major fungal species responded to the treatments differently. With F. solani effects were essentially the same as total colony number except that at 12 weeks the effects of treatment were only detected from plots receiving the high rate of prochloraz. With T. basicola, treatment with the high rate of prochloraz resulted in a significant reduction in colony numbers, while at the low rate, effects were only detected at 12 weeks. Benomyl treatment did not result in reduced root invasion by the fungus. Numbers of colonies of Acremonium sp., F. oxysporum, and G. roseum were reduced by prochloraz and benomyl treatments at some of the harvests. Numbers of colonies of Cy. scoparium were only reduced by prochloraz at 3.46 g/m^2, and benomyl at 18 weeks. C. destructans and V. dahliae were not isolated until 12 weeks, and the numbers of colonies were not affected by any of the chemical treatments.

The relative abundance of each of the major fungal species varied with the treatments. In the roots of plants growing in control plots, F. solani was generally the most abundant, followed by T. basicola. The proportion of V. dahliae was relatively small during the first 18 weeks. In the plots drenched with prochloraz at 3.46 g/m^2 the relative proportion of fungal colonies isolated from roots was greater than in the controls for V. dahliae and less for T. basicola. By 45 weeks V. dahliae had become the predominant fungus in roots from plots receiving this treatment followed by F. solani. Verticillium dahliae was also found abundant in the benomyl and prochloraz 1.15 g/m^2 treatments.

The progressive invasion of root tissue by fungi is seen more clearly in results expressed on a per root basis (Table 5-4-4). The total number of colonies per root increased 15 - 30 fold from 6 to 12 weeks, 2.5 - 6 fold from 12 to 18 weeks, and 6 - 11 fold from 18 to 45 weeks. The effects of fungicidal drenches on total fungal populations, and on those of the major fungal species described above, were also evident in the "per root" data. In addition, effects of benomyl and prochloraz at the low rate were detected at 6 weeks.

Table 5-4-4. Mean numbers of colonies ($x10^3$) **per root** in total, and of the major fungal species isolated from 6-, 12-, 18-, and 45 week-old-red clover plants growing in a field plot drenched with benomyl or prochloraz immediately after sowing.

Fungi	Control	Benomyl 3.46 g/m²	Prochloraz 1.15 g/m²	Prochloraz 3.46 g/m²
Six weeks				
Total	2.72 a[1]	1.07 b	1.12 b	0.96 b
Acremonium sp.	0.24 a	0.11 b	0.04 b	0.09 b
Cy. scoparium	0.01 a	0.00 a	0.02 a	0.01 a
C. destructans	0.00	0.00	0.00	0.00
F. oxysporum	0.44 a	0.18 a	0.18 a	0.20 a
F. solani	1.15 a	0.36 b	0.40 b	0.37 b
G. roseum	0.18 a	0.07 a	0.07 a	0.06 a
T. basicola	0.48 a	0.26 b	0.26 b	0.15 b
V. dahliae	0.00	0.00	0.00	0.00
Others	0.22 a	0.11 a	0.18 a	0.09 a
Twelve weeks				
Total	40.44 a	36.83 a	32.52 ab	20.53 b
Acremonium sp.	1.37 a	1.55 a	0.75 a	0.83 a
Cy. scoparium	2.11 a	3.52 a	3.85 a	2.28 a
C. destructans	0.79 a	0.67 a	0.66 a	0.46 a
F. oxysporum	3.35 a	2.34 a	1.96 a	1.77 a
F. solani	12.61 a	14.29 a	14.85 a	6.65 b
G. roseum	3,54 a	3.33 a	2.17 a	1.69 a
T. basicola	11.67 a	7.46 ab	6.17 ab	4.02 b
V. dahliae	2.81 a	2.57 a	1.64 a	1.68 a
Others	2.21 a	1.11 a	0.47 a	1.15 a
Eighteen weeks				
Total	217.41 a	90.26 b	90.62 b	62.99 b
Acremonium sp.	17.44 a	4.81 b	4.21 b	5.35 b
Cy. scoparium	7.96 a	5.05 a	5.81 a	5.08 a
C. destructans	3.94 a	2.29 a	2.41 a	3.80 a
F. oxysporum	13.41 a	4.81 b	4.87 b	4.56 b
F. solani	89.23 a	31.01 b	26.15 b	15.07 b
G. roseum	20.65 a	8.15 b	8.93 b	6.24 b
T. basicola	28.10 a	19.34 ab	16.03 ab	4.72 b
V. dahliae	7.86 a	4.82 a	14.30 a	9.63 a
Others	28.80 a	10.02 b	8.12 b	8.55 b
Forty five weeks				
Total	1272.00 a	767.10 ab	958.70 ab	696.20 b
Acremonium sp.	74.98 a	36.11 a	91.60 a	40.81 a
Cy. scoparium	18.55 a	9.93 ab	2.04 b	2.38 b
C. destructans	142.65 a	106.40 a	160.38 a	135.98 a
F. oxysporum	27.94 a	14.70 a	20.27 a	12.32 a
F. solani	386.67 a	191.14 b	165.07 b	184.91 b
G. roseum	53.46 a	28.56 a	18.17 a	16.58 a
T. basicola	215.50 a	176.76 ab	164.61 ab	79.64 b
V. dahliae	163.15 a	237.29 a	166.82 a	190.06 a
Others	196.00 a	49.20 ab	169.70 ab	33.20 b

[1]: See Table 5-4-3 for explanation.

5.4.4 Discussion

The demonstration in this experiment that a single drench application of prochloraz at a rate of 3.46 g/m^2 increased yield (Fig. 5-4-1; Table 5-4-2), and decreased fungal invasion of the roots of red clover (Tables 5-4-3; 5-4-4) indicated that prochloraz can be a suitable fungicide for use in the assessment of effects of soilborne pathogens on red clover growth under field conditions. The results also provided further evidence to that of Skipp et al. (1986), that fungi in soils in the plant breeder's evaluation block were damaging to red clover plants.

Increased seedling emergence resulting from benomyl or prochloraz (low rate) treatment could be the result of control of soilborne fungi other than oomycetes, such as species of Fusarium and Rhizoctonia. However, no significant increases were detected from the high rate of prochloraz treatment. As discussed in Experiment 4, this probably indicated that some growth-retarding effects on red clover occurred at this concentration.

Although it is known that EBI fungicides have a potential to increase plant yields by protecting plants from injury due to drought, chilling, and ozone (Fletcher, 1985), it is unlikely that the big increases (28 - 95%) over a relatively long period (45 weeks) were due to the side effects of the fungicide (Table 5-4-2). Roots from plots treated with the high rate of prochloraz also yielded fewer fungal colonies (Table 5-4-3; 5-4-4), and showed less severe root rot symptoms. This suggested that the fungicide had reduced invasion of root tissue by fungi and thus allowed increased growth through a reduction in root disease.

Effects of fungicide drenches on root growth were detected later than those on shoot growth (Fig. 5-4-1; Table 5-4-2). Root rot symptoms did not become obvious until 12 weeks after sowing. Root disease appeared to develop more slowly and take longer to show severe effects on plant growth under field conditions than in the glasshouse (Experiment 2).

Numbers of colonies per gram (Table 5-4-3) and per root (Table 5-4-4) in total, and of F. solani, were reduced by all the chemical treatments throughout the experimental period. These effects could be due to a reduction in the inoculum level of fungi in the soil, to inhibition of fungi in the roots when they contacted fungicide absorbed by the plant, or to a combination of both. Experiment 3

showed that prochloraz was fungicidal at high concentrations, and Experiment 4 showed that application of prochloraz to the soil at rates of 1.15 and 3.46 g/m^2 reduced the total number of fungal CFU markedly over a period of 4 weeks. Benomyl is also known to have direct effects on fungal inoculum in the soil. Its effects on soil micro-organisms lasted 6 months at rate of 2.24 g/m^2 under glasshouse conditions (Siegel, 1975). Benomyl has high systemic activity whereas prochloraz has only limited systemic activity (Birchmore et al., 1977). The toxic derivative of benomyl (MBC or carbendazim) is known to be rapidly translocated from roots to leaves (Fuchs et al., 1972). Fungitoxicity is the result of the fungicide concentration in the plant, at the site of action, over time. It is unlikely that systemic fungicides, particularly benomyl, were retained by root tissue in sufficient concentration to protect roots from pathogenic fungi. Therefore, the reduction of fungal invasion of roots seemed most likely to have been the result of a reduction in the level of propagules of pathogenic fungi in the soil.

Prochloraz has rarely been used as a soil drench. The only experiment in which the fungicide has been used as a drench was reported by Roberston et al. (1987 c) in a glasshouse trial to control root rot of peas caused by F. solani f.sp. pisi and Phoma medicaginis var. pinodella. They found that the fungicide reduced the root rot, but had no effect on the yield. This may be due to the application of fungicide too late. The present experiment confirmed the results of Experiment 4, and it is suggested that prochloraz soil drench treatment could be used to control some root diseases, as well as being used experimentally to study the effects of root pathogens.

Benomyl has been reported to increase yield, and decrease root invasion, of subterranean clover by F. avenaceum over a period of 30 weeks after drenching 3 times at a rate of 2.5 g/m^2 (Greenhalgh and Clarke, 1985). Increased yield and persistence of red clover over a period of 15 months has been reported following a single application at the rate of 15.3 g/m^2 (Leath et al., 1973). In the present experiment, benomyl reduced invasion by some fungi (Tables 5-4-3; 5-4-4), but showed little effect on plant growth (Fig.5-4-1). Similarly, Skipp et al. (1986) reported that benomyl failed to reduce symptoms of root disease and fungal invasion of the tap root stele of red clover growing in pots containing field soil from the plant breeder's evaluation block, and suggested that this may be because

the rate of benomyl used was too low. Perhaps the absence of any consistent yield increase in the present experiment was also due to the use of too low rate. Higher rates or more applications may be necessary.

The percentage of plants with stele browning (Table 5-4-1) was related to the numbers of colonies of V. dahliae per g roots (Table 5-4-3) and per root (Table 5-4-4). This supported previous observations and isolation studies (Skipp et al., 1986) that V. dahliae was the main fungus responsible for stele browning in red clover tap roots. V. dahliae reduced the yield of red clover seedlings in a glasshouse inoculation test (Skipp et al., 1986). There was no indication from the present field experiment that V. dahliae had been controlled by the fungicide drenches. Therefore the present experiment provided no information on its possible effects in the field. In addition to V. dahliae, several other fungi, T. basicola, Cy. scoparium, and F. solani, found in roots of red clover plants from the evaluation block (Table 5-4-3. 5-4-4), had all proved to be pathogenic in a glasshouse test, and many others had the ability to invade red clover roots (Skipp et al., 1986; Chapter 2). It was not possible to ascertain from the present data whether any one species was more important than the others. Further experiments are reported in Chapter 6 on the effects of inoculation of red clover plants with pure cultures of these fungi under field conditions.

It is concluded from the results of this experiment that application of a prochloraz drench at a rate of 3.46 g/m^2 reduced invasion by soilborne pathogenic fungi present in the plant breeder's evaluation block and thus permitted increased growth of red clover plants over a prolonged period. Use of benomyl at same rate had some effect on root invasion but none on yield.

CHAPTER 6
A STUDY OF THE EFFECTS OF DISEASES ON THE GROWTH OF RED CLOVER BY USE OF INOCULATION METHODS

6.1 INTRODUCTION

An increase in yield of red clover following a single drench treatment with prochloraz described in Experiment 5 above, appeared to be mainly due to control of a complex of several root-invading fungi. However, it was not possible to apportion damaging effects to the individual fungal species because prochloraz has a broad spectrum of antifungal activity with the major root-invading fungi from red clover showing similar responses to the fungicide (Experiment 3 and 4).

Plant inoculation and soil infestation techniques have often been employed to study the pathogenicity and importance of root-invading fungi of forage legumes (Chapter 2), particularly in experiments in the glasshouse and under controlled environment conditions. A few field experiments have been conducted on lucerne (Cormack, 1937; Kushner and Crittenden, 1967; Faris and Sabo, 1981; Johnson et al., 1982; Turner and Van Alfen, 1983), and sweet clover (Cormack, 1937), but no similar investigations have been conducted on red clover. Experiments reported in this chapter studied effects of fungal isolates from diseased roots from the plant breeder's evaluation block on the persistence and growth of red clover : -

1. In field plots.
2. In the glasshouse and microplots.

6.2 EXPERIMENT SIX: EFFECTS OF ROOT-INVADING FUNGI ON PERSISTENCE AND GROWTH OF RED CLOVER PLANTS IN FIELD PLOTS

6.2.1 Introduction

In a previous glasshouse experiment (Skipp et al., 1986) it was found that 5-week-old red clover seedlings inoculated with conidia of F. solani, T. basicola, or V. dahliae by root dipping (see Chapter 2) produced less shoot dry weight than uninoculated control plants, and that all plants inoculated with Cy. scoparium were dead 8 weeks after inoculation. Root dipping has also been used to inoculate plants in a field study (Johnson et al., 1982). In this experiment red clover plants were inoculated by root dipping and transplanted to field plots to examine effects of major root-invading fungi on:

1. survival; and
2. growth.

6.2.2 Materials and methods

The experimental area was situated in the pasture block. The seedbed was prepared as described in Experiment 5. The experiment was carried out as a randomised complete block design with 4 replicates. The experimental area was surrounded by a 1 m wide path. The blocks were 1 m apart and each was divided into 1 x 1 m plots spaced 50 cm apart.

Plastic sheets were laid on the surface of each of the four 1 x 12 m blocks. The sheets were sealed by putting their edges into trenches (about 10 cm wide x 10 cm depth) around each block and covering with soil. The blocks were then fumigated with "Israel bromine" (98% methyl bromide and 2% chloropicrin; Bromine Compounds Ltd) at the rate of 113 g/m^2 on the 8th of Dec.1986. The plastic sheets were left in place for 1 week following fumigation and then removed for 8 days prior to planting to allow aeration.

Isolates of seven fungal species were obtained from roots of red clover during the previous experiments. They were Cylindrocarpon destructans, Cylindrocladium scoparium, Fusarium oxysporum, F. solani, Gliocladium roseum, Trichocladium basicola, and Verticillium dahliae. The cultures were maintained on PDA medium at 20°C in the dark until used. Inocula of conidial suspensions in distilled water were prepared from 9-day-old cultures, filtered through four layers of muslin and adjusted to a concentration of about 10^6 conidia per ml except for Cy. scoparium which only contained 2.5×10^4 conidia per ml.

Five-week-old red clover plants grown in the glasshouse in 100 mm diam. pots containing a peat-sand potting mix were removed and their roots washed carefully under running tap water. The plants were divided into eight groups and each consisted of similar numbers of plants. The roots of the plants were soaked in the inoculum suspension for 1 h, one group per fungal species. The remainder were soaked in distilled water and used as controls. Shoots were trimmed with scissors about 5 cm above the crown. All plants were then transferred to the same-sized pots containing peat-sand potting mix (10 seedlings per pot), and grown in the glasshouse for another week.

Twenty five plants, as 5 x 5 pattern at a 15 x 15 cm spacing, were planted into each plot on the 23rd of Dec. 1986. Another five extra plants were planted at one side of each plot for symptom assessment and fungal isolation. The plants were hand-watered daily during the first 10 days after planting, and hand-weeded as necessary thereafter.

6.2.2.1 Assessment of symptoms and isolation of fungi from the roots

Seven weeks after planting, the extra plants at one side of each plot were dug out, washed free from soil, and external and internal symptoms of the roots were noted. One plant per replicate was taken at random, its lateral roots trimmed off, and the tap root used for fungal isolation. The root bark was removed from plants inoculated with V. dahliae to expose the stele cylinder. Isolations were also made from the petioles of those plants inoculated with V. dahliae which showed marginal chlorosis on the leaves. The tap roots, stele

cylinders, and petioles were surface sterilised in 0.3% sodium hypochlorite solution for 1 min, rinsed in sterile water and cut into 1 - 2 mm long segments (Skipp et al., 1986). Ten segments from each root were placed on ABPDA and incubated at 20°C in the dark until fungi could be identified.

6.2.2.2 Measurement of survival and growth of plants

Measurements were taken on five occasions (8, 16, 39, 49, and 62 weeks after planting) when the height of green canopy had reached about 30 cm. On each occasion the number of plants in each plot was counted. One row of plants along each side of each plot was treated as a guard row. The numbers of shoots were counted on the plants in the central area of each plot. The plants were then cut to 30 - 40 mm height with electric shears, and dried overnight at 90°C. Dry weights per plant were determined.

Some plants inoculated with T. basicola or Cy. scoparium had died soon after planting. The large differences in the number of surviving plants per plot of the different treatments meant that growing conditions, in particular the amount of inter-plant competition, were not comparable between the control and these two treatments, so no yield data were collected from the plants inoculated with either of the pathogens.

6.2.2.3 Soil temperatures and rainfall

Soil temperatures at 10 and 20 cm depth, and rainfall records for the experimental period, were obtained from the Palmerston North DSIR meterological station, which is adjacent to the pasture block.

6.2.2.4 Statistical analysis

Analysis of variance (LSD test) was performed after arcsine transformation for count data. A similar analysis was carried out for the weight data but these were not transformed.

6.2.3 Results

6.2.3.1 Root symptoms and fungal reisolation

Disease symptoms seen on roots 7 weeks after planting in the field were: control - both tap roots and lateral roots were well developed and white internally and externally; C. destructans, F. oxysporum, and G. roseum - similar to control; F. solani - a few scattered brown lesions on the tap roots, some lateral roots were brown, and there were some discrete light brown or discoloured regions in the stele; V. dahliae - lateral roots were less well developed, dark brown discoloration was present throughout the whole longitudinal section of the stele tissues (Plate 6-1 b), no visible lesions or discoloration were seen externally; T. basicola - the lower parts of the tap roots were rotted and sloughed off, necrotic dark lesions were present, but stele tissue remained cream in colour; Cy. scoparium - tap roots were stunted, only a few lateral roots had developed, and there were some dark brown lesions in the cortex, stele tissue remained white.

Marginal pale yellow lesions, and areas of necrosis and wilting were seen on some leaves of the plants inoculated with V. dahliae (Plate 6-1 a). No leaf symptoms were seen on the other plants.

In all treatments the fungus used as inoculum was reisolated from a high proportion of the roots (Table 6-2-1). Verticillium dahliae was also recovered from petioles of inoculated plants. Species of Fusarium (other than F. oxysporum, and F. solani), Penicillium, Trichoderma, and zygomycetes were also isolated from the segments cut from the roots in all treatments. Percent frequency of isolation of these non-inoculated fungi did not differ significantly among the treatments ($P > 0.05$).

Table 6-2-1. Percent frequency of isolation of fungal colonies from 1-2 mm segments cut from the surface sterilised tap roots and petioles of red clover plants 7 weeks after transplanting into the field.

Treatments	Parts of plants	Fungi isolated				
		Ino[1]	Pen[2]	Tri[2]	Zyg[2]	Fsp[2]
Control	Roots	---	35.0	5.0	10.0	22.5
C. destructans	Roots	47.5	17.5	5.0	15.0	10.0
Cy. scoparium	Roots	65.0	15.0	2.5	7.5	15.0
F. oxysporum	Roots	75.0	15.0	5.0	10.0	---
F. solani	Roots	93.3	7.5	2.5	17.5	---
G. roseum	Roots	60.0	22.5	---	5.0	7.5
T. basicola	Roots	72.5	10.0	2.5	22.5	17.5
V. dahliae	Steles	87.5	22.5	2.5	10.0	7.5
	Petioles	92.5	12.5	---	12.5	---

[1]: Ino, Fungi inoculated;

[2]: Pen, Penicillium spp.; Tri, Trichoderma spp. Zyg, Zygomyces; Fsp, Fusarium spp..

6.2.3.2 Plant survival

From the 25 plants placed in each field plot about 20 - 21 plants of the control, and those inoculated with C. destructans, F. oxysporum, F. solani, and G. roseum, were present 62 weeks after planting. Slightly fewer (18) plants inoculated with V. dahliae remained in the plots, but this was not significantly different from the control (Fig. 6-2-1). Numbers of plants inoculated with Cy. scoparium or T. basicola were significantly lower than those in the control plots at all harvests (Fig. 6-2-1).

6.2.3.3 Plant growth

Plants inoculated with V. dahliae had produced significantly less shoot dry weight than control plants when harvested after 8 weeks, but no significant differences were found thereafter except at 39 weeks, where the weight was

significantly higher (Table 6-2-2 A). Plants inoculated with C. destructans, F. oxysporum, F. solani, or G. roseum had produced similar amounts of dry matter as control plants at all harvests (Table 6-2-2 A). Significantly fewer shoots than controls were produced from the plants inoculated with V. dahliae at 8 weeks, F. solani or G. roseum at 16 and 39 weeks, and F. oxysporum at 39 weeks, (Table 6-2-2 B).

Table 6-2-2. Mean dry weights (A) and numbers (B) of shoots per red clover plant harvested from field plots on five occasions.

A. Dry weights (g)

Fungal inoculum	Weeks after planting				
	8	16	39	49	62
Control	3.86 a[1]	11.87 ab	9.51 b	19.52 a	21.85 a
C. destructans	3.38 ab	13.62 a	8.84 b	19.48 a	17.48 a
F. oxysporum	3.91 a	10.72 b	7.83 b	16.00 a	20.80 a
F. solani	3.65 ab	11.20 b	9.11 b	15.77 a	16.42 a
G. roseum	3.86 a	11.77 ab	7.01 b	15.92 a	20.48 a
V. dahliae	2.00 b	10.01 b	14.82 a	21.33 a	26.13 a

B. Numbers of shoots

Fungal inoculum	Weeks after planting				
	8	16	39	49	62
Control	8.38 a	16.12 a	19.55 a	23.70 ab	18.33 ab
C. destructans	8.18 a	16.51 a	17.05 ab	24.32 ab	16.00 b
F. oxysporum	7.03 a	14.11 ab	15.10 b	20.17 b	17.75 ab
F. solani	6.57 ab	12.86 b	14.57 b	21.15 ab	15.75 b
G. roseum	7.20 a	13.14 b	13.90 b	22.15 ab	19.08 ab
V. dahliae	4.89 b	13.81 ab	17.95 a	25.35 a	22.38 a

[1]: For each sampling date, means followed by the same letters were not significantly different at 5% probability level.

Plate 6-1 a-b Leaf (a) and root (b) symptoms on red clover plants inoculated with Verticillium dahliae by the root dip method after 12 weeks growth in the field plots. Note the marginal pale yellow lesions and areas of necrosis and wilting on some leaves (arrows), and dark brown discolouration throughout root stele.

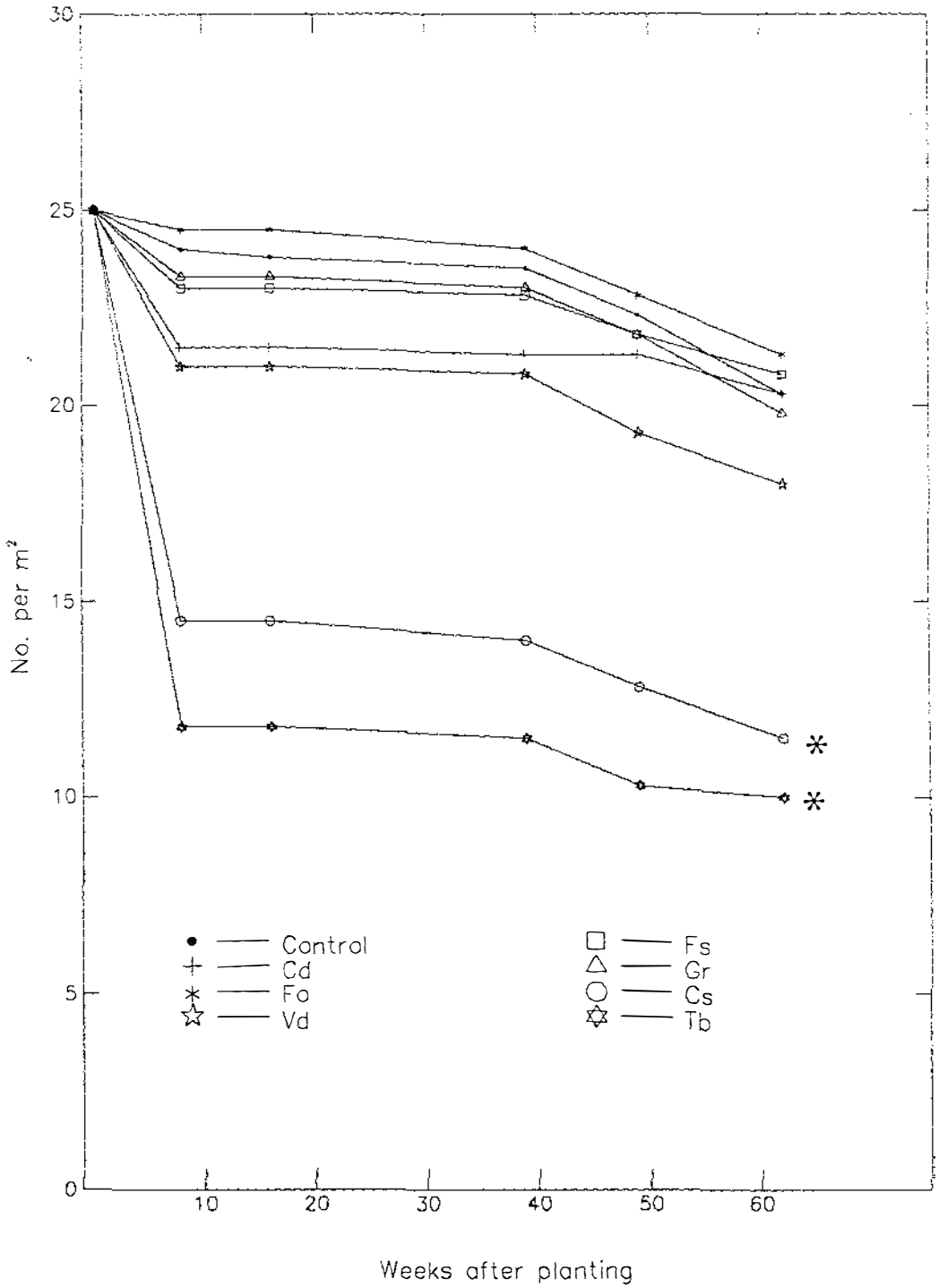


Figure 6–2–1. Mean numbers of red clover plants per m^2 over a 62 week period after planting inoculated plants in field plots. * indicated that means at all harvests were significantly different from the control at 5% probability level in an analysis of transformed values (arcsine). Cd, C. destructans; Cs, Cy. scoparium; Fo, F. oxysporum; Fs, F. solani; Gr, G. roseum; Tb, T. basicola; Vd, V. dahliae

6.2.3.4 Soil temperatures and rainfall

Mean daily soil temperatures at 10 and 20 cm depth and daily rainfall for the experimental period were similar to the 30 year average except for the period 9-16 weeks after planting, when soil temperatures at both depths were 1°C lower, and rainfall was 1.1 mm higher, than those of 30 year average (Appendix 6-2-1).

6.2.4 Discussion

The high mortality of plants inoculated with T. basicola in this experiment suggests that this fungus could affect survival of red clover in the field in other areas where the pathogen occurs naturally such as the Waikato and North Auckland (R.A. Skipp and M.J. Christensen, pers. comm.) where the climate is similar to that recorded for the experimental period (Appendix 6-2-1).

Cylindrocladium scoparium had been found to be more pathogenic than T. basicola in previous glasshouse tests (Skipp et al., 1986), but this was not apparent here (Fig. 6-2-1), possibly because a low level of inoculum of Cy. scoparium had been used. Cylindrocladium scoparium has not been recorded in red clover from pastures.

The density of the swards planted in this experiment was similar to that normally used for grazing trials on red clover and plants were sufficiently close to allow competition (R.J.M. Hay pers. comm.). The method was suitable to study effects on growth caused by relatively weak pathogens such as species of Fusarium, which did not kill plants, but not the more virulent pathogens, T. basicola and Cy. scoparium, which killed the plants rapidly thus changing the plant density. As reported for prairie grass infected with the head smut fungus Ustilago bullata Berk., plants remaining after a proportion of the population had been killed by disease were no longer comparable with the control plots (Falloon and Hume, 1988).

Reduced shoot dry matter production of plants inoculated with V. dahliae

8 weeks after planting into the field (Table 6-2-2 A) supports the findings of previous glasshouse studies (Skipp et al., 1986) that the fungus is pathogenic to red clover. The yield reduction was associated with the production of fewer shoots by the inoculated plants (Table 6-2-2 B). Number of shoots is known to be an important yield component of red clover (Hay, 1985). No yield reduction was detected later in the experiment despite the appearance of leaf symptoms and stele browning (Plate 6-1) which suggested that the invading fungus had affected the physiology of the host plant. The effects of V. dahliae on growth of red clover need to be studied over a longer period to establish whether the fungus has deleterious effects on the growth or long term persistence of the plant.

Verticillium dahliae has been isolated from roots of red clover plants grown in both Islands of New Zealand (R.A. Skipp and M.J. Christensen, pers. comm.). It can attack more than 100 species of host plants in New Zealand (Pennycook, 1989) and causes considerable economic losses in some crops (Smith, 1965). Although isolates obtained from red clover in the present study were not tested for pathogenicity on other hosts, other work (Milton and Isaac, 1976) has demonstrated that isolates from red clover can attack other crops. It is suggested that red clover should not be rotated with other crops which are known susceptible to V. dahliae such as potatoes and tobacco. C. destructans, F. oxysporum, F. solani, and G. roseum caused no detectable reduction in dry matter production but did reduce the numbers of shoots produced during early stages of growth (Table 6-2-2).

In conclusion, the results of Experiment 6 demonstrated that Cy. scoparium, T. basicola, and V. dahliae are pathogenic to red clover under field conditions. However, the field plot technique as used here suffers from the disadvantages in that it does not :

1. provide prolonged exposure of plant roots to inoculum;
2. indicate effects on establishment; and
3. easily allow measurement of effects under conditions of changing plant density.

6.3 EXPERIMENT SEVEN : EFFECTS OF ROOT-INVADING FUNGI ON THE GROWTH OF RED CLOVER PLANTS IN THE GLASSHOUSE AND MICROPLOTS

6.3.1 Introduction

Although the plot experiment described above (Experiment 6) confirmed the field pathogenicities of several fungi to red clover, the disadvantages listed above suggested that this type of experiment might not give the most reliable or relevant information about the possible effects of particular fungi on red clover growth. Field microplots established in clay tiles offered a suitable alternative. As discussed in Chapter 2 the technique has been used to study interactions of fungi with nematodes (Martin et al., 1982; Rowe et al., 1985; Riedel et al., 1985; Francl et al., 1987; Starr et al., 1989), and environmental factors (Chun and Lockwood, 1985). It has also been used to study root morphology of white clover by plant breeders in Grasslands Division, DSIR (Caradus and Woodfield, 1986; Woodfield and Caradus, 1987). To date, microplots have not been used to assess yield loss of red clover from root-invading fungi.

Although it is believed that the health of the plant will affect the symbiotic balance of plant and rhizobium (Tu and Ford, 1984), little information is available on effects of soilborne fungi on nodulation and nitrogen fixation of forage legumes apart from a paper by Sawada (1983) who reported that lucerne grown in soil infested with F. oxysporum had fewer nodules than plants in sterilised soil.

The objectives of this experiment were to assess effects of root-invading fungi on:

1. establishment of seedlings in the glasshouse and field microplots;
2. nodulation and nitrogen fixation of plants in the glasshouse;
3. growth of plants in the glasshouse and field microplots.

6.3.2 Materials and methods

Isolates of Cy. scoparium, F. oxysporum, F. solani, and T. basicola obtained from the roots of red clover during the previous experiments were used in both glasshouse and field microplot studies. The cultures were maintained on PDA medium at 20°C in the dark. Cultures for inoculum were prepared by inoculating moist, sterilised, cracked whole corn kernels (Healtheries of N.Z. Ltd) with 9-day-old cultures of the test fungi and growing for 2 weeks at 20°C in the dark.

Seed used in both glasshouse and field microplot studies was inoculated with rhizobium immediately before sowing by soaking the seeds in 4-day-old cultures of rhizobium (Rhizobium trifolii Dangeard 1926, Biotechnology Division Culture Collection NZP578) for 30 min.

6.3.2.1 Glasshouse studies

Soil from the pasture block, which had been fumigated and inoculated with cultures of the different test fungi on corn kernel medium, as described for the microplot experiment (see below), was transferred to the laboratory. The mixtures of fumigated soil and corn kernel inoculum were placed into unperforated plastic pots (95 mm diam. x 110 mm), at the rate of 450 g per pot, 15 pots per treatment. Ten seeds were sown into each pot and covered to a depth of 10 mm with 50 g fumigated soil. The pots were watered to 60% WHC and placed into temperature controlled waterbaths at 20°C. Soil moisture content in the pots was maintained at 60% WHC by weighing daily and adding water as required.

A. Counts of numbers of seedlings

Two weeks after sowing, the numbers of seedlings per pot were counted from 10 pots of each treatment chosen at random. The numbers of seedlings in the same pots were recounted 6 weeks later (8 weeks after sowing).

B. Assay of acetylene reduction and nodulation

Assays were undertaken 4, 8, and 12 weeks after sowing. On each occasion, 5 pots per treatment were used. Number of seedlings per pot was counted. All seedlings, and the soil adhering to their roots, were removed from each pot, excess soil shaken off carefully, and placed in a sealed glass jar. The volume of the jars used was 500 ml at 4 and 8 weeks, and 1000 ml at 12 weeks. Ten percent of gas (by volume) was withdrawn from the sealed jars containing the plants and replaced with acetylene using a hypodermic syringe. After 60 min. incubation, duplicate 1 ml gas samples were taken from each jar with separate disposable plastic syringes. The sampled gas was sealed within the syringes by stabbing the syringe needles into a rubber bung. 0.5 ml of sampled gas from one of the duplicate syringes was used to assay for ethylene content using a gas chromatograph (Series 204 Pye Unicam (Phillips) Ltd) in the Soil Fertility Laboratory of Grasslands Division, Palmerston North.

A blank test consisting of a jar containing 10% acetylene in air but without plant material was included on each occasion. This provided a check of whether contaminating sources of ethylene had been introduced into the acetylene or released from the other sources. A second control was used at 8 and 12 weeks. This consisted of a jar containing plants but without acetylene added to test for the possibility of ethylene production occurring by means other than through nitrogenase activity (i.e. directly from the plant or inoculum). Very little ethylene was produced in these controls (less than 3 nmoles/h/plant) and no difference was detected between the treatments. No correction was made for this source of ethylene in the final calculation.

After the gas sample had been withdrawn, the real gas volume of each jar was measured by displacement with water. The amount of ethylene produced (μmole/h/pot) = ethylene sample CU* x (Vol. gas in jar / Vol. gas assayed in GC) x incubating time (hour) x K** - Blank CU x (Vol. gas in blank jar / Vol. gas assayed in GC) x K

*: CU = Chart units used to measure peak height (mm)

**: K = Conversion factor obtained using a standard ethylene gas mixture to calibrate the chromatograph. In this test K was obtained from Grasslands Division.

Ethylene production per plant = ethylene production per pot / no. of plants per pot.

At 8 and 12 weeks, two roots from each treatment were taken and the number of nodules per root were counted.

C. Measurements of fresh weights

After each acetylene assay, the plants were washed free from soil. Shoots and roots were separated with a scalpel and blotted dry. Fresh weights of shoots and roots per plant were determined.

Fungi were reisolated from roots after 4 weeks by taking two plants from each pot and processing them by the root segment method to confirm the presence of the inoculated fungus.

6.3.2.2 Field microplot studies

The experimental area was situated in the pasture block. The seedbed was prepared as in Experiment 5 and divided into five, 1.2 x 16 m strips, 1.5 m apart. The basic replicate was a single microplot (see below). Each treatment consisted of 20 replicated microplots arranged as a randomised complete block design among the strips. The microplots, 60 cm apart, were established in each strip by placing open-ended, unglazed, clay drain tiles (38 cm long x 10 cm internal diameter) in holes dug to 30-35 cm depth with a post hole borer. The top edge of each tile was about 5 cm above soil level (Plate 6-2).

Plate 6-2 a-b Establishment of microplots.
a. General view of the experimental site; digging the holes for the clay tiles with a post hole borer.
b. Filling the microplots with fumigated soil plus fungal inoculum.

The strips (soil + tiles) were sealed with plastic sheets and then fumigated with "Israel bromine" as described in Experiment 6 on the 9th of Nov. 1987. Eight days after fumigation the sheets were removed and plots allowed to aerate for 7 days. Fumigated soil was collected from the strips, weighed, and mixed. Required amounts of corn kernel medium cultures of the individual test fungi were added to the soil to give inoculum levels of 0.1 and 1% (i.e. g inoculum per 100 g fresh field soil). The inoculum and soil were mixed thoroughly by hand. The infested soil (about 3.8 kg per tile) was then placed into each appropriate tile. Fumigated field soil alone and fumigated field soil + 1% sterilised corn kernel medium were used as controls. The remaining infested soil was placed in plastic bins [50 x 32 x 22 (depth) cm] for transfer to the laboratory and used for the glasshouse study (see above). The soil in the tiles was allowed to settle for 3 days, and then the soil level was adjusted to about 5 cm below the top edge of the tiles.

Twenty seeds were sown into each tile on the 26th of Nov. 1987 and covered with approximately 50 g fumigated soil. Seeds were also sown (at 60 cm intervals) around each strip as guard rows.

A. Measurement of survival and growth of plants.

Six weeks after sowing the numbers of seedlings in each tile were counted, and seedlings were then thinned to one per tile. The excess seedlings were collected and their tap roots used for fungal reisolation as described above.

Plant growth was measured on 6 occasions at 20, 46, 54, 59, 67, and 76 weeks after sowing. Each time, the numbers of surviving plants in the total of 20 tiles were counted. The length of the longest shoot in the each tile was measured. Plants were then cut to 8-10 cm with hand shears, dried overnight at 90°C, and the mean dry weights per plant were determined. On the last harvest shoots were removed at crown level.

On two of the six occasions, 59 and 67 weeks after sowing, 10 plants were randomly selected from the guard rows, and the length and dry weight of shoots per plant were determined as above. The data were compared with those from

control microplots (soil without amended corn kernel).

Percentage accumulated yields per plant as compared with the control were calculated.

B. Assessment of symptoms and measurement of dry weight of roots

At the last harvest (76 weeks after sowing), the tiles (including the plant and the soil) were dug out. The roots were removed carefully by washing off the soil with a jet of tap water. They were then washed free from soil, and brought to the laboratory for assessment of symptoms. After examining the external symptoms, the tap roots were cut through longitudinally to assess crown rot and stele browning. The symptoms were scored for root rot, crown deterioration and stele browning, respectively, using the indices given in Table 6-3-1.

Segments bearing lesions were cut from roots of control plants, and from plants inoculated with F. oxysporum, and F. solani. The cortex and stele were separated, and both parts were used for fungal isolation by the root segment method. No isolation was made from roots of plants infected with Cy. scoparium or T. basicola, because of the complete deterioration of the tissues.

Dry weight of the roots was then determined as above.

C. Growth of plants after transplanting into microplots

Experiment 6 had showed that Cy. scoparium can kill seedlings of red clover rapidly, thus it was unlikely that growth measurements could continue for very long in microplots inoculated with this fungus. Attempts were therefore made to prolong the period available for growth measurement by using more mature plants transplanted into growth medium inoculated with the fungus. Seven weeks after the experiment had been set up, 5-week-old red clover plants grown in pots containing a peat-sand potting mix in the glasshouse were removed from their pots and their roots carefully washed free of potting mix. One plant was transplanted into each of 10 control microplots which had been established for this purpose among the 5 strips of the experimental block. Plants were also

transferred to 10 microplots of each Cy. scoparium treatment in which seedlings had already been killed by the pathogen. The plants were watered daily with a hose with a rose attachment during the first 10 days after planting. Measurement of plant growth was undertaken at the same time as for the other microplots i.e. 13, 39, 47, 52, 60, and 69 weeks after planting.

During the whole experimental period (25th of Nov. 1987 to 11th of May 1989) the plots were hand weeded as necessary.

Table 6-3-1. Scoring indices for external and internal root symptoms of plants grown in the field microplots 76 weeks after sowing.

Root rot index
0 Roots white - without lesions
1 Some small brown lesions
2 Smaller lateral roots rotten and/or superficial lesions > 1 cm long on tap root or large lateral roots
3 Tap root or larger lateral roots rotted through to stele in some places
4 Extensive rotting of whole depth of tap root and large lateral roots
5 Main roots dead (excluding adventitious roots)
Crown deterioration index
0 Pale cream throughout
1 Internal breakdown up to 4 mm^2
2 Internal breakdown > 4 mm^2, no cavity
3 Cavity without channel to outside
4 Cavity and channel
5 Cavity extending into root stele
Stele browning index
0 White
1 Faint flecking or trace of browning in outer stele
2 Brown - outer stele only
3 Brown - whole stele
4 Dark brown - whole stele

6.3.2.3 Statistical analysis

Analysis of variance (LSD test) was performed after arcsine transformation for seedling emergence data. A similar analysis was carried out for the weight data obtained in the glasshouse except that data were not transformed. Analysis of variance was carried out for weight and height data obtained in field microplots as unbalanced data.

6.3.3 Results

In all treatments the fungus used as inoculum was reisolated from 4-week-old plants grown in the pots and 6-week-old plants grown in the microplots.

6.3.3.1. Glasshouse studies

A. Seedling emergence

Numbers of seedlings per pot were significantly fewer in the pots containing 0.1% corn kernel cultures of F. solani or T. basicola, and either inoculum level of Cy. scoparium than those in control pots 2 weeks after sowing (Fig. 6-3-1). All seedlings in the pots containing Cy. scoparium had died 8 weeks after sowing. Seedlings established in the pots inoculated with any inoculum level of F. solani or T. basicola were fewer than those in the controls at 8 weeks (Fig. 6-3-1).

B. Acetylene reduction and nodulation

Ethylene production did not differ significantly among the treatments at 4 weeks, however, the amount of ethylene produced by plants infected with T. basicola was much lower than that produced by control plants and no ethylene was detected from plants infected with Cy. scoparium (Table 6-3-2 A). Plants infected

with F. solani or T. basicola produced less ethylene than controls at 8 weeks, but no significant differences were found at 12 weeks (Table 6-3-2 A).

Nodulation of red clover seedlings was adversely affected by the pathogens (Table 6-3-2 B). Plants infected with T. basicola produced fewer nodules than the controls.

Table 6-3-2. Mean ethylene production (A) and number of nodules (B) per red clover plant grown in the pots at 20°C and 60% WHC on three occasions after sowing.

A. Ethylene production (μmoles/h/plant).

Fungi and inoculum level[1]	Weeks after sowing 4	8	12
Control	0.13 a[2]	16.33 a	17.44 a
Control II	0.15 a	10.47 abc	10.54 a
Cy. scoparium (0.1%)	0.00 a	---[3]	---
Cy. scoparium (1%)	---	---	---
F. oxysporum (0.1%)	0.36 a	15.84 ab	13,06 a
F. oxysporum (1%)	0.33 a	9.76 abc	14.20 a
F. solani (0.1%)	1.03 a	8.47 c	16.25 a
F. solani (1%)	0.24 a	9.57 bc	18.19 a
T. basicola (0.1%)	0.01 a	7.46 c	11.26 a
T. basicola (1%)	0.07 a	1.21 d	12.56 a

B. Number of nodules.

Fungi and inoculum level[1]	Weeks after sowing 8	12
Control	130.2 a[2]	272.1 a
Control II	104.6 ab	234.9 ab
Cy. scoparium (0.1%)	---[3]	---
Cy. scoparium (1%)	---	---
F. oxysporum (0.1%)	133.8 a	182.4 b
F. oxysporum (1%)	81.2 b	196.0 ab
F. solani (0.1%)	91.9 ab	233.5 ab
F. solani (1%)	73.4 b	186.9 b
T. basicola (0.1%)	36.4 c	95.7 c
T. basicola (1%)	23.2 c	99.7 c

[1]: Control, Fumigated soil only; Control II, Fumigated soil + 1% sterilised corn kernel (w/w); 0.1%, Fumigated soil + 0.1% w/w of inoculated corn kernel medium. 1%, Fumigated soil + 1% w/w of inoculated corn kernel medium.

[2]: For each sampling date, means followed by the same letters were not significantly different at 5% probability level.

[3]: Plant dead

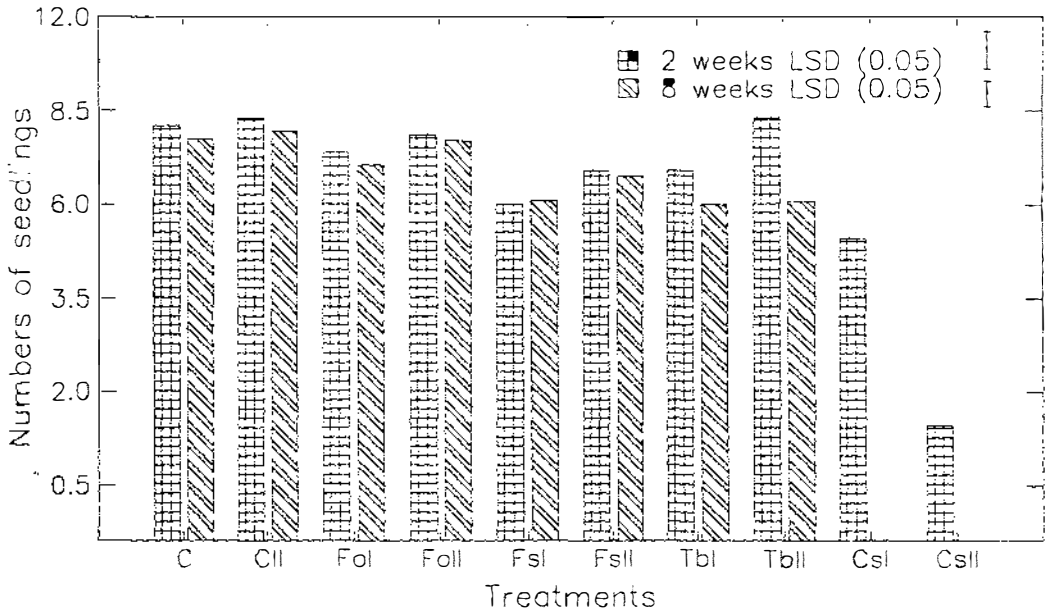


Figure 6-3-1. Mean numbers of seedlings per pot 2 and 8 weeks after sowing 10 seeds into each pot containing fumigated soil plus 0.1 or 1% of corn kernel cultures of different fungi. The pots were kept in water baths in a glasshouse at 20°C and 60% WHC. Statistical analyses were performed after arcsine transformation. C, Control; Fo, F. oxysporum; Fs, F. solani; Tb, T. basicola; Cs, Cy. scoparum; I and II indicated that 0.1% and 1% corn kernel bearing the inoculum had added, while CII was soil + 1% sterilised corn kernel medium.

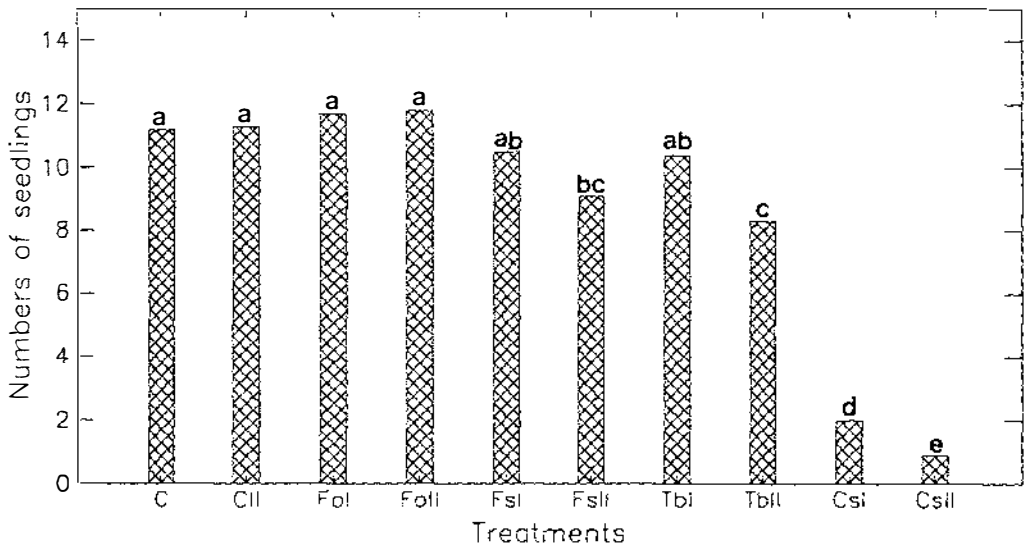


Figure 6-3-2. Mean numbers of seedlings per microplot 6 weeks after sowing 20 seeds into each microplot containing fumigated soil plus 0.1 or 1% of corn kernel cultures of different fungi. Bars with the same letters were not significantly different in an analysis of transformed values (arcsine). For abbrevations see Fig. 6-3-1.

C. Shoot and root weights

Mean fresh weights of shoots per plant differed among the treatments (Table 6-3-3 A). Plants grown in the pots inoculated with either level of T. basicola had produced less herbage than the control (Table 6-3-3 A) at each harvest, similarly, plants from the high F. solani inoculum treatment (1%) had produced less herbage than the control at 4 and 8 weeks.

Root fresh weights were reduced by either inoculum level of T. basicola and the 1% inoculum of F. solani, as compared with control, at all harvests (Table 6-3-3 B).

Plants grown in the pots containing fumigated soil + 1% corn kernel medium (Control II) had produced less shoot material than the control lacking corn kernel medium at 4 weeks, and less root material, at all the harvests.

6.3.3.2 Field microplot studies

A. Survival

Numbers of seedlings established in the microplots inoculated with either inoculum level of Cy. scoparium, or the 1% inoculum level of T. basicola or F. solani, were significantly lower than controls 6 weeks after sowing (Fig. 6-3-2).

Plants infected with Cy. scoparium or T. basicola continued dying. By 20 weeks, percentage survival of plants was 70 for T. basicola (1%), 25 for Cy. scoparium (0.1%), while no plants survived in the microplots inoculated with Cy. scoparium at the high level of corn kernel inoculum (1%). In the other treatments 85 -100% plants remained alive. By the end of experiment (76 weeks) percentage survival of plants for each treatments were: control, 75; control + 1% sterilised corn kernel, 80; F. oxysporum (0.1%), 75; F. oxysporum (1%), 65; F. solani (0.1%), 50; F. solani (1%), 70; T. basicola (0.1%), 50; T. basicola (1%), 45; Cy. scoparium (0.1%), 20.

Table 6-3-3. Mean fresh weights of shoots (A) and roots (B) per red clover seedling grown in pots at 20°C and 60% WHC on three occasions after sowing.

A. Shoots (grams).

Fungi and inoculum level[1]	Weeks after sowing 4	8	12
Control	0.18 ab[2]	0.97 ab	3.15 a
Control II	0.07 de	0.86 bc	2.81 ab
Cy. scoparium (0.1%)	0.01 f	---[3]	---
Cy. scoparium (1%)	---	---	---
F. oxysporum (0.1%)	0.20 a	1.21 a	3.43 a
F. oxysporum (1%)	0.13 bc	0.86 bc	2.89 a
F. solani (0.1%)	0.20 a	0.84 bc	3.75 a
F. solani (1%)	0.05 e	0.64 c	3.47 a
T. basicola (0.1%)	0.12 dc	0.73 c	2.06 bc
T. basicola (1%)	0.04 e	0.45 d	1.91 c

B. Roots (grams).

Fungi and inoculum level[1]	Weeks after sowing 4	8	12
Control	0.13 a[2]	0.52 a	1.86 a
Control II	0.07 bc	0.34 bc	1.34 c
Cy. scoparium (0.1%)	0.01 e	---[3]	---
Cy. scoparium (1%)	---	---	---
F. oxysporum (0.1%)	0.12 a	0.55 a	1.55 bc
F. oxysporum (1%)	0.09 ab	0.36 bc	1.49 bc
F. solani (0.1%)	0.13 a	0.42 ab	2.07 a
F. solani (1%)	0.05 cd	0.28 c	1.43 bc
T. basicola (0.1%)	0.06 bc	0.27 c	0.84 d
T. basicola (1%)	0.02 de	0.16 d	0.90 d

[1], [2], [3] : See Table 6-3-2 for explanation.

Table 6-3-4. Mean length (A) and dry weight (B) of shoots per red clover plant grown in the microplots measured on six occasions after sowing.

A. Length (cm).

Fungi and inoculum level[1]	Weeks after sowing 20	46	54	59	67	76
C	31.5 a[2]	39.9 ab	52.2 a	56.4 a	49.3 a	26.8 a
C II	25.5 b	42.1 a	51.4 ab	56.1 a	48.6 a	27.1 a
Cs (0.1%)	12.6 d	25.4 c	40.2 d	59.3 a	43.3 a	22.5 a
Cs (1%)	---[3]	---	---	---	---	---
Fo (0.1%)	31.0 a	41.7 a	51.4 ab	53.7 a	48.7 a	25.1 a
Fo (1%)	21.4 bc	36.9 abc	48.4 abc	56.1 a	50.0 a	26.9 a
Fs (0.1%)	27.7 ab	36.7 abc	48.1 abc	51.3 a	45.7 a	22.9 a
Fs (1%)	22.4 bc	33.8 bc	45.7 cd	57.8 a	46.0 a	28.0 a
Tb (0.1%)	21.4 bc	35.1 bc	46.8 bcd	50.8 a	42.9 a	25.5 a
Tb (1%)	18.9 c	31.3 c	41.3 d	55.6 a	46.2 a	22.0 a

B. Dry weight (gram).

Fungi and inoculum level[1]	Weeks after sowing 20	46	54	59	67	76
C	25.4 a[2]	39.4 a	61.7 a	64.0 a	58.1 a	45.4 a
C II	10.3 c	39.3 a	56.6 ab	59.2 ab	52.1 a	45.8 a
Cs (0.1%)	1.7 d	6.2 c	27.5 d	39.0 cd	23.2 c	20.5 bc
Cs (1%)	---[3]	---	---	---	---	---
Fo (0.1%)	17.9 b	28.0 ab	56.7 ab	53.5 abc	38.3 bc	31.6 b
Fo (1%)	9.1 c	20.2 bc	44.6 bcd	60.4 ab	45.3 ab	30.0 b
Fs (0.1%)	16.4 b	30.9 ab	49.5 bcd	44.6 bcd	31.6 bc	31.6 b
Fs (1%)	8.3 c	16.0 bc	40.9 cd	57.9 ab	45.4 ab	33.9 b
Tb (0.1%)	9.1 c	18.0 bc	37.2 cd	34.9 d	26.4 c	16.4 c
Tb (1%)	5.1 d	11.8 c	34.1 d	39.4 cd	24.5 c	16.5 c

[1]: C, Control; Fo, F. oxysporum; Fs, F. solani; Tb, T. basicola; Cs, Cy. scoparium; Others see Table 6-3-2.

[2], [3]: See Table 6-3-2 for explanation.

Plate 6-3 a-b Red clover plants in microplots 54 weeks after sowing.
a. General view.
b. Reduced growth in microplots inoculated with Trichocladium basicola (Ce) compared with a control plant (rear).

B. Shoot lengths and dry weights

Mean shoot lengths of plants infected with Cy. scoparium (0.1%), T. basicola (0.1 or 1%) (Plate 6-3), or F. solani (1%) at 20 and 54 weeks, and of those infected with Cy. scoparium (0.1%) or T. basicola (1%) at 46 weeks, were significantly shorter than controls (Table 6-3-4 A), but no significant differences were found thereafter.

Shoot dry weights of plants grown in the microplots inoculated with Cy. scoparium (0.1%), or T. basicola (0.1 or 1%), were consistently lower than controls (Table 6-3-4 B). Yields were also lower at some harvests in microplots inoculated with F. solani and F. oxysporum.

Similar lengths and dry weights of shoots per plant were obtained from plants grown in the guard rows and in the microplots containing soil without added corn kernel medium (Table 6-3-5, Plate 6-3).

Table 6-3-5. Mean height and dry weight of shoots per red clover plant grown in the microplots containing soil without added corn kernel medium and in the guard rows 59 and 67 weeks after sowing.

Weeks	Treatments	Height (cm)	Dry weight (g)
59	Guard row	58.2 a[2]	68.9 a
	Microplot	56.4 a	64.0 a
67	Guard row	54.3 a	62.6 a
	Microplot	49.2 a	58.1 a

[2] : See Table 6-3-2 for explanation.

The cumulative yield of plants infected with Cy. scoparium or T. basicola was reduced by more than 50% in comparison with plants from uninoculated control microplots (Fig. 6-3-3). Reduction in cumulative yield in microplots inoculated with F. oxysporum and F. solani, was about 25 and 30%, respectively. Addition of 1% corn kernel to the soil (Control II) caused a 10% reduction.

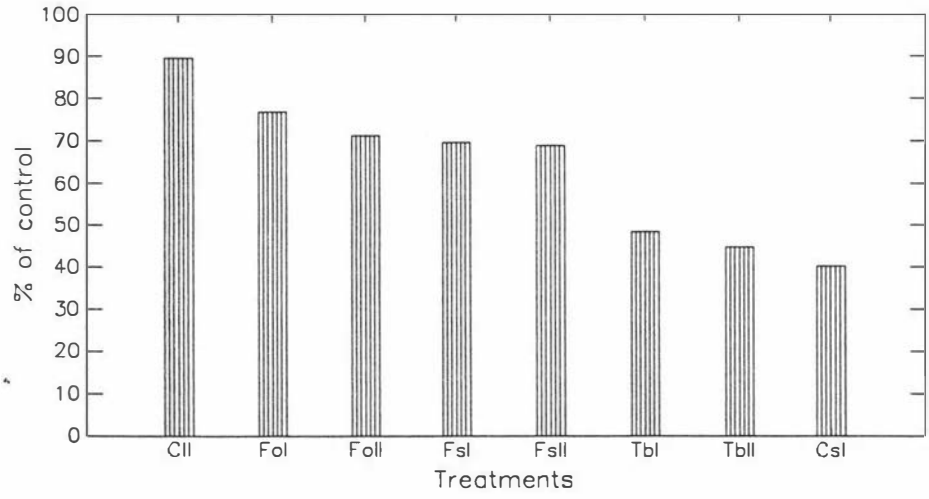


Figure 6–3–3. Percentage cumulative yield per red clover plant in microplots containing fumigated soil plus 0.1 or 1% of corn kernel cultures of different fungi as compared with control. For abbrevations of treatments see Fig. 6–3–1.

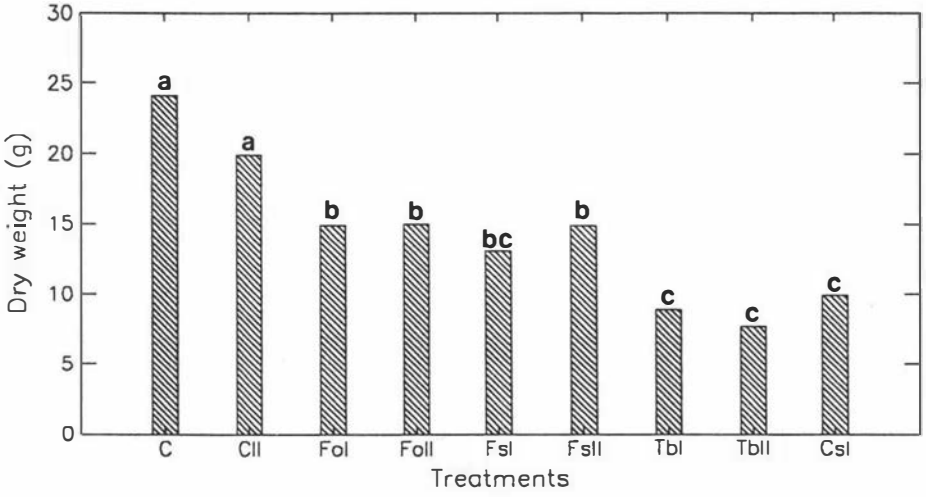


Figure 6–3–4. Mean dry root weight per red clover plant in microplots containing fumigated soil plus 0.1 or 1% of corn kernel cultures of different fungi, 76 weeks after sowing. Bars with the same letters were not significantly different at 5% probability level. For abbrevations of treatments see Fig. 6–3–1.

C. Root dry weights and symptoms

Root dry weights of the plants grown in the microplots containing any fungal inoculum were significantly lower than those of the controls 76 weeks after sowing (Fig. 6-3-4).

Varying degrees of external and internal symptoms were seen on the roots (Table 6-3-6). Tap roots and major lateral roots of most plants infected with T. basicola or Cy. scoparium were completely rotted and sloughed off, adventitious and lateral roots had developed around the crown and upper parts of the tap roots, and dark brown lesions were seen on the remaining parts of tap roots and the major lateral roots. In contrast, plants grown in the control microplots or in those inoculated with F. oxysporum or F. solani had well developed tap roots. However, superficial brown lesions were present on the tap roots and major lateral roots, and some smaller lateral roots were rotten. Internal breakdown was common and appeared as a conical brown necrotic area in the pith of the crown. Cavities developed in the central tissue of the upper tap roots of plants from all treatments (Table 6-3-6). Symptoms seen in the stele of tap roots and the major lateral roots of plants infected with different pathogen were as follows : control and F. oxysporum, most were white or cream in colour, but traces of browning were seen in the outer regions; F. solani, browning of outer areas was common, but occasionally the whole stele was brown; Cy. scoparium or T. basicola, disorganised and brown in colour (Table 6-3-6).

Fungi isolated from lesioned tissue on the roots of control plants were F. culmorum, Codinaea fertilis, Gliocladium roseum, species of Penicillium, Cladosporium, Chaetomium, Rhizoctonia (binucleate), and infrequently, F. solani. Fusarium spp. were the predominant species in the roots from microplots inoculated with F. oxysporum or F. solani, together with the fungi which were common in control plants (listed above). The major isolate from the brown stele tissue of roots inoculated with F. solani was this fungus, together with a few colonies of Codinaea fertilis and Geotrichum sp..

Table 6-3-6. Mean root rot, crown rot, and stele browning indices of red clover plants grown for 76 weeks in the microplots.

Fungi and inoculum level[1]	Root rot[4]	Crown rot	Stele browning
Control	2.10 c[2]	2.60 a	1.00 c
Control II	2.10 c	3.20 a	1.60 bc
Cy. scoparium (0.1%)	4.40 a	2.20 a	4.00 a
Cy. scoparium (1%)	---[3]	---	---
F. oxysporum (0.1%)	2.40 bc	2.80 a	1.30 bc
F. oxysporum (1%)	2.60 bc	2.70 a	0.90 c
F. solani (0.1%)	3.00 b	3.50 a	2.20 b
F. solani (1%)	2.50 bc	2.90 a	1.50 bc
T. basicola (0.1%)	4.30 a	3.80 a	4.00 a
T. basicola (1%)	4.20 a	4.00 a	4.00 a

[1], [2], [3]: For explanation see Table 6-3-2.

[4]: For details see Table 6-3-1.

D. Shoot lengths and dry weights of transplanted plants

Plants in the microplots containing the high inoculum level of Cy. scoparium (1%) were dead during the first 4 weeks after transplanting. The shoot lengths of surviving plants from microplots inoculated with the lower rate of Cy. scoparium (0.1%) were shorter than those of plants from control microplots on most occasions (Table 6-3-7 A); they had also produced less dry matter than controls at all harvests (Table 6-3-7 B).

Table 6-3-7. Mean height (A) and dry weight (B) of shoots per red clover plant on 6 occasions after transplanting 5-week-old plants into the microplots.

A. Height (cm)

Weeks after planting	Fungi and inoculum level[1] C	CII	Cs (0.1%)	Cs (1%)
13	35.3 a[2]	30.6 a	15.9 b	---[3]
39	46.6 a	39.0 a	29.9 b	---
47	56.7 a	55.9 a	38.9 b	---
52	54.0 a	60.0 a	53.9 a	---
60	55.2 a	56.0 a	42.4 b	---
69	30.3 a	24.0 a	22.5 a	---

B. dry weight (g).

Weeks after planting	Fungi and inoculum level[1] C	CII	Cs (0.1%)	Cs (1%)
13	17.4 a[2]	13.0 a	2.6 b	---[3]
39	37.1 a	27.3 a	7.6 b	---
47	72.2 a	69.4 a	23.7 b	---
52	71.8 a	72.0 a	30.2 b	---
60	65.1 a	63.4 a	17.4 b	---
69	75.0 a	78.2 a	16.5 b	---

[1], [2], [3]: For explanation see Table 6-3-2.

6.3.4 Discussion

Seedling establishment was reduced by Cy. scoparium, T. basicola, and F. solani both in the glasshouse (Fig. 6-3-1) and field microplots (Fig. 6-3-2). Stand reduction caused by F. solani in the glasshouse has been reported elsewhere (Kilpatrick et al., 1954; Fulton and Hanson, 1960; Chi, 1965; Elliott et al., 1969).

However, the effects of T. basicola have been less well documented

although there have been brief reports of the possible role of Thielaviopsis basicola (= Trichocladium basicola) (Hey, 1946), or a species of Thielaviopsis (Tverskoiet al., 1950) in red clover seedling failure. Effects of Cy. scoparium on seedling survival of red clover have not been previously reported, but isolates from spruce (Bugbee, 1962; Bugbee and Anderson, 1963), and Agonis flexuosa (Willd.) Schau. (Bertus, 1976), caused damping-off of red clover and/or lucerne in host range tests. Cylindrocladium scoparium was the most virulent of the three seedling pathogens from roots of red clover and it killed seedlings in the pots rapidly (Fig. 6-3-1). Such rapid killing may reflect the presence in the pots of conditions favourable for development of the pathogen (Experiment 2).

The acetylene reduction test provides a direct method of measuring nitrogenase activity, which reflects the capacity of nodules to fix atmospheric nitrogen (Hardy et al., 1968). This technique has not been previously employed to assay changes in nitrogen fixation of forage legumes resulting from infection by soilborne fungi but it has been used to study effects of infection of white clover mosaic virus on red clover (Khadhair et al., 1984). There was some indication in the present results that the nitrogenase activity of red clover was reduced by T. basicola (8 weeks; Table 6-3-2 A). It is known that ethylene can be produced by soil micro-organisms (Lieberman, 1979), or by plant tissues in response to various stress factors (Primrose, 1979; Boller, 1988). This possible source of error was eliminated by use of appropriate controls.

Nodulation of red clover as well as ethylene production was reduced by T. basicola (Table 6-3-2 B). This effect commonly occurs following virus (Guy et al., 1980; Tu and Holmes, 1980; Khadhair et al., 1984), or fungal (Sawada, 1983) infection on forage legumes. Reduced nodulation may have been associated with the rotting of the tap and lateral roots by T. basicola (Table 6-3-6), or changes in root exudate which reduced the attraction of Rhizobium to the roots (Vance and Johnson, 1981; Tu and Ford, 1984). Reduction in nodule number can often be accompanied by a compensatory increase in nodule size (Tu et al., 1970; Nutman, 1958). Thus the production of similar amounts of ethylene by roots of red clover infected with T. basicola and controls (Table 6-3-2 A) at 12 weeks reflects greater activity by nodules on diseased roots.

The discrepancy between ethylene production (Table 6-3-2 A) and numbers of nodules (Table 6-3-2 B) supports the suggestion that nodule number is not a good index of the benefit which the host is receiving from the Rhizobium symbiosis (Burton, 1985), and as a single factor is an inadequate indication of the effect of fungal invasion on nitrogen fixation.

The survival of plants infected with Cy. scoparium or T. basicola was poor. In the present experiment and Experiment 6 the stands declined most rapidly during the first 20 weeks. This suggested that plants were very vulnerable at early stages of their growth. Infected plants which survived beyond this stage mainly relied on the development of lateral and adventitious roots. This seems to be a common situation for red clover whose tap roots have completely rotted (Taylor et al., 1962; Cressman, 1967; R.A. Skipp and M.J. Christensen, pers. comm.), and it also has been seen on subterranean clover (Barbetti et al., 1986 a). The ability of red clover to produce vigorous lateral roots and adventitious roots when the tap roots are destroyed could be a useful index for selection of more persistent plants.

Productivity of red clover was reduced markedly by both Cy. scoparium and T. basicola (Table 6-3-3, 6-3-4, 6-3-7, Fig. 6-3-3, 6-3-4) as found in previous studies (Skipp et al., 1986; Experiment 6). Yield reduction also occurred in microplots inoculated with F. oxysporum and F. solani (Table 6-3-4 and Fig. 6-3-3 and 6-3-4) in contrast to Experiment 6 where a different inoculation method had been used. Sowing seeds into soil inoculated with corn kernels bearing the fungal inoculum allowed the plants to have more prolonged exposure to the pathogen than following the root dipping method.

Incorporation into soil of fungal inoculum grown on cereal grains or the seeds of other plants has been commonly used in studies on effects of soilborne pathogens on plant growth (Chapter 2). In most of these studies, only unamended soil has been used for the control. In the present experiment, a second control, soil + sterilised corn kernels (Control II), was introduced. Addition of sterilised corn kernel medium to fumigated soil caused a reduction in the growth of red clover plants in comparison with the unamended control. The effect was particularly marked during the early stage of growth (Table 6-3-3 and 6-3-4). It is unlikely that the soil properties had been changed by adding the small amount

of corn kernel medium (B.E. Clothier pers. comm.), and a more probable explanation was that the medium provided a substrate for the growth of micro-organisms which inhibited growth of red clover seedlings. A separate experiment showed that the corn kernel medium became heavily colonised by fungi such as species of Mucor and Penicillium. These fungi can cause discoloration of red clover seedlings (Fezer, 1961) and affect plant growth at an early stage (Lynch, 1976). This seems to be a common problem when nutrient substrate is added to the soil (Ostazeski, 1957; Pettit et al., 1969), and raises the question of what are the most valid controls for inoculation experiments. Presumably the corn kernel medium used in Control II was more readily colonised by microbial contaminants than that present in the inocula because: (a) there were no fungal competitors already present and (b) nutrients had not been depleted by fungal inoculum. Thus it seems likely that growth medium effects would have been greater in the control II than in the inoculum treatments. Comparison of data from inoculum treatments with the control lacking nutrients would appear to be more appropriate than with Control II. More suitable inoculum and controls would result from addition of minimal amounts of nutrients to the soil.

Of the fungi isolated from the roots of control plants at the termination of the experiment, some were common root invaders of ryegrass and white clover (Skipp and Christensen, 1989 a and b), and others were ubiquitous rhizosphere fungi. It is likely the source of contamination was root debris from the non-fumigated area of the block (originally a ryegrass-white clover pasture; Chapter 3), which over a long period of exposure could have been spread to the control microplots by wind, rain splash, or during activities of field management etc. Fungal invasion of roots of control plants grown in the field seems almost unavoidable. The emphasis of this type experiment should be on successful establishment of the test fungi rather than the complete sterility of control plants.

The field microplot system used in the present experiment, although laborious, combines the advantages of the traditional glasshouse and field study methods, which allows for the precise experimental control of the inoculum level, with growth of plants under natural field conditions. Most previous work in which microplots have been established in clay tiles concerned annual crops (Martin et al., 1982; Rowe et al., 1985; Riedel et al., 1985; Francl et al., 1987), or perennial

crops grown for one season (Caradus and Woodfield, 1986; Woodfield and Caradus, 1987). In the present experiment, red clover plants were grown in the microplots for 76 weeks. A yield comparison detected no difference between plants grown inside and outside microplots at a late stage of the experiment (Table 6-3-5). This indicated that the tiles did not restrict plant growth and development, and that this type of microplot could be used for longer term studies.

In conclusion, the present experiment demonstrated that the seedling establishment, survival, and productivity of red clover were adversely affected by the major root-invading fungi of red clover from the plant breeder's evaluation block. Nodulation and nitrogen fixation were also affected by one of the fungi .

CHAPTER 7

EXPERIMENT EIGHT: LIGHT AND ELECTRON MICROSCOPY STUDIES ON INVASION OF RED CLOVER ROOTS BY TRICHOCLADIUM BASICOLA, AND EFFECTS OF BENOMYL AND PROCHLORAZ.

7.1 INTRODUCTION

Trichocladium basicola attacks roots of a wide range of plants including the forage legumes, red clover (Skipp et al., 1986), lucerne (Close et al., 1982), birdsfoot trefoil (Ostazeski, 1966), and sainfoin (Sears et al., 1975) and it was a major pathogen of red clover roots in the plant breeder's evaluation block (Chapters 4 - 6). As discussed in Chapter 2, Light microscopy and scanning electron microscopy studies have been carried out on invasion by T. basicola of roots of plants other than red clover, but little information is available on host cell reaction to the presence of the fungus.

Application of prochloraz, and to a lesser extent benomyl, to field plots in the plant breeder's evaluation block reduced invasion of roots by T. basicola and some other fungi (Experiment 5). Ultrastructural studies should help provide an understanding of the effects of fungicides on the fungus, and the host/fungus relationship. Ultrastructural studies have been carried out on effects of benomyl on fungi in culture and in host plants (see Chapter 2), but no information is available on its effect on T. basicola, or on the effects of prochloraz on the fine structure of any fungus.

The objectives of this experiment were to provide information using both light microscopy and transmission electron microscopy on : -

1. invasion of red clover roots by T. basicola and reaction of host cells to fungal invasion.

2. effects of prochloraz and benomyl on red clover seedlings, and T. basicola.

7.2 MATERIALS AND METHODS

7.2.1 Fungus

An isolate of T. basicola obtained from red clover roots during a previous experiment was used. Cultures were established from a single endoconidium and maintained on PDA at 20°C in the dark.

7.2.2 Fungicides

Prochloraz (Sportak 45% EC, FBC Ltd) and benomyl (Benlate 50% WP, Du Pont Ltd) were used in the experiments. In the study of effects of fungicides on fine structure of the fungus grown in vitro, the concentrations used were 0.01 and 0.06 μg a.i. per ml of culture medium for prochloraz and benomyl, respectively. In the study of effects of fungicides on fungal invasion and development in the host, the concentrations used were 1 and 10 μg a.i. per ml of culture medium for prochloraz, and 10 μg a.i. per ml of culture medium for benomyl.

7.2.3 Seedlings and inoculation

Seedlings were produced following the method of Christensen et al. (1988). Plates (9 cm diam.) of 2% water agar, with or without fungicides were prepared. Ten surface-sterilised red clover seeds (10 min in 0.2% sodium hypochlorite solution, followed by five rinses in sterile water) were placed on the surface of the agar in a row across the centre of each plate. Plates were bound together with adhesive tape in batches of 5 and placed on edge in a constant environment cabinet (20°C and 16 h light/day at 46.7w/m^2 light intensity) with the rows of seeds aligned horizontally to ensure that the roots of the germinating seedlings grew across the surface of the agar towards the edge of the plate.

Fungal cultures were inoculated by streaking PDA plates with a suspension

of endoconidia and incubated at 20°C in the dark for 8 - 10 days. Endoconidia were harvested by flooding the surface of the medium with sterile distilled water. The endoconidial suspension was filtered through four layers of sterilised muslin and the concentration adjusted to 10^6 spores per ml.

Five days after sowing, seedlings of uniform length, and free from microbial contamination, were collected, rinsed with sterilised water (5 changes), and blotted dry with sterilised filter paper. The seedlings were inoculated by dipping the roots in the endoconidial suspension for 30 min, and then were transferred to water agar plates (5 seedlings per plate). The plates were bound and replaced in the constant environment cabinet.

7.2.4 Effects of the fungicides on the fine structure of the fungus grown in culture

Plates containing corn meal agar (CMA Difco), with or without fungicide, were prepared as described in Experiment 3. They were inoculated with three, 4 mm diam. agar plugs taken from the margin of 10-day-old fungal colonies on PDA medium, and incubated in the dark at 20°C for 9 days. Small pieces (approx. 2 x 5 mm) of agar and mycelium were taken from the colony margin and processed for transmission electron microscopy (see below).

7.2.5 Assessment of disease symptoms

Areas of lesions on roots of 20 seedlings per treatment were measured 5 and 8 days after inoculation, and results were expressed as the percentage of the root surface covered by the lesions.

7.2.6 Sampling for histological studies

For studies on conidial germination and penetration by light microscopy of cleared and stained whole roots, 5 roots were sampled per treatment, 16 and 24 h after inoculation. For studies on fungal development within the host by light

and transmission electron microscopy 5 roots per treatment were sampled 1, 3, 5, and 8 days after inoculation. Sampled roots were cut into 1 cm length segments before processing (see below), the top 1 cm segment of each root being used for quantitative studies.

7.2.7 Light microscopy

7.2.7.1 Conidial germination and penetration

Each 1 cm length segment of red clover root was placed in a well of a Milton-Boerner Micro Test Slide, and covered with a combined clearing/staining solution overnight. The solution contained 0.005 g Trypan blue (Aldrich), 15 ml distilled water, 45 g chloral hydrate, 15 g phenol, 12.5 ml 90% lactic acid ,and 30 ml 95% ethanol (R.A. Skipp, pers. comm.). The stained segments were placed on microscope slides and mounted in lactic acid - glycerol solution (lactic acid : glycerol : water, 1:2:1 by weight).

The total number of endoconidia, and their % germination, was determined for each segment examined. After segments were removed from the staining wells, the number of endoconidia remaining in the Trypan blue solution, and their germination was also determined. Results are presented as percentage germination of all endoconidia per treatment. Numbers of penetrations are expressed as a percentage of the total number of germinated spores per segment. The proportion of penetration sites at which papillae were formed in host cells is expressed as a percentage of the total number of penetrations per segment. Twenty hyphae within the tissue of each segment were further examined and the number of cells per hypha counted.

7.2.7.2 Fungal development within the host

Segments were processed using a modification of a method developed for clearing and staining ryegrass leaves for examination of rust fungi (R.A. Skipp, pers. comm.). The roots were cleared for 1 - 2 days in the above clearing solution

(from which Trypan blue was omitted). After clearing, the segments were stained in a solution of chlorazole black (Sigma, 1 mg/ml) in chloral hydrate (chloral hydrate : distilled water, 2.5 : 1 by weight) for 6 - 8 h, rinsed in 50% ethanol and destained in chloral hydrate for 2 h before mounting on a microscope slide with lactoglycerol.

Numbers of endoconidiophores and chlamydospores per 1 mm length of root were counted on three 1 mm length units per segment. Roots were observed using a Leitz Dialux 20 microscope conjoint with a Leitz Combiphot - automatic system camera. Other observations were made with a Reichert microscope fitted with a differential interference contrast lens.

7.2.8 Transmission electron microscopy (TEM)

Small pieces (0.5 - 1 mm) of root tissue, or agar medium bearing fungus, were fixed in 3% glutaraldehyde + 2% formaldehyde in 0.1 M phosphate buffer (pH 7.2) (Karnovsky, 1965) for 2 - 3 h at room temperature. Vacuum-infiltration was used if the specimens tended to float; specimens were transferred to fresh primary fixative immediately after vacuum-infiltration. The primary fixed specimens were washed three times for 30 min in buffer and post-fixed for 0.5 - 1 h in 1% osmium tetroxide (OsO_4) at room temperature. Following three buffer washes, the fixed material was dehydrated in a graded acetone series (25%, 50%, 75%, 95%, 100%, 100%), infiltrated with Polarbed 812 epoxy resin, and cured in silicone rubber moulds for 48 h at 60°C to complete embedding.

One μm sections for light microscopy were cut using a glass knife, and transferred to a drop of distilled water on a microscope slide. The slides were placed on a hot plate to evaporate the water drop and cause sections to adhere to the glass surface. A drop of 0.05% toluidine blue in 0.1 M phosphate buffer (pH 7.2) was added, and the slides heated at approximately 80 - 90°C for 10 sec. For TEM, sections (ca. 90nm; silver to pale gold interference colour) were cut with a DDK diamond knife on a ultramicrotome (Reichert Ultracut E) and mounted on 400 mesh copper grids. Grid-mounted sections were double-stained for 5 - 7 min with saturated uranyl acetate in 50% ethanol, followed by lead citrate (Venable

and Coggeshall, 1965), washed with distilled water and examined with a transmission electron microscope (Philips EM 201 C).

7.2.9 Statistical analysis

Analysis of variance was carried out after square root transformation. Means were compared among different treatments for each sampling time using an LSD test.

7.3 RESULTS

7.3.1 Invasion of red clover roots by T. basicola and effects on host cells

7.3.1.1 Course of infection

The sequence and timing of infection by T. basicola of both chemical-treated (benomyl or prochloraz) and non-treated red clover seedlings was similar. The account refers to both unless otherwise stated.

Most conidia germinated within 24 h to produce a short germ tube. This either penetrated an epidermal cell directly by means of a slender penetration peg, without the formation of appressorium (Plate 7-1 a), or branched dichotomously to produce two hyaline hyphae. The hyphae grew on the root surface for some distance, then either penetrated epidermal cells as described previously, or became superficial, ramifying mycelium. Some conidia developed a cylindrical or knob-like structure (Plate 7-1 b, c) instead of producing a short germ tube. These structures produced superficial hyphae, penetrating hyphae and/or endoconidiophores (Plate 7-1 d).

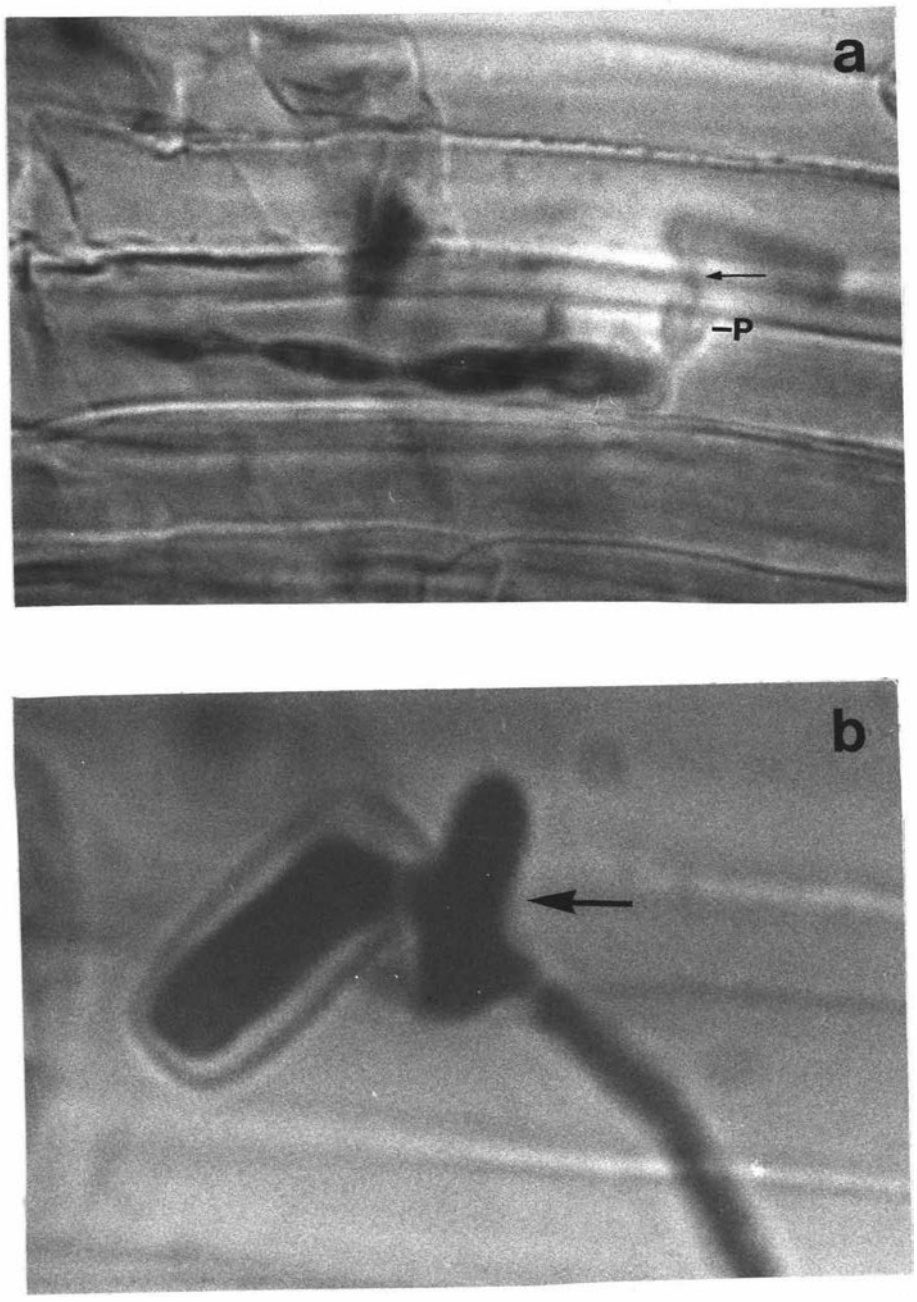


Plate 7-1. a - d. Germination and development of endoconidia on the root surface.

a. A slender penetration peg has developed from an endoconidium on the root surface (arrow), and directly penetrated the epidermal cell. Note the papilla structure (P) and a chain of beaded fungal cells within the tissue (16 h, prochloraz 1 μg/ml, x 1475).

b. A swollen cylindrical structure (arrow) has arisen from an endoconidium and a long hyaline hypha has grown across the root surface (1 day, control, x 3200).

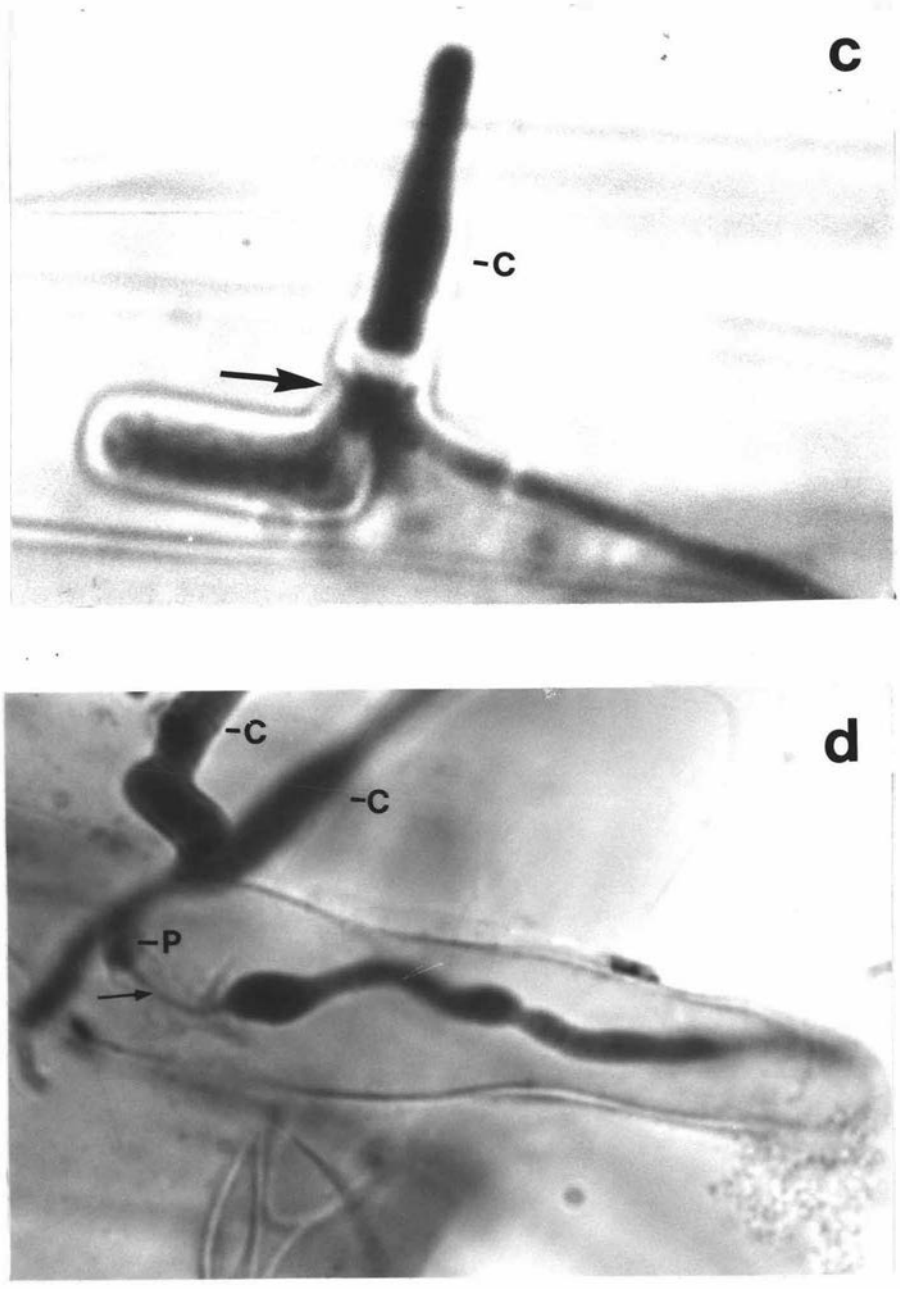
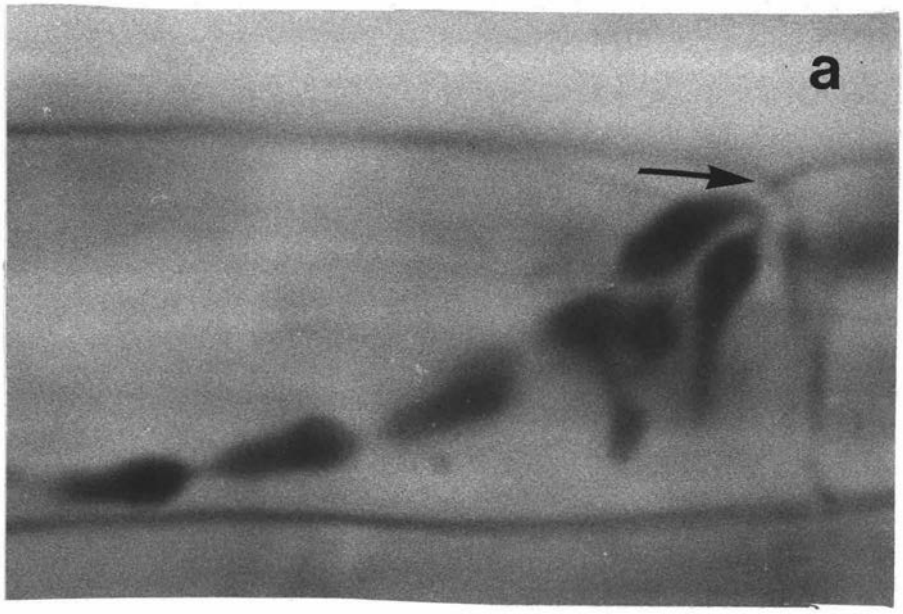


Plate 7-1 cont. **c.** A short endoconidophore (C) and long, septate hypha developed from a knob-like structure (arrow) on an endoconidium on the root surface (1 day, control, x 2560).

d. An endoconidium has germinated on the root surface to form two endoconidiophores (C) and a hypha. A penetration peg (arrow) arising from the hypha has penetrated the root hair and formed an intracellular hypha. Note the papilla (P) surrounding the penetration peg (1 day, control, x 1525).

After penetration, the tip of the penetration peg enlarged into a pyriform, or sometimes a spherical structure, from which developed a chain of thin-walled cells which were constricted at their septa (Plate 7-2 a). These were the characteristic "beaded hyphae" of T. basicola (Christou, 1962). The average diameter of beaded hyphae was 3.0 μm (2.3-4.2 μm). The ultrastructure of the cytoplasm and cell wall of the intracellular beaded hyphae (Plate 7-2 b) was similar to that described by DelVecchio et al. (1969) . Beaded hyphae continued growth by increasing the number of cells along their length, and by profuse branching. Branching also occurred at the base of the first cell formed after entry of the penetration peg (Plate 7-2 a). There were up to 13 cells per beaded hypha 24 h after inoculation. The hyphae eventually filled the whole lumen of a host cell (Plate 7-2 c). When a beaded hypha penetrated an adjacent host cell wall, it often became closely appressed to the host cell and formed a blunt infection peg which penetrated the host cell wall (Plate 7-3 a). The wall of penetrating hypha within the host cell wall was indistinct, sometimes with an irregular outline. Slight dissolution of the host cell wall occurred around the penetrating hypha (Plate 7-3 b). Penetration of walls occurred in a similar manner in cells which were moribund (Plate 7-3 c).

As development of beaded hyphae proceeded, they became narrower, and were less obviously constricted at the septa. The average diameter of the straight, non-constricted hyphae which developed from the beaded hyphae was 1.9 μm (1.4-2.5 μm). They had a similar ultrastructure to the beaded hyphae and grew singly in the cortical cells parallel to the long axis of the root. Neighbouring cortical cells were penetrated following formation of slender penetration pegs (Plate 7-4 a) which gave rise to beaded hyphae in the cell lumen as described previously. Thus, invasion by the fungus increased both in area and depth.



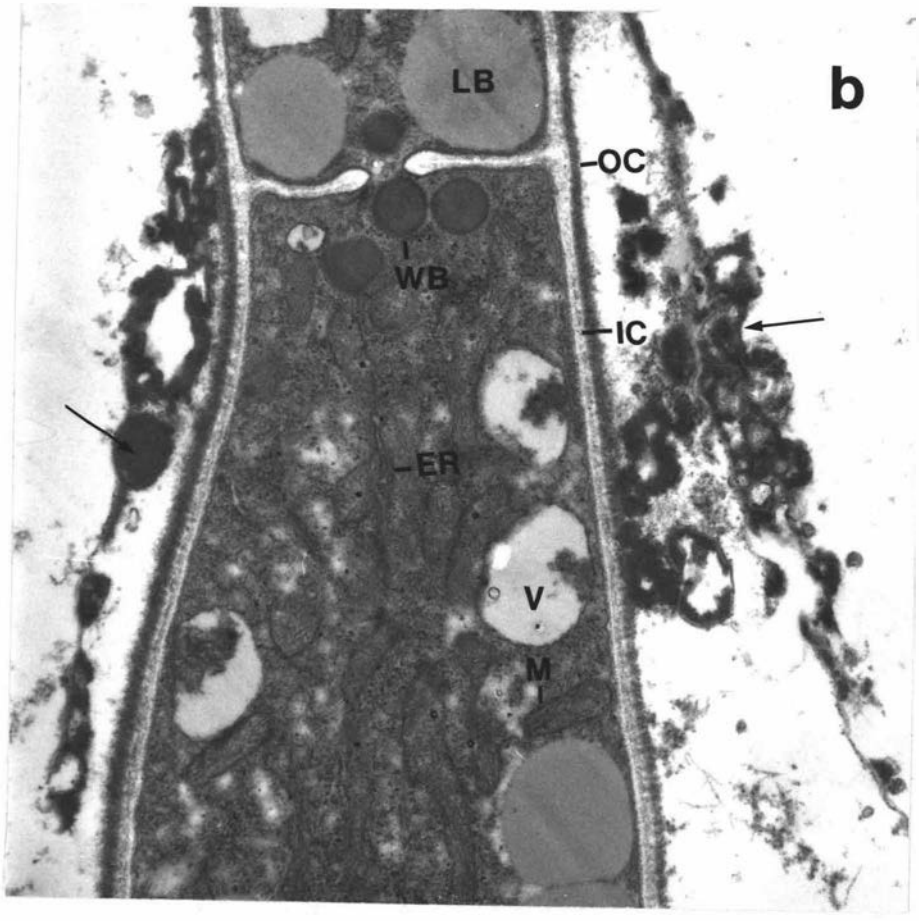


Plate 7-2. a - c. Development of intracellular beaded hyphae.

a. A pyriform cell has formed in an epidermal cell at the tip of a penetration peg (arrow), and produced several other cells each constricted at the septum to form a typical beaded hypha (1 day, benomyl 10 μg/ml, x 905).

b. A septate beaded hypha inside a cortical cell. The cell wall is composed of an electron-dense outer layer (OC) and an electron-lucent inner layer (IC). The cytoplasm contains mitochondria (M), vacuoles (V), and endoplasmic reticulum (ER). Woronin bodies (WB) occur near the septum, close to the septal pore. Note the disorganisation of cytoplasm (arrows) of the host cell (5 days, control, x 20000).

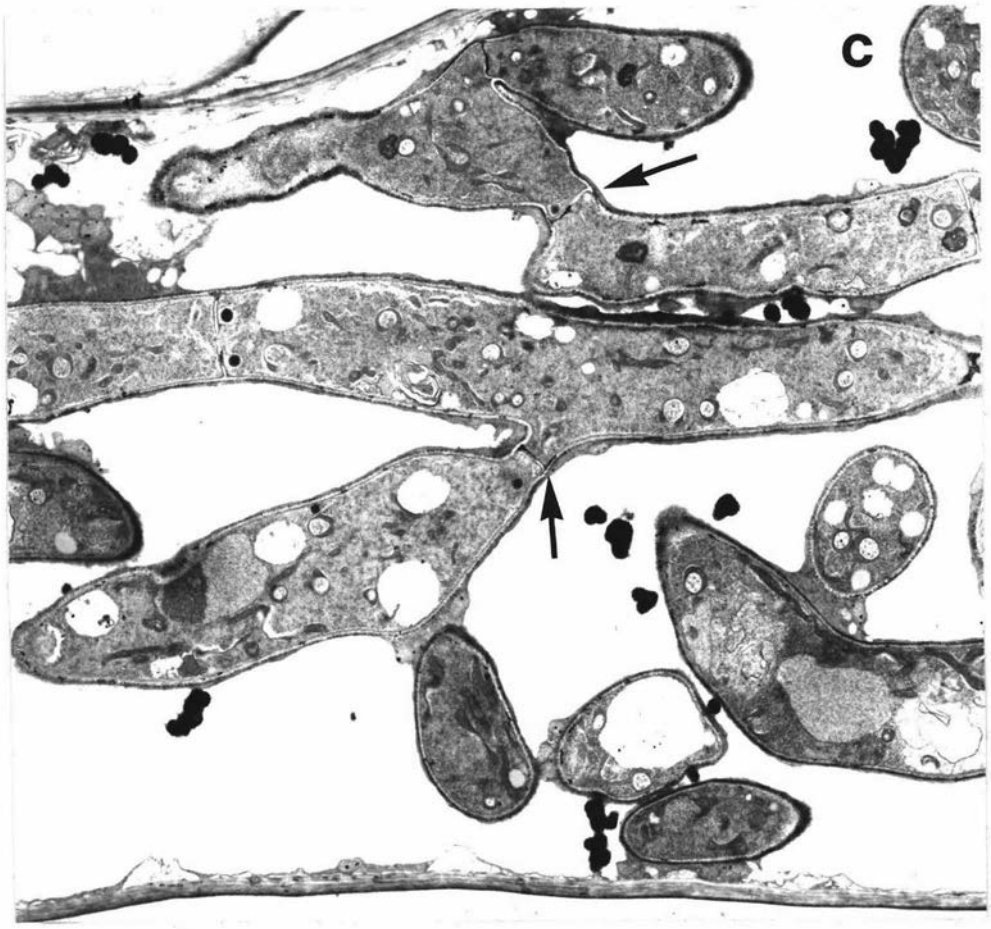


Plate 7-2 cont. **c.** Beaded hyphae filling the lumen of a cortical cell. Note constrictions in hyphae at septa (arrows) (8 days, prochloraz 10 μg/ml, x 5000).

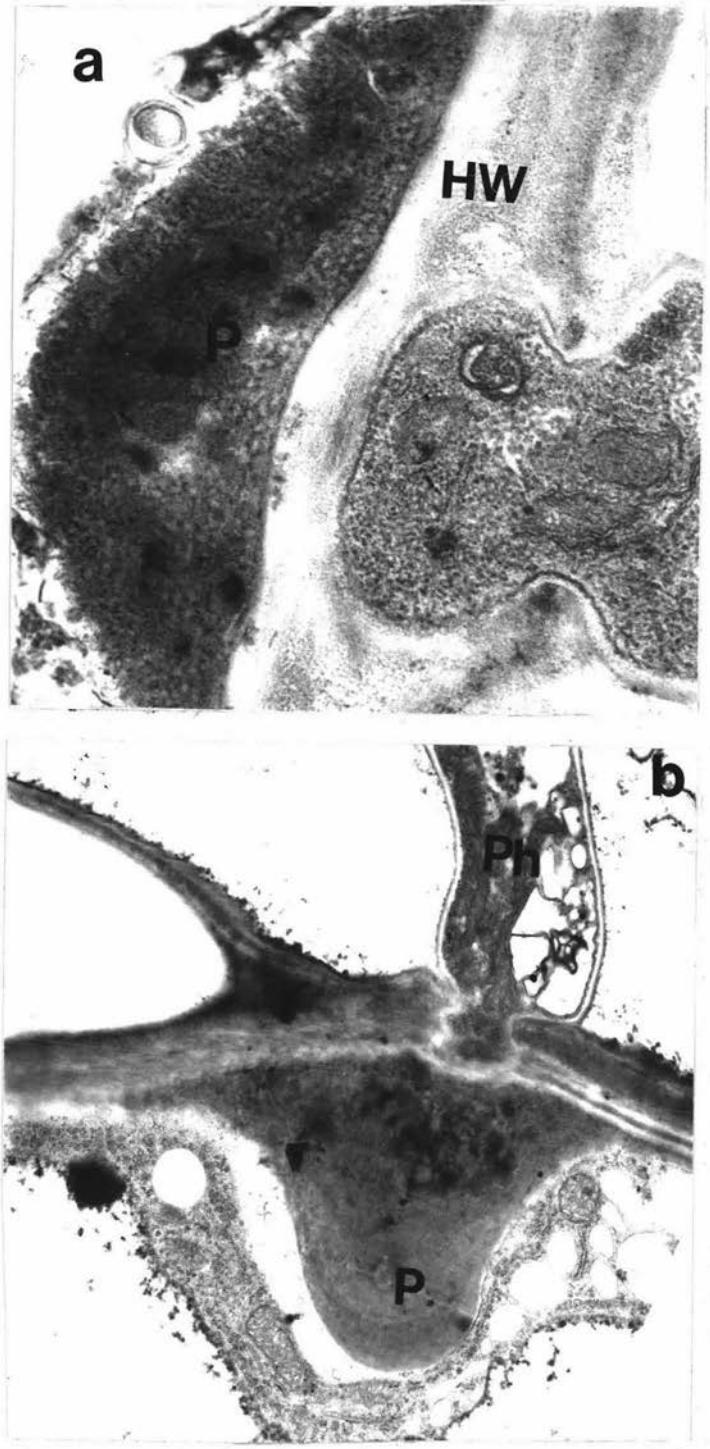


Plate 7-3. a - c. Penetration of adjacent cells.

a. A blunt penetration peg in the process of penetrating a host cell wall (HW). A hemispherical papilla (P) has formed (3 days, control, x 31800).

b. A penetration peg with irregular outline originating from a hyphal tip (Ph). Dissolution of the host cell wall has occurred around the penetration peg. Note the regions having different staining properties in the papilla (P) (1 day, prochloraz 10 μg/ml, x 11200).

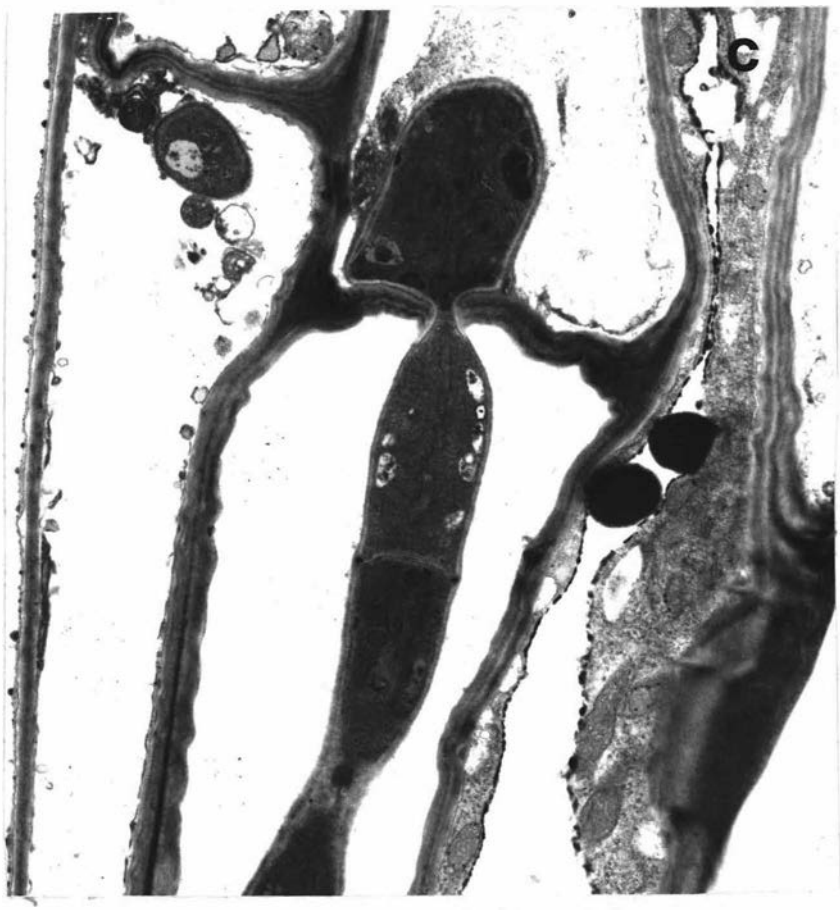


Plate 7-3 cont. c. An intracellular hypha passing through a cell wall into an adjacent cortical cell. The hypha is constricted at the penetration site, attains normal dimensions soon after leaving the cell wall, and forms a beaded hypha in the newly invaded cell. Note disappearance of the host cell contents and absence of a papilla at the penetration site (3 days, control, x 8700).

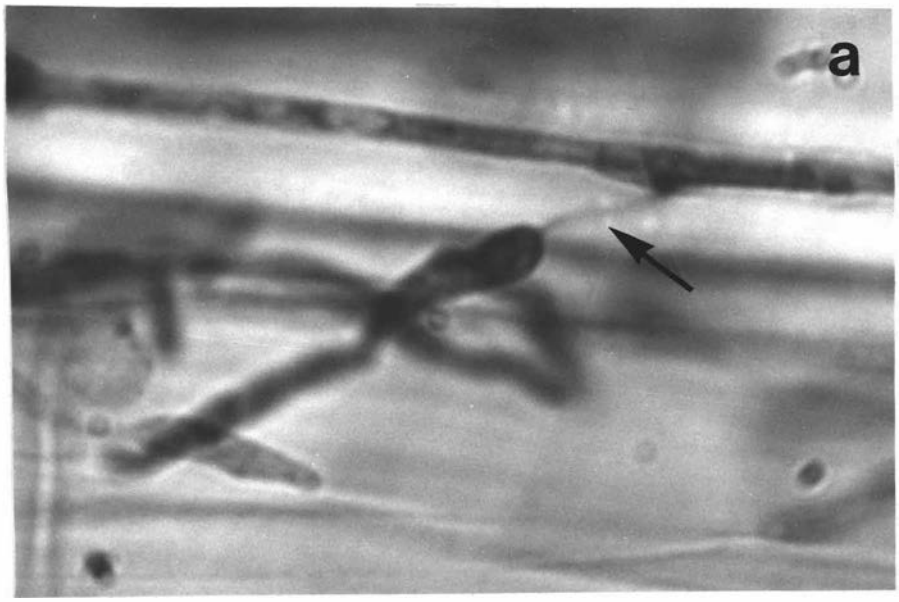
Three days after inoculation, a third type of hypha was seen to have developed from the intracellular straight hyphae. This was non-constricted, thick-walled, brown in colour, and was much wider (average diameter 4.7 μm, 2.8 - 8.5 μm) than the other two types of hypha. The thicked-walled, pigmented hyphae grew both horizontally and vertically within the cells. Eventually, they emerged from the root and formed endoconidiophores and chlamydospores on the root surface. These hyphae were named reproductive hyphae (Christou, 1962). No intercellular hyphae or endoconidiophores were observed within plant tissues. Five to eight days after inoculation, root surfaces were covered with endoconidia and chlamydospores (Plate 7-4 b). However, even at this advanced stage of infection, only a few chlamydospores had been formed within the plant tissue. They were smaller (usually 2 -3 cells) than those formed on the surface and occurred singly rather than in clusters (Plate 7-4 c).

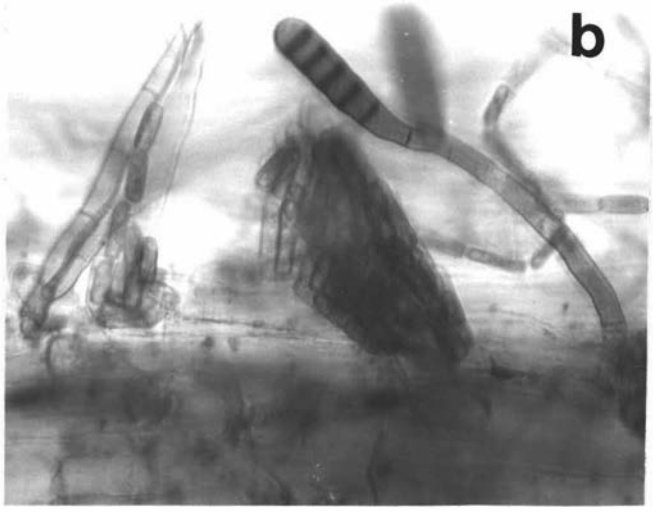
All regions of the root were invaded by T. basicola except the root cap. Root hairs were sometimes infected. Initial penetration took place at the junction between the root hair and the tap root surface, shortly after the conidium had germinated (Plate 7-1 d).

Tissues colonised by the fungus included the epidermis, cortex and endodermis. Occasionally, hyphae were detected in the vascular system.

7.3.1.2 Reaction of host cells to fungal invasion

Where a fungal hypha was closely appressed to the wall of a host cell, as during attempted to penetration (see above), the plasmalemma of the host cell became irregularly invaginated (Plate 7-5 a). Varying amounts of wall apposition material were seen between the plasma membrane and cell wall at individual sites of penetration. Continued accumulation of apposition material resulted in the formation of hemispherical (Plates 7-3 a, 7-5 b) or conical (Plate 7-3 b) papillae. Layers could be distinguished within papillae, differing in texture and staining properties, which probably indicated successive deposition of material of differing composition (Plate 7-5 b).





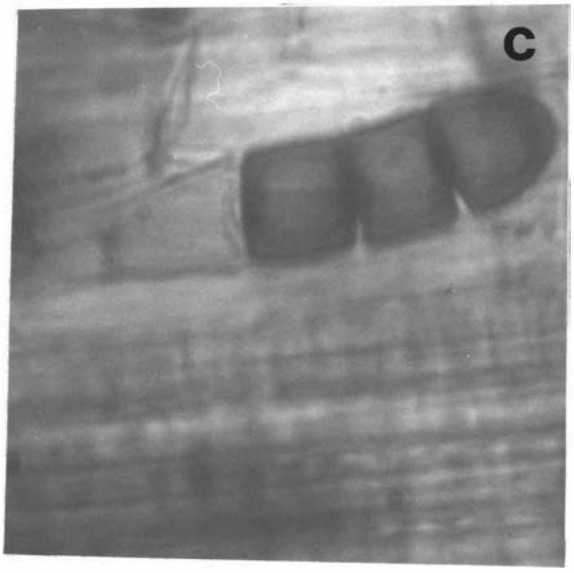


Plate 7-4. a - c. Formation of intracellular straight hyphae, endoconidia and chlamydospores.

a. A straight hypha in the upper cell has produced a slender, peg-like structure (arrow), which developed into beaded hypha in the lower cell (3 days, control, x 1640).

b. Masses of endoconidia and chains of chlamydospores on the root surface. Note the chain of chlamydospores is composed of five thick walled dark cells plus 1 thin walled hyaline basal cell (5 days, control, x 425).

C. A chlamydospore has formed within cortical tissue close to the vascular system, and appears to be separating. Note the spore has only three brown cells and one basal cell (8 days, control, x 2560).

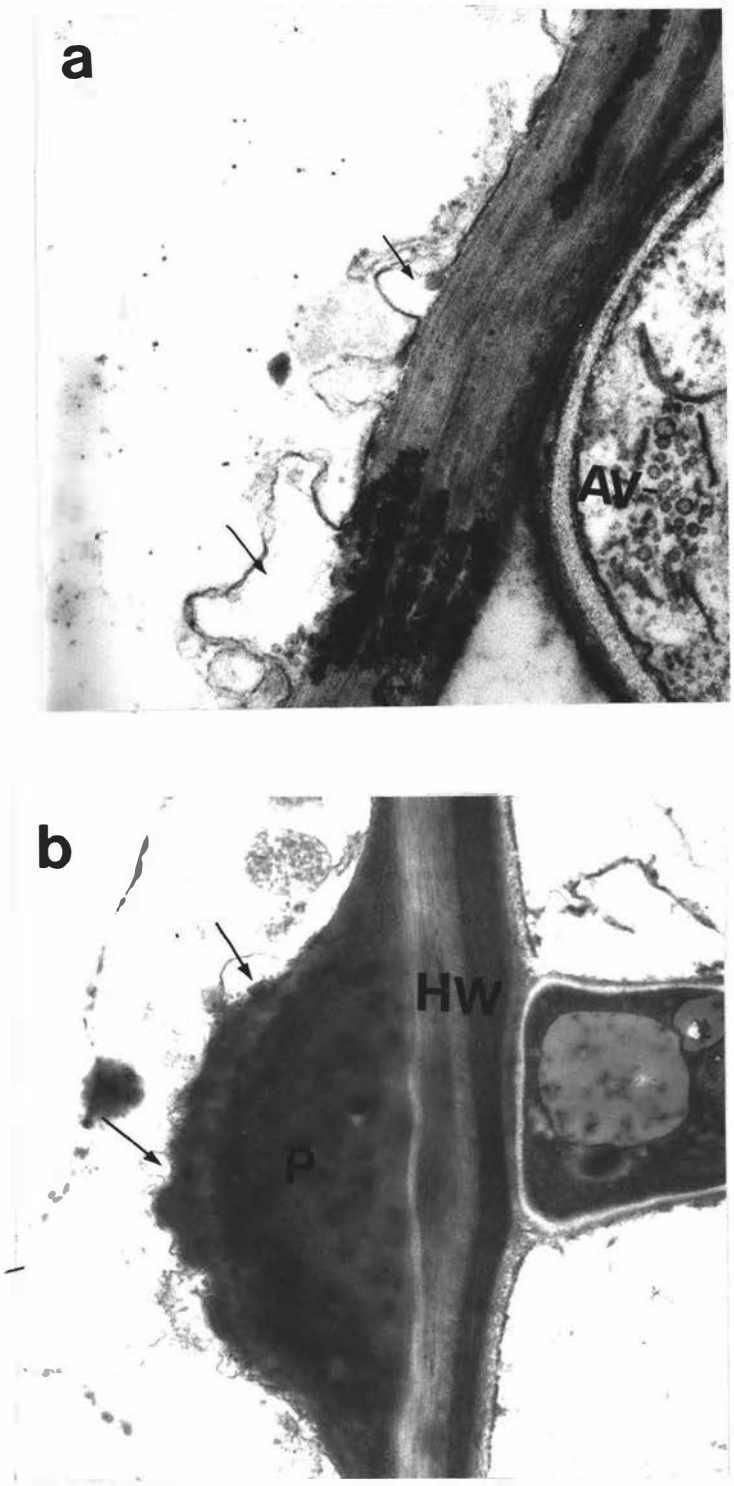
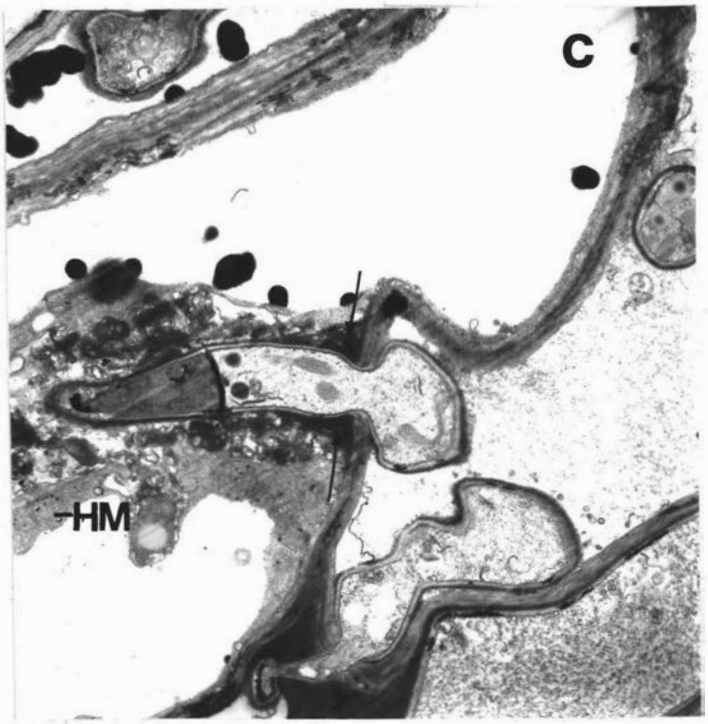


Plate 7-5. a - d. Response of host cells to the fungal invasion.

a. A host cell wall adjacent to a closely appressed hypha showing invagination of the plasma membrane in the adjacent cell (arrows). Note the presence apical vesicles (AV) at the hyphal apex (8 days, control, x 48600).

b. A hemispherical papilla (P) with a layered structure has formed at a point where a hypha is abutting the wall (HW) in the adjacent cell. Note the invagination of the plasmalemma around the papilla (arrows) (3 days, control, x 21200).



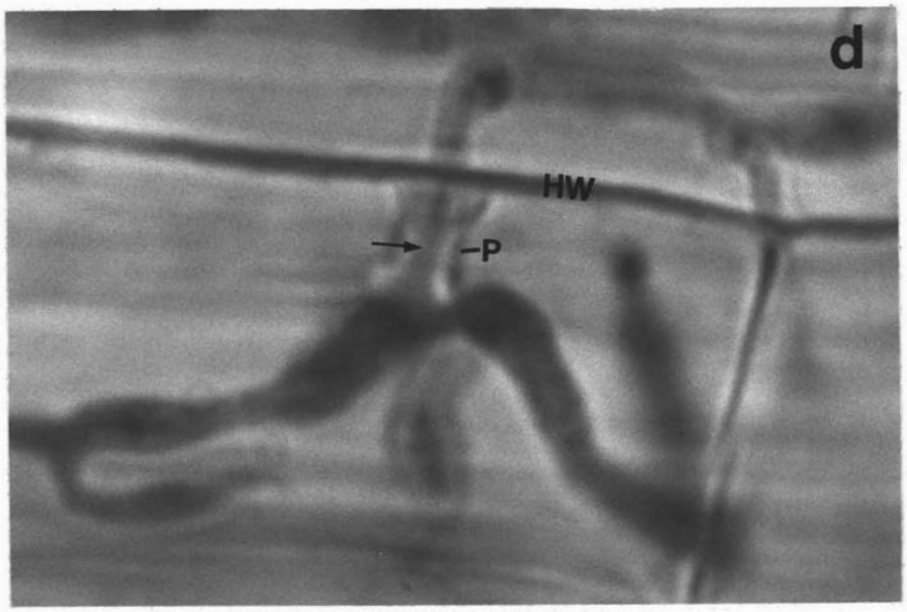


plate 7-5 cont. **c**. Site of fungal penetration between 2 cortical cells. In the newly-invaded cell the hypha is surrounded by cytoplasm containing abundant mitochondria (HM) and vesicles. Note the presence of a matrix layer (arrows) consisting of electron-dense papilla material at the penetration site (8 days, control, x 7800).

d. A papilla (P) has been penetrated by a slender hypha (arrow). A cluster of beaded hyphae in a digitate form have developed in the newly invaded cell (5 days, control, x 2560).

Although papilla formation was common in invaded host cells, it did not seem to halt invasion. Narrow hyphae grew through the deposit then emerged and expanded within the cell lumen (Plates 7-3 d, 7-5 c, d).

At an early stage of infection, the cytoplasm of host cells containing an infection hypha appeared similar to that in uninfected cells (Plate 7-6 a), forming a thin dense layer lining the fungal cell wall (Plates 7-3 d, 7-6 b). In some cells, the invading hypha was surrounded by a mass of host cell cytoplasm, which had become rich in mitochondria and vesicles (Plate 7-6 c). Such changes appeared to be associated with papilla deposition. Approximately 24 h after inoculation, breaks became visible in the tonoplast and plasmalemma of infected cells, which suggested that cell contents had began to degenerate (Plate 7-6 d). Three days after inoculation, cell contents had become greatly disorganised and membranes of host nuclei had broken (Plate 7-6 e). By 5 days, the contents of invaded host cells had become completely disorganised, forming an electron-dense matrix in which no organelles could be recognised (Plate 7-6 f).

Each infected cell followed a similar sequence of events; an initial tolerance of apparently benign parasitism followed by gradual degeneration and finally cell death. Thus in one cell could have disorganised cytoplasm with only membranous debris remaining, while an adjacent cell retained the ultrastructural characteristics of uninfected, or recently- infected cells (Plate 7-6 g).

Macroscopic symptoms first became evident 3 days after inoculation as scattered light brown flecks on the root surface. The flecks coalesced into dark brown to black lesions 5 - 8 days after inoculation. The outer epidermis often sloughed off and sometimes the whole cortical tissue collapsed (Plate 7-6 h).

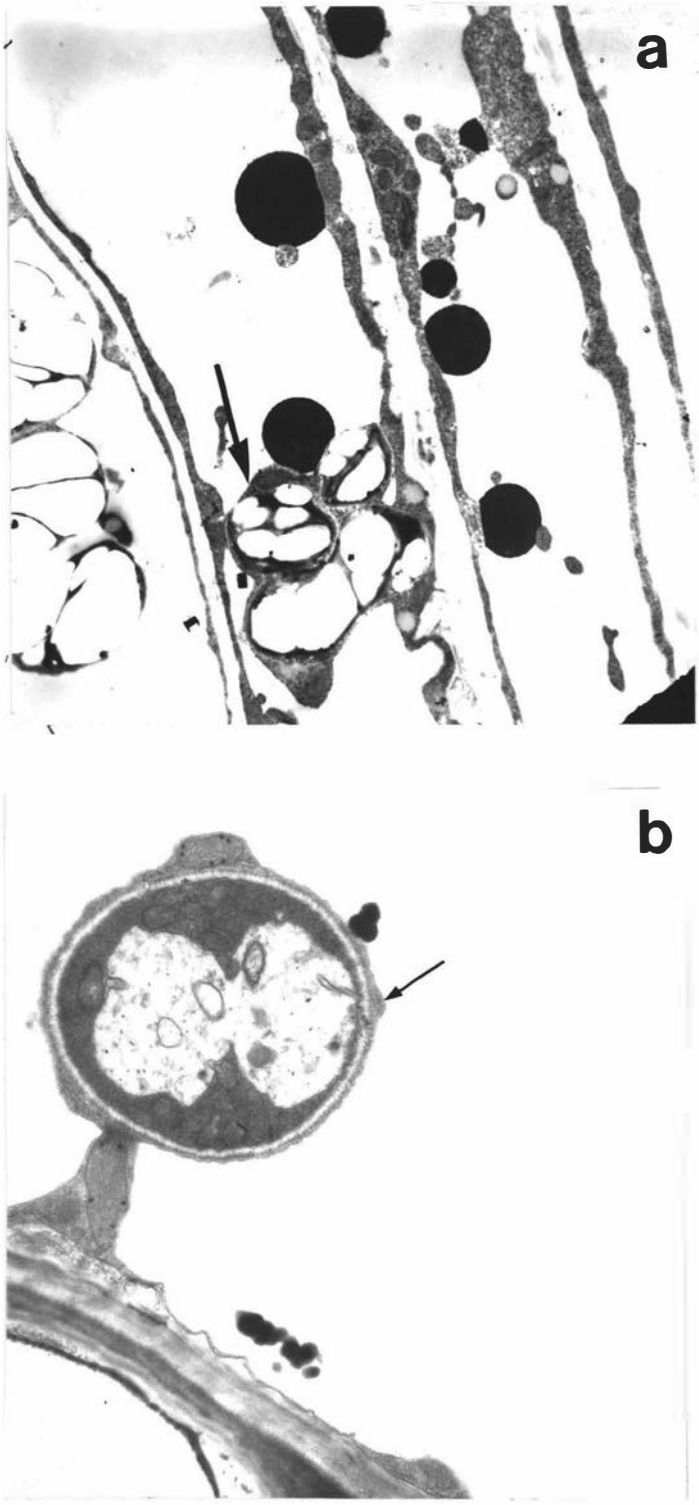


Plate 7-6. a - h. Degeneration of infected cells.

a. A longitudinal section of host cells from uninoculated root tissue. Cells are lined with a thin layer of cytoplasm. Note the tannin like material (dark spot), and plastids (arrow) (x 5200)

b. A host cortical cell containing a transverse section of a hyphal cell. The thin layer of the cytoplasm of the host cell (arrow) surrounding the hypha is similar in appearance to that in Plate 7-6 a (3 days, prochloraz 1 μg/ml, x 21200).

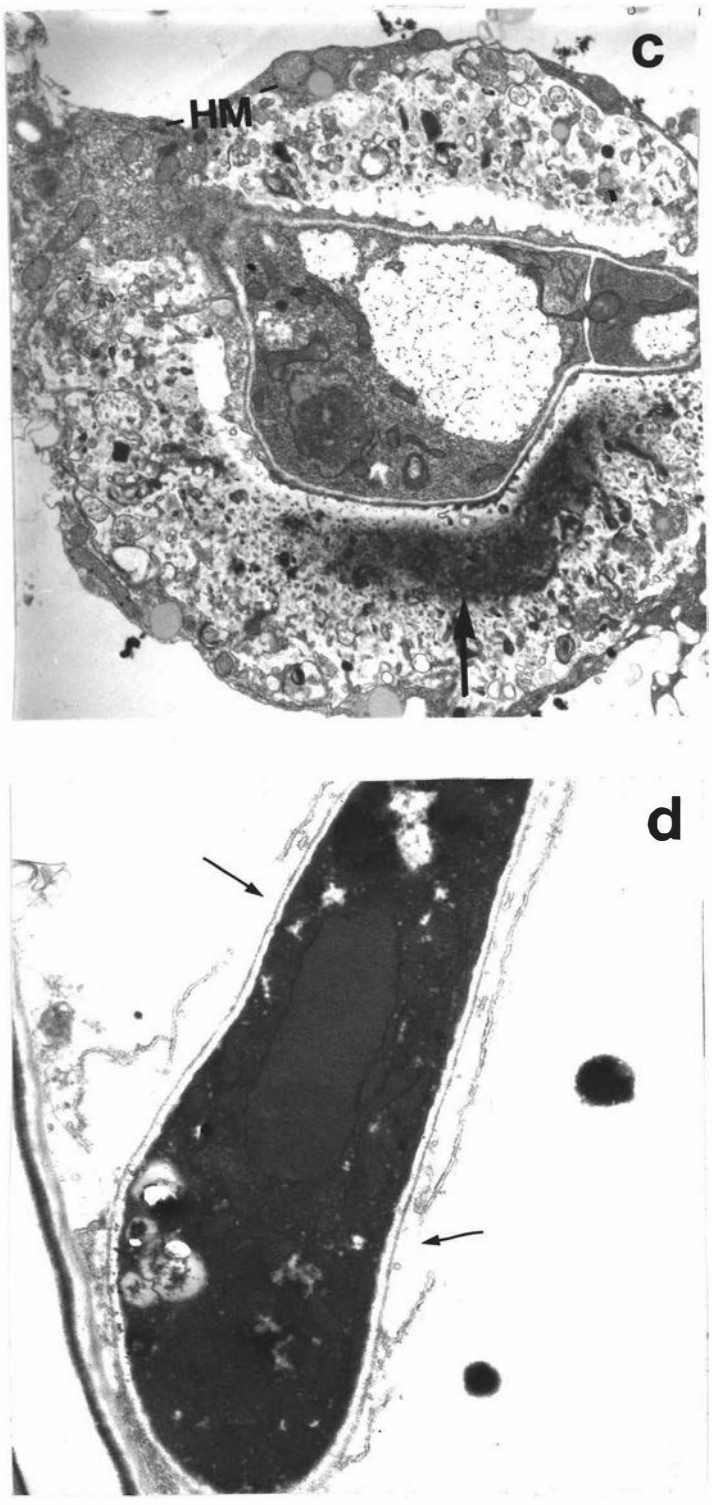
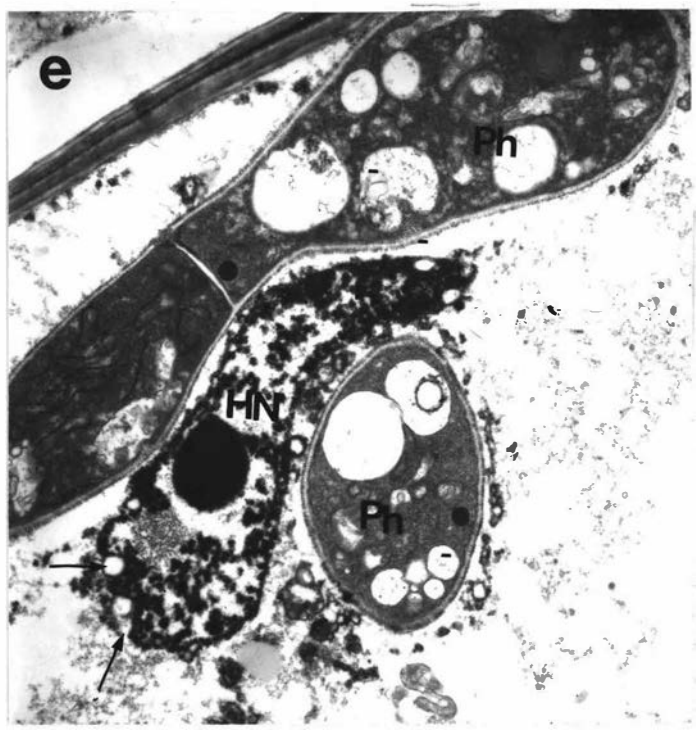


Plate 7-6 cont. **c**. A cortical cell containing a hypha. The host cytoplasm enveloping the hypha contains many mitochondria (HM), vesicles. Note the deposition of papilla materials on the host cytoplasm (arrow) (3 days, benomyl 10 μg/ml, x 7800).

d. Breaks in the plasmalemma (arrows) of a cortical cell containing beaded hypha. Note the uniform thickness of the fungal cell wall throughout (1 day, control, x 15300).



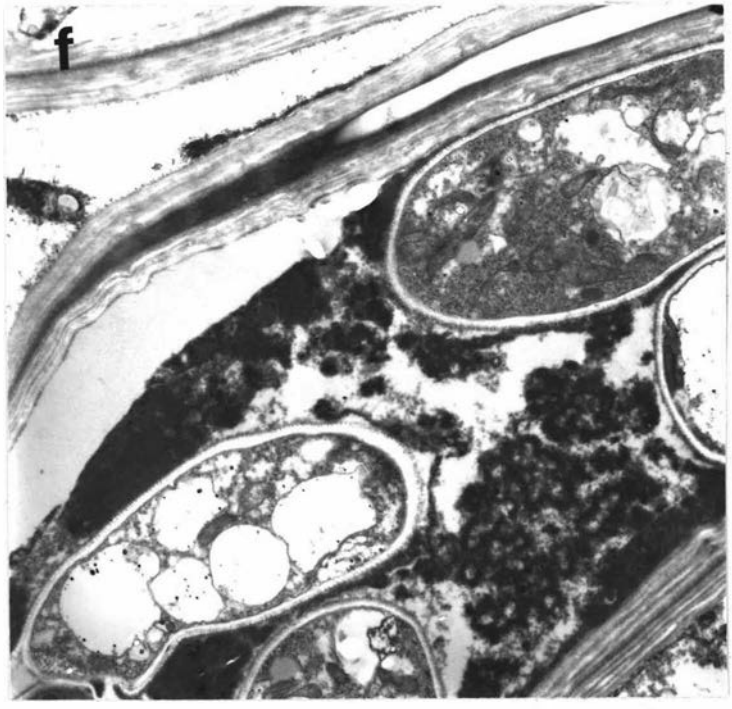
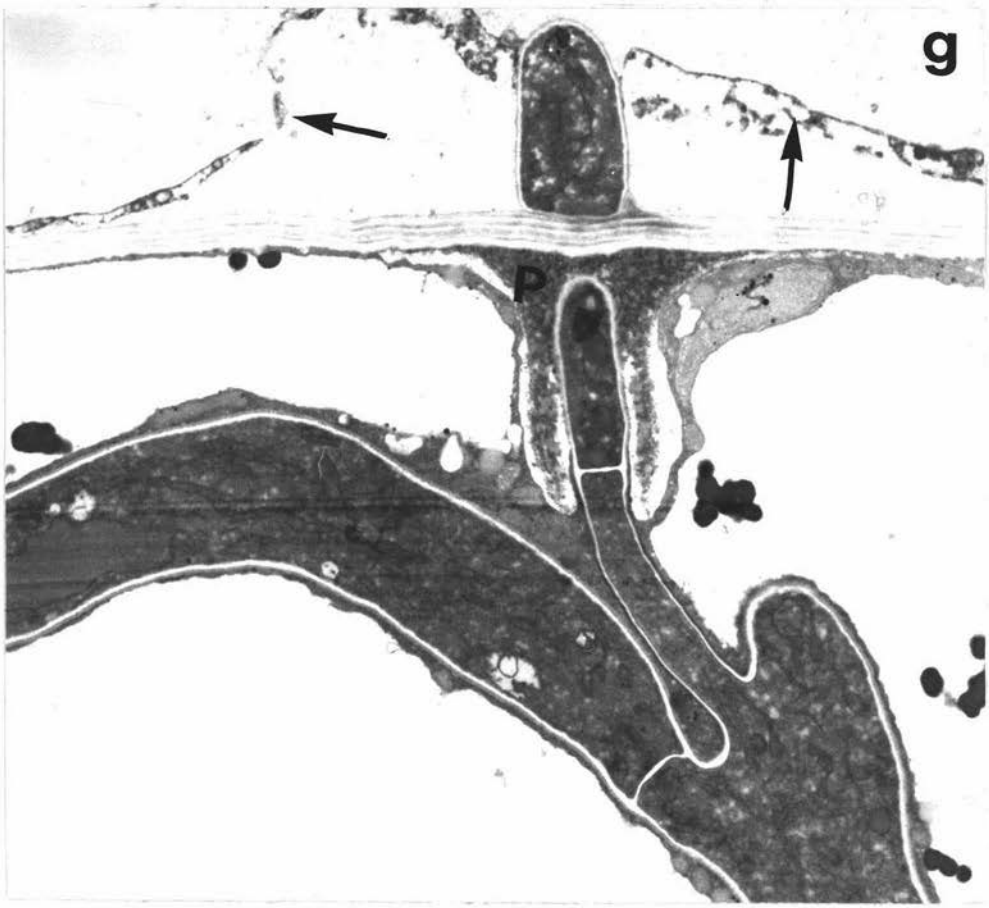


Plate 7-6 cont. **e.** A cortical cell containing hyphae (Ph) seen in longitudinal and transverse section, showing a disorganised nucleus (HN) and a broken nuclear membrane (arrows). Note the cell contents are disorganised (3 days, benomyl 10 μg/ml, x 7800).

f. A disorganised cortical cell filled with hyphae and electron-dense material (5 days, control, x 7800).



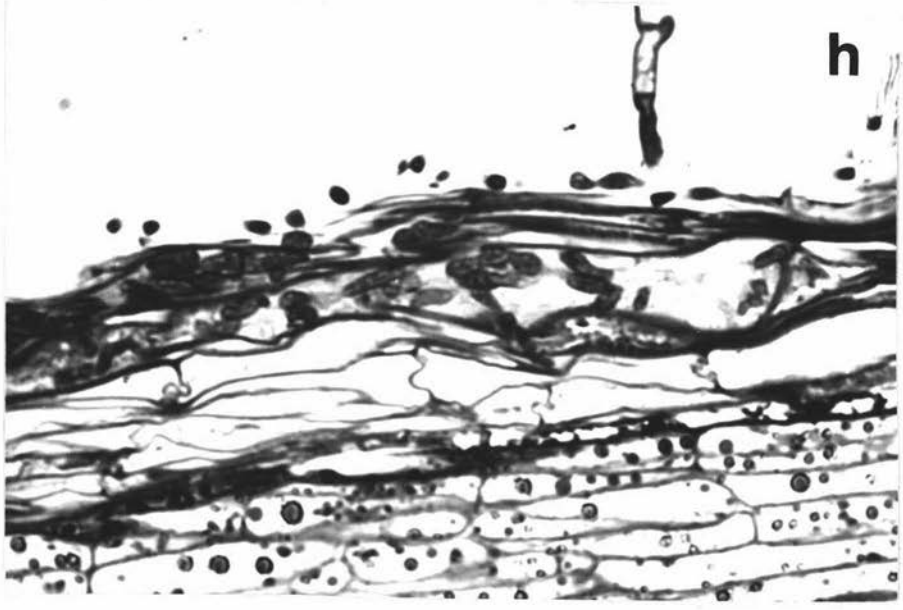


Plate 7-6 cont. **g.** Two adjacent cortical cells. The upper one containing a hypha is dying as indicated by its dissipated loose contents and membranous debris (arrows). The lower one containing newly-invaded hyphae appears to have coherent cytoplasm which envelops the hyphae. Note the expansion of the hypha after growth through a papilla (P) (5 days, prochloraz 1 μg/ml, x 8100).

h. A longitudinal semi-thin section of root tissue containing abundant hyphae and collapsed cells (5 days, control, x 425).

7.3.2 Effects of fungicides on red clover seedlings and T. basicola.

7.3.2.1 Effects of fungicides on root rot development and papilla formation.

Percentage area of root surface covered by lesions caused by T. basicola was significantly lower on seedlings grown on agar containing prochloraz (1 and 10 μg/ml), than on untreated seedlings or those grown on agar containing benomyl, both at 5 and 8 days after inoculation (Fig. 7-3-1).

More papillae were produced by seedlings treated with either benomyl or prochloraz than untreated seedlings at both 16 and 24 h after inoculation (Fig. 7-3-2). No differences were found between the chemical treatments.

No protoplasmic abnormality was detected in cells of red clover seedlings treated with benomyl or prochloraz when examined by light and electron microscopy.

7.3.2.2 Effects of fungicides on spore germination, hyphal penetration, and growth on the root surface

Percentage germination of endoconidia on the root surface of red clover seedlings was similar among the treatments (Table 7-3-1) although benomyl treatment gave the lowest germination at 16 h.

Less hyphal penetration occurred, and there were fewer cells per beaded hypha in the seedlings treated with prochloraz, than in control seedlings or those treated with benomyl (Table 7-3-1).

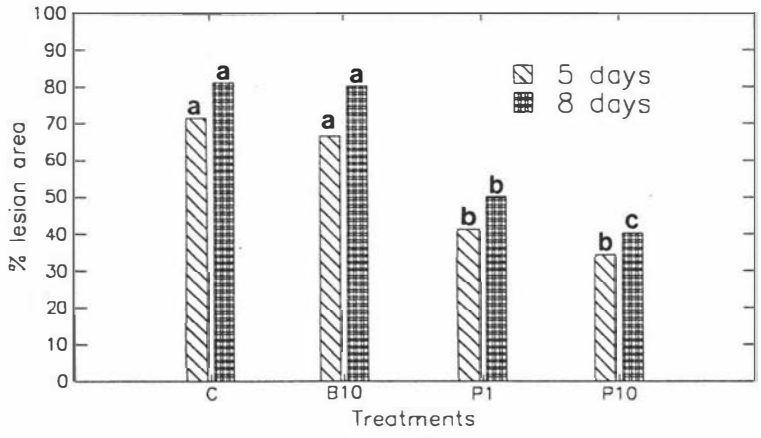


Figure 7–3–1. Mean percentages of root surface covered by lesions 5 and 8 days after inoculation with T. basicola. Columns for the each date followed by the same letters were not significantly different at 5% probabilty level in an analysis of transformed values (sq. rt.). C, control; B10, benomyl 10 ppm; P1, prochloraz 1 ppm; P10, prochloraz 10 ppm.

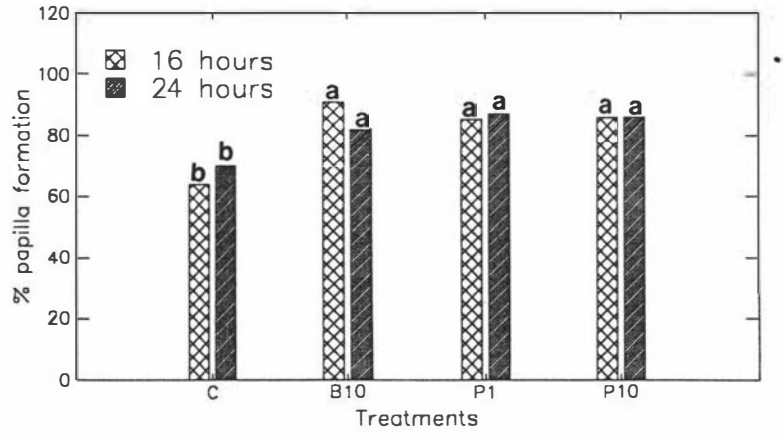


Figure 7–3–2. Mean percentage infections where papillae were formed in cells of red clover roots 16 and 24 h after inoculation with T. basicola. Columns for the each time followed by the same letters were not significantly different at 5% probabilty level in an analysis of transformed values (sq. rt.). See Fig.7–3–1 for abbrevations.

Table 7-3-1. Mean percentage spore germination, hyphal penetration, and mean number of cells per invaded hypha of T. basicola on roots of red clover seedlings grown on water agar amended with benomyl or prochloraz.

Treatments	Germination %	Penetration %	No.cells
	16 h after inoculation		
Control	79.72 ab[1]	57.16 a	5.34 a
Benomyl 10 µg/ml	71.23 b	51.77 ab	5.29 a
Prochloraz 1 µg/ml	87.07 a	36.66 bc	3.86 b
Prochloraz 10 µg/ml	76.97 ab	21.61 c	2.32 c
	24 h after inoculation		
Control	86.66 a	61.17 a	7.99 a
Benomyl 10 µg/ml	91.62 a	58.32 a	7.08 a
Prochloraz 1 µg/ml	90.01 a	29.68 b	4.23 b
Prochloraz 10 µg/ml	88.10 a	25.62 b	3.95 b

[1]. Means followed by the same letters were not significantly different at 5% probability level in an analysis of transformed value (sq. rt.).

Chemical treatment affected sporulation of T. basicola on the root surface. Numbers of endoconidophores produced per 1 mm of roots treated with benomyl, or either concentration of prochloraz were significantly less than those on untreated roots from 5 days after inoculation (Table 7-3-2). No differences were detected among the chemical treatments.

No chlamydospores were found on roots until 5 days after inoculation when fewer were present on roots treated with prochloraz or benomyl, than on control roots (Table 7-3-2).

Table 7-3-2. Mean numbers of conidiophores and chlamydospores per 1 mm length on the surface of roots of red clover seedlings grown on water agar amended with benomyl or prochloraz.

Treatments	Days after inoculation 1	3	5	8
		Conidiophores		
Control	0.53 a[1]	25.33 a	59.27 a	66.53 a
Benomyl 10 µg/ml	0.20 a	15.33 ab	36.87 b	38.73 b
Prochloraz 1 µg/ml	0.40 a	7.87 b	33.67 b	35.67 b
Prochloraz 10 µg/ml	0.13 a	10.00 b	32.40 b	34.87 b
		Chlamydospores		
Control	0.00	0.00	11.00 a	38.13 a
Benomyl 10 µg/ml	0.00	0.00	2.27 b	37.53 a
Prochloraz 1 µg/ml	0.00	0.00	1.73 b	14.73 b
Prochloraz 10 µg/ml	0.00	0.00	2.60 b	16.73 b

[1] : See Table 7-3-1.

7.3.2.3 Effects of fungicides on the fine structure of the fungus grown in culture and in the host.

Similar abnormalities were found in fungal ultrastructure following treatment with prochloraz or benomyl in culture and in the host. Reference is to both unless otherwise stated.

A. Effects of benomyl

Lomasome formation was common in cells of mycelium treated with benomyl, and the plasmalemma became more convoluted (Plates 7-7 a, 7-7 b) than in cells of untreated hyphae, in which it was homogeneous, and closely and regularly appressed to the cell wall (Plate 7-2 b). In addition, membraneous

complexes formed from endoplasmic reticulum (ER) occurred in some cells of mycelium from treated cultures (Plate 7-7 c). In the most extreme situation, internal fine structure was lost completely and only vesicles and fragments of membranes remained, and the plasmalemma and lomasomes became electron opaque (Plate 7-7 d). However, in cells of most hyphae treated with benomyl, organelles remained intact and the cytoplasm showed little sign of disorganisation.

B. Effects of prochloraz

The cell walls of hyphae from treated mycelium were generally of greater and more uneven thickness than those of controls (Plates 7-8 a, b, compared with Plate 7-6 d). In some cells, several additional wall layers developed, with the inner layers usually being undulated (Plates 7-8 c, d) and the outer layers sometimes appearing fragmented (Plate 7-8 e).

Mitochondria in some cells within host tissue became swollen and their cristae were disorganised (Plate 7-8 c).

Necrotic hyphae were common in culture (Plates 7-8 b, d) and in the host (Plate 7-8 f). Similar necrosis was seen in hyphae and endoconidia on the root surface.

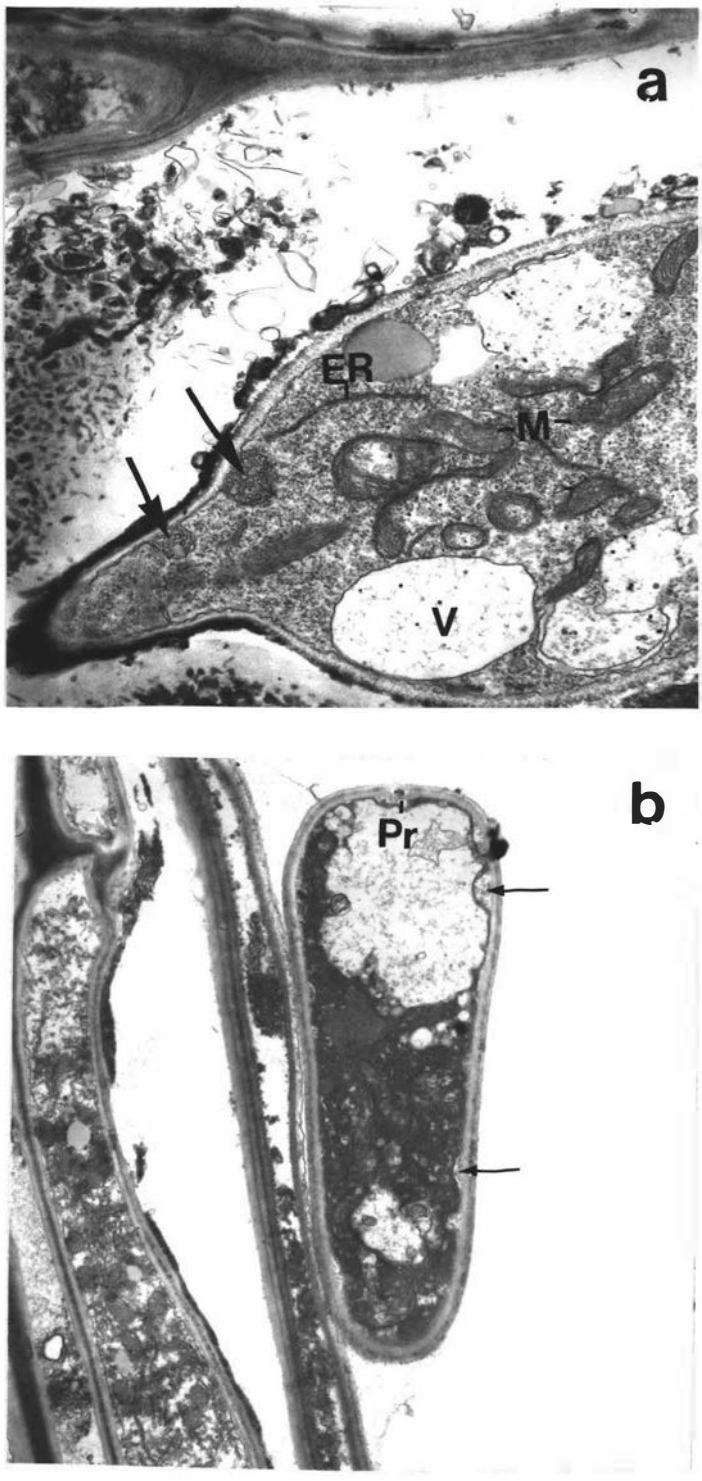


Plate 7-7. a - d. Fine structure of fungal cells treated with benomyl.

a. A growing hypha with several lomasomes (arrows) in a cortical cell. Other organelles including mitochondria (M), endoplasmic reticulum (ER), and vacuoles (V) in the fungal cell appear to be normal. Note electron-dense material in the disorganised cortical cell (3 days, benomyl 10 μg/ml, x 15300).

b. An endoconidium with several lomasomes (arrows) on the root surface. At one end of the conidium the cell wall protrudes into the cytoplasm (Pr). The cell wall of the conidium consists of 2 layers (1 day, benomyl 10 μg/ml, x 7800).

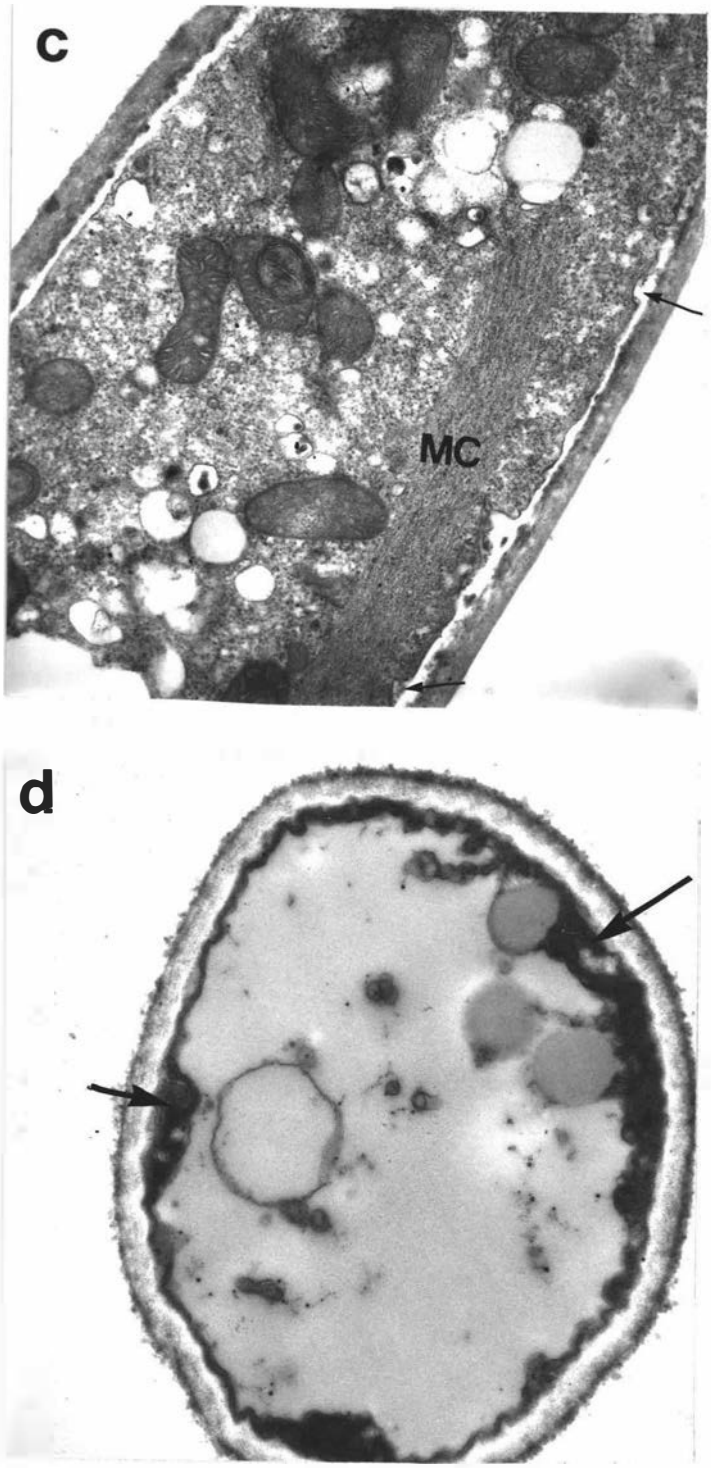


Plate 7-7 cont. **c**. A hypha from a culture grown on medium containing benomyl in which lomasomes are visible (arrows), and endoplasmic reticulum has organised into a membrane complex (MC). Other organelles appear to be normal (9 days, benomyl 0.06 μg/ ml, x 15300).

d. A hypha from a culture grown on medium containing benomyl shows convolutions of the plasmalemma due to the formation of lomasomes (arrows). The cytoplasm is electron-opaque and no organelles are recognisable (9 days, benomyl 0.06 μg/ml, x 21200).

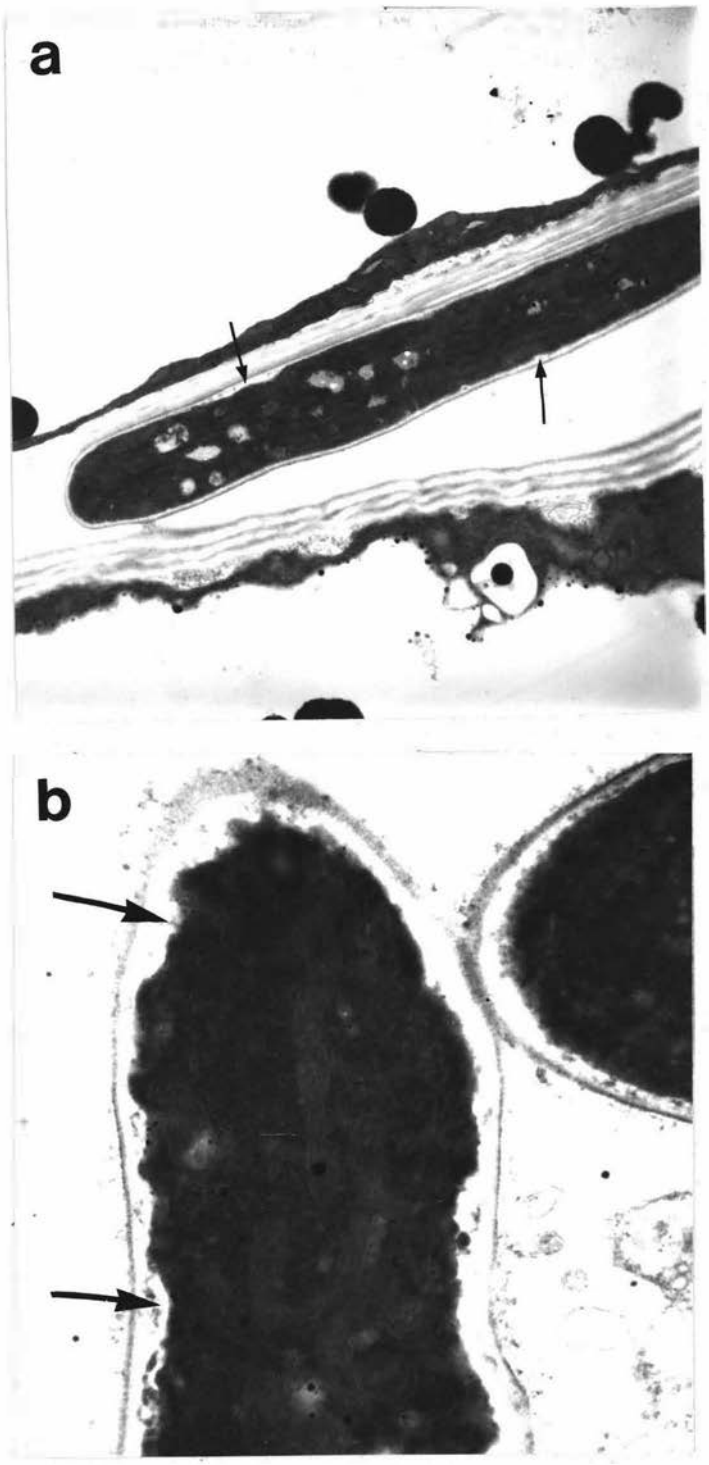


Plate 7-8. a - f. Fine structure of fungal cells treated with prochloraz.

a. A hypha with uneven thickness of cell wall (arrows) in a cortical cell of the host. Note the contents of fungal cell appeared unchanged (5 days, prochloraz 1 μg/ml, x 7800).

b. A hypha from a culture grown on medium containing prochloraz shows uneven thickening walls (arrows) and necrosis of the cell contents (9 days, prochloraz 0.01 μg/ml, x 21200).

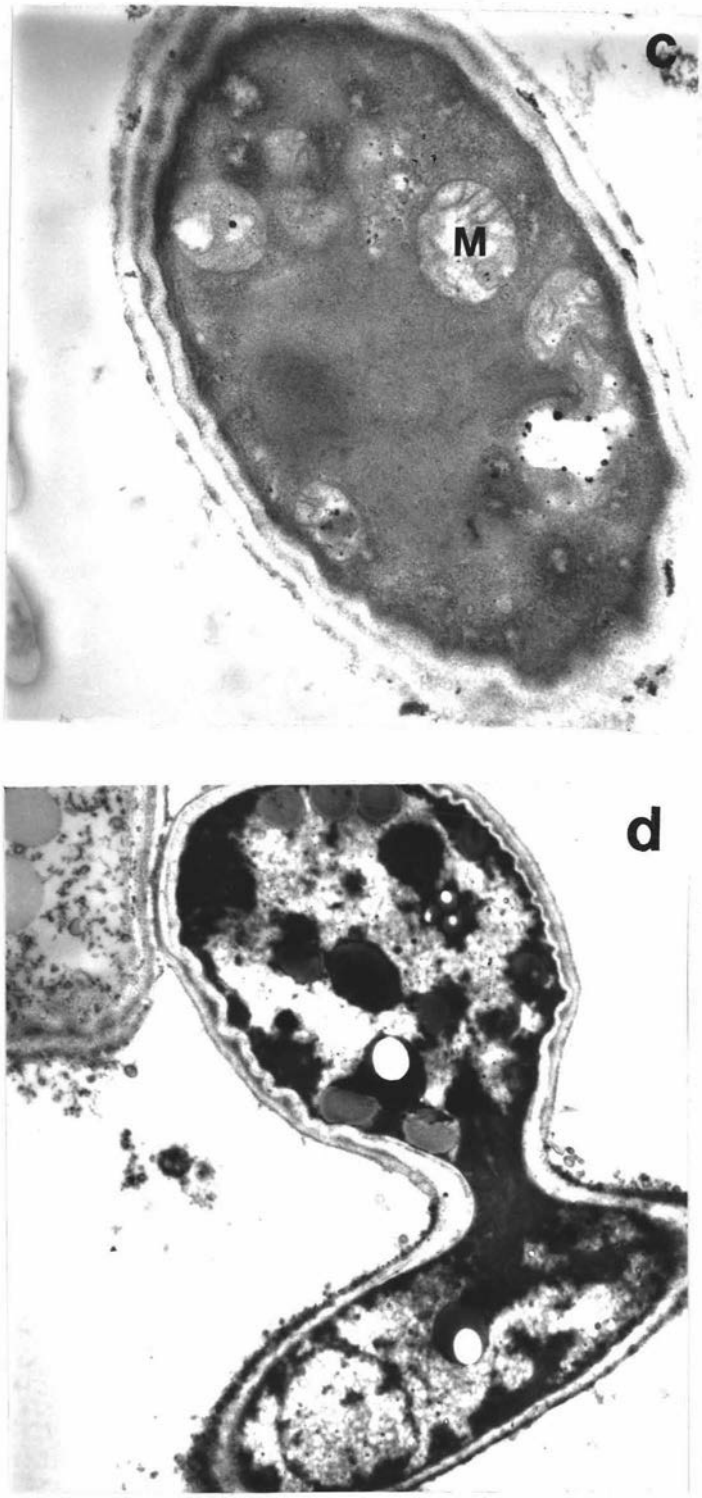
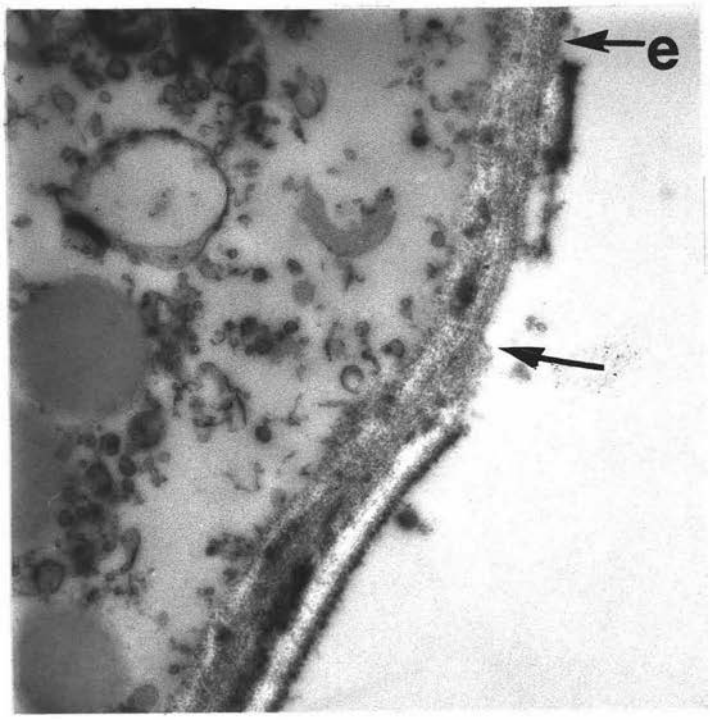


Plate 7-8 cont. **c.** A hypha growing on the root surface containing distended, disorganised mitochondria (M). The cell wall has several layers, the inner layer being undulating. (3 days, prochloraz 1 μg/ml, x 21200).

d. A hypha from a culture grown on medium containing prochloraz. The cell wall is similar in appearance to that in Plate 7-8 c. Note the cell contents are disorganised (9 days, prochloraz 0.01 μg/ml, x 31800).



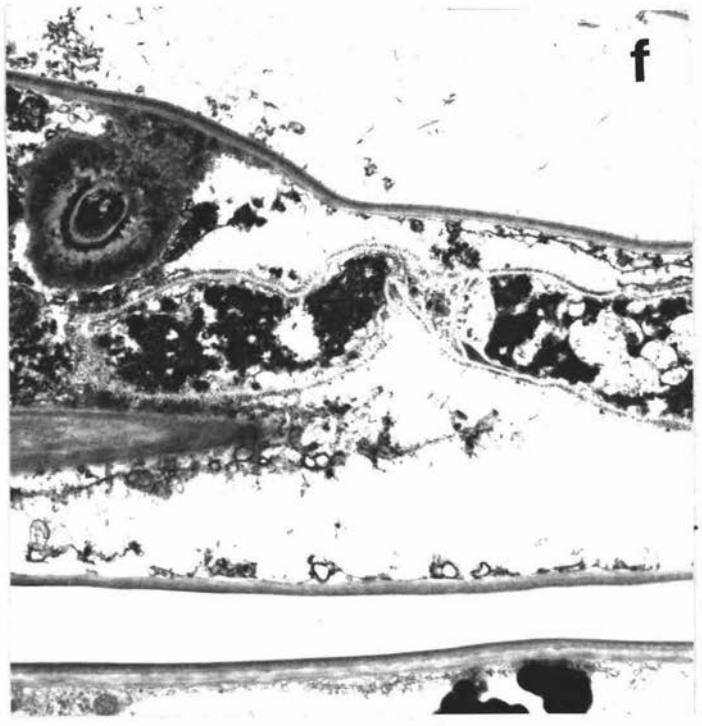


Plate 7-8 cont. **e**. Part of a hypha from a culture grown on medium containing prochloraz shows an unevenly thickened cell wall comprising several layers, the outer of which is fragmented (arrows). The cell contents are disorganised (9 days, prochloraz 0.01 μg/ml, x 31800).

f. A necrotic hypha in the cortical cell of the host. Note a papilla (P) containing a hyphal section and disorganisation of the host cell contents (3 days, prochloraz 10 μg/ml, x 7800).

7.4 DISCUSSION

The use of inoculated seedlings grown on agar, and a new method for clearing and staining whole root segments without the need for heating, has helped provide information about pre-penetration events which was not available in the literature. Endoconidia germinated to produce long hyphae that developed into ramifying mycelium, or conidiophores which expelled masses of endoconidia on the root surface. These events occur in culture (Stover, 1950) but the formation of the cylindrical or knob-like (Plates 7-1 b, c) structures has not been reported previously.

Direct penetration of red clover roots by T. basicola occurred without the formation of appressoria and is consistent with other reports (Christou, 1962; Pierre and Wilkinson, 1970; Wick and Moore, 1983; Lim and Cole, 1984), but is contrary to Mauk and Hine's (1988) finding that appressoria are involved in infection of cotton roots by T. basicola.

The development of intracellular hyphae with a characteristic beaded appearance following penetration has been reported for other hosts in the studies cited above. However, colonisation of red clover roots by the 'straight' and 'reproductive' hyphae which developed from beaded hyphae was exclusively intracellular. In bean (Christou, 1962; Pierre and Wilkinson, 1970) and citrus (Tsao and Van Gundy, 1962), 'straight and /or 'reproductive' hyphae were intercellular as well as intracellular.

There was no previous information available about whether host cell penetration is achieved mechanically or enzymically, but T. basicola produces wall-degrading enzymes during infection of bean roots (Lumsden and Bateman, 1968). Penetration of cortical cell walls seemed to be mediated by enzymic degradation because a sharp, clean pore, without curled edges, was made at the site of penetration. Dissolution of the host cell wall around the penetrating hyphae was also indicated by changes in its staining properties, which probably resulted from chemical modification by enzymes. As with obligate biotrophic fungi, degradation of the cell wall was highly localised around the infection peg,

suggesting that the enzymes responsible are bound to the hyphal wall, or are rapidly inactivated (Keon et al., 1987).

The most common host response to penetration was the aggregation of host cytoplasm and the formation of papillae. These formed in the paramural space adjacent to the invading hypha through a process which involved invagination of the host plasmalemma (Plate 7-5 a), and aggregation of electron-dense material (Plate 7-5 b). Papilla formation is a well known response of living plant cells to attempted penetration by fungi, or to mechanical wounding caused by insertion of microneedles or feeding by nematodes (Aist, 1976; 1983). The formation of papillae in plant roots in response to fungal invasion has been reported for white clover infected by T. basicola (Lim and Cole, 1984) and other fungi (Skipp and Christensen, 1982), and other host and pathogen interactions (Griffiths, 1971; Skipp et al., 1974; Bishop and Cooper, 1983).

The chemical nature of papillae has been of interest largely because of the possible implications regarding to any role in resistance. No attempt has been made to identify the chemical constituents of the papilla in the present study. However, the most common chemical components found in other studies are callose and lignin (Aist, 1976). Other constituents include protein, pectin, silicon, etc. Some workers have reported that invading hyphae became completely encased by papillae (Griffiths, 1971; Bishop and Cooper, 1983; O'Connell et al., 1985), and thus were prevented from entering the cell lumen. This was not the case in the present study where hyphae were often seen to have grown through papilla deposits (Plates 7-5 c, e, 7-6 g). However, observations indicated that formation of some papillae occurs prior to host cell wall penetration and they may function to slow colonisation of host tissue.

The beaded hyphae which formed in epidermal and cortical cells following direct penetration of the cell wall resembled haustoria, in that they completely invaginated, but did not rupture, the host plasmalemma and were enveloped by a thin layer of apparently unaffected host cytoplasm. The sequential occurrence of this benign phase followed by gradual degeneration and death of each infected host cell reflected the dual biotrophic and necrotrophic activities of the fungus within the same infection site. These findings suggest that T. basicola behaves as

a hemibiotroph like Collectrochum lindemuthianum (Skipp and Deverall, 1972; O'Connell et al., 1985; Keon et al., 1987). However, the biotrophic phase of T. basicola is short-lived. Breaks in the plasmalemma of red clover cells became visible at 24 h, and macroscopic symptoms appeared 3 days after inoculation.

Pre-treatment of seedlings with benomyl or prochloraz is a suitable technique to study effects of fungicides on fungal invasion and on fine structure. The fungicide in the WA must travel systemically through the host to reach the fungus, thus allowing investigation of fungicidal activity in host tissue. The observations from root tissue were further confirmed by the results obtained from cultures treated with either fungicide at concentrations which inhibited, but did not completely stop, mycelium growth.

Seedlings treated with either chemical produced more papillae in response to infection than those which had not been treated (Fig. 7-3-2). As discussed above, papillae were actively deposited by the host protoplast. Benomyl has cytokinin-like activity (Gayed, 1970; Skene, 1972), and EBI fungicides can induce plant to produce cytokinin (Fletcher, 1985) although generally they act as growth retardants. It is not clear whether the increased proportion of infections with papillae reflected stimulation of papilla production or a slowing of fungal penetration which allowed more cells to respond by producing papillae.

Neither fungicide eliminated fungal invasion at the concentrations used. However, disease symptoms were greatly reduced by prochloraz treatment (Fig. 7-3-1), and the treatments affected fungal growth on and in root tissues (Table 7-3-1 and 7-3-2). These effects were accompanied by changes in fungal ultrastructure which appear to have been induced by the fungicides.

Lomasomes, formed abundantly in fungal cells of mycelium treated with benomyl (Plate 7), may have been the result of fixation artefacts as suggested by Bracker (1967). However, it seems more likely that they reflected effects of benomyl on fungal cells since all specimens were processed using the same procedure, and lomasomes were abundant only in benomyl-treated mycelium. Similar increases in lomasome formation have been reported for Botrytis fabae grown in cultures containing benomyl at a sublethal concentration (Richmond and Pring, 1971). Lomasomes only occur in walled cells where they are most

frequently found in areas active in wall synthesis (Bracker, 1967; Hawes and Beckett, 1977). The change in appearance of endoplasmic reticulum seen in some cells of benomyl treated mycelium in vitro may be related to lomasome formation (Marchant et al., 1967).

Disorganisation of cell contents was found in benomyl-treated mycelium from cultures (Plate 7-8 d), but not in root tissue treated with much higher concentrations of the same fungicide. Benomyl decomposes to methyl 2 - benzimidazolecarbamate within plants, which is rapidly exported from roots to leaves via the xylem (Peterson and Edgington, 1970). Thus the concentration of fungicide remaining in the tissue at the time of infection may have been much lower than that encountered in the agar plates.

The most striking effect of prochloraz on the fine structure of the fungus was the irregular and randomly distributed thickening in cell walls (Plate 7-8 a , b). Similar results have been reported for other EBI fungicides in a variety of fungi grown in culture (Stiers et al., 1980; Borgers et al., 1981; Hippe and Grossmann, 1982; Richmond, 1984; Hippe, 1984 a and b; Dahmen et al., 1988; Sancholle et al., 1988), and in the host (Pring, 1984; Smolka et al., 1988).

One unusual finding from the present study was the formation of additional layers of cell wall in some cells, and the subsequent separation and fragmentation of these layers (Plate 7-8 c-d). These effects have also been observed in Coccidioides immitis Rixf. and Gilch treated with miconazole (Borgers et al., 1981), and Taphrina deformans treated with propiconazole (Sancholle et al., 1988). Thus effects which occurred in cells of T. basicola appear to be common to EBI fungicides rather than to prochloraz alone.

Cell wall thickening may have been the first detectable abnormality in fungal cells affected by prochloraz in a series of changes which led to cell death. This is supported by the findings that some fungal cells with unevenly thickened cell walls had relatively unchanged cytoplasm (Plate 7-8 a), whereas others had completely disorganised cell contents (plate 7-8 b). Borgers et al. (1981) reported that cell death in fungal cultures occurred within 24 h of treatment with imidazole chemicals, even at concentrations which did not completely inhibit growth. In the present study, cultures were incubated for 9 days before being processed for

electron microscopy. Even though the fungus grew slowly during this period it is not surprising that cells examined under the electron microscope were necrotic. These observations support the view that prochloraz has fungicidal activity as indicated in Chapter 5.

The occurrence of necrotic hypha cells on the root surface and in the root tissue, treated with prochloraz (Plate 7-8) suggested that fungicidal concentrations of prochloraz were present in root tissues. This is consistent with the reduced fungal growth and symptom development on prochloraz-treated roots (Fig. 7-3-1; Table 7-3-1; 7-3-2).

Differences in the ultrastructural abnormalities of the fungus when treated with prochloraz or benomyl were probably related to the differing modes of action of the two fungicides. As suggested in other studies (Richmond and Pring, 1971; Simons, 1975; Dahmen et al., 1988; Sancholle et al., 1988), however, abnormalities caused by either of the fungicides could be interpreted as secondary effects.

Although many cells from treated mycelium appeared abnormal, particularly after exposure to prochloraz, some hyphae showed no such effects and probably functioned normally. This variation in response may have resulted from uneven distribution of fungicide within the root or differing sensitivity of individual hyphal cells.

In summary, T. basicola penetrated roots of red clover directly and colonised tissue by beaded and straight hyphae, which lived hemibiotrophically within epidermal and cortical cells. Endoconidia and chlamydospores were produced on the root surface. Pre-treatment of seedlings with benomyl or prochloraz did not change the sequence or timing of fungal penetration, but reduced fungal growth. Changes in fungal ultrastructure resulting from benomyl treatment were an increased frequency of lomasome production, and occasionally a disorganisation of cell contents. Changes resulting from prochloraz treatment included thickening and fragmentation of cell walls, and necrosis of hyphal cells.

CHAPTER 8
CONCLUDING DISCUSSION

8.1 MEASUREMENT OF EFFECTS OF ROOT-INVADING FUNGI ON RED CLOVER GROWTH

While the impact of root pathogens in pasture remains poorly understood, there is little incentive to seek improved productivity through plant breeding or other measures aimed at control of root disease. This study was primarily undertaken to provide the means to study the effects of soilborne, root-invading fungi on plant growth and persistence in pasture by manipulating microbial populations in soil and roots, while monitoring the experimentally-induced changes in populations of target and non-target organisms.

The study showed the value of using fungicide soil drenches to modify levels of viable fungal biomass in soil, and in roots. Prochloraz proved most suitable for this purpose of the 11 fungicides tested. It had a broad spectrum of antifungal activity, was highly active against all the major root-invading fungi found in the plant breeder's evaluation block, had low toxicity to Rhizobium trifolii, and had the least retardant effect on red clover growth. Efficacy in soil was demonstrated in glasshouse and field experiments (Experiment 4 and 5) where populations of root-invading fungi in soil, and in red clover roots, were lower following drench treatment with prochloraz than in untreated controls. In the field trial (Experiment 5) quantitative differences in internal mycoflora of red clover roots in treated and untreated plots were still detectable 45 weeks after a single fungicide application following sowing. Maintenance of reduced populations for a prolonged period made it possible to establish relationships between root invasion by fungi and resultant plant yield. Use of more than one fungicide drench could prolong effects on fungal populations even further to facilitate longer term studies on red clover and other perennial pasture plants.

Throughout the study, root maceration and plating was used to quantify fungal invasion of red clover roots. The method detected a similar range of fungi to that found by plating segments, but yielded more colonies and showed less

variation. Using standardised amounts of tissue and blending times (2 g and 60 or 120 sec), differences in fungal populations in roots subjected to different treatments were readily detected. This method appears suitable for routine studies on infection of roots by complexes of root-invading fungi.

Experiment 5 illustrated some of the possible side effects arising from fungicide application. These included retardant effects on plant growth (Experiment 3; 4), and increased populations of bacteria and fungicide-insensitive fungi such as Penicillium spp.(Experiment 4) in treated soil. Furthermore, the fungicide application method does not provide information about damaging effects caused by individual fungal species. This is more readily studied by the microplot method where exposure of red clover plants to the environmental stress of field conditions should also allow expression of chronic damage caused by weak pathogens.

One problem which arose in the microplot experiment was that the amount of fungal biomass, and of nutrients present in the inoculum, influenced the amount of root damage. As seen in Experiment 7, the presence of nutrient in the inoculum medium can stimulate growth of contaminant fungi particularly in fumigated soil. These fungi may influence plant growth or invasion by the test organism. However, while it may be difficult to estimate possible losses in production which might occur in the field from microplots, the method did provide evidence of the field pathogenicity of several fungi, and indicated their relative aggressiveness.

Red clover is mainly reliant on a tap root for sustained productivity. Methods developed and tested during this study on red clover would be readily applicable to other tap-rooted forage plants such as lucerne and sainfoin and, more generally, to many horticultural and field crops. Further applications include microplot investigations on the interactive effects of nematodes and soilborne pathogens, and an extension to pure swards and mixed pastures of studies on the relationships between plant yield and levels of fungal inoculum in soil and root tissue.

8.2 INVASION OF RED CLOVER ROOTS BY FUNGI

The study also provided information about the internal mycoflora of red clover roots, the method, site and timing of fungal infection, the role of individual pathogens in the development of symptoms of root disease, and the influence of environmental factors on root invasion.

Of the 40 fungal species found in roots of red clover grown in the plant breeder's evaluation block, those most frequently isolated were Fusarium solani, Verticillium dahliae, F. oxysporum, Trichocladium basicola, Cylindrocladium scoparium, Cylindrocarpon destructans, and Gliocladium roseum (Experiments 1; 2; 4; and 5; see also Skipp et al., 1986). Many of these fungi have also been reported from red clover and other forage legumes elsewhere in New Zealand and in other countries (Chapter 2). The similarity of the root-invading mycoflora of red clover and other forage legumes in different parts of the world may reflect the ubiquitous distribution of some soilborne fungi, and a suitability of roots of the plants as a substrate for growth of those fungi. This in turn may explain why root rot of forage legumes occurs wherever they are grown.

Verticillium dahliae, T. basicola, and Cy. scoparium have not previously been considered as part of the root rot complex of red clover or other forage legumes, although they have been occasionally isolated from roots of these crops (Chapter 2). Isolates of these fungi were among the most pathogenic obtained from plants grown in the plant breeder's evaluation block (Experiment 6 and 7; Skipp et al., 1986) and many plants grown in soil from the block became infected by them. This raises the question of whether the evaluation block, which had been maintained as a red clover monoculture to encourage development of high inocula of pathogenic fungi, had an unusual mycoflora. Verticillium dahliae and T. basicola have been found in roots of red clover from farms in New Zealand (R.A. Skipp and M.J. Christensen, pers. comm.) which may suggest that in this country, the root rot complex of red clover may involve more fungal species than are considered elsewhere. All three fungi have a wide host range (Pennycook, 1989) thus pasture soils containing red clover may harbour pathogens which could

seriously affect subsequent crops of other plants.

Invasion of red clover roots by fungi can occur at an early stage of plant growth (Skipp et al., 1986; Experiments 1; 2; 4; 5; 6; 7; 8) and the extent of this early colonisation may have a critical influence on the course of root deterioration in the life of the plant. Detailed studies with T. basicola (Experiment 8) showed that seedling roots could become infected within 1 day of germination of endoconidia on the surface. Under favourable conditions cells of the epidermis, cortex, and endodermis were colonised by beaded and straight hyphae which formed visible lesions, and initiated formation of endoconidia and chlamydospores, about a week later. Fusarium solani, F. oxysporum, and other Fusarium species, are also able to invade uninjured roots of red clover (Chi et al., 1964; Siddiqui and Halisky, 1968 b). In a preliminary experiment in the present study, F. solani was seen to penetrate epidermal cells directly without the formation of appressoria. Following penetration, the hyphae branched and grew extensively through the epidermal and cortical tissue. Eventually, the cortex was completely colonised, and xylem tissue was also invaded. Similarly, conidia of V. dahliae germinated and penetrated epidermal cells directly within 24 h of inoculation. Seven days after inoculation, hyphae were seen in the xylem tissue, and microsclerotia had formed in the cortex. Other fungi isolated from red clover roots were also found to be able to penetrate uninjured tissue and form necrotic lesions on roots of red clover seedlings (Skipp et al., 1986).

As seedling growth proceeds, most direct fungal penetration of uninjured roots probably occurs through young tissues of the root tips, and newly developed lateral roots. This is supported by observations that some plants from field soil were seen to have lost the lower part of their tap root through root rot while the upper part remained intact, presumably as secondary thickening countered lesion development. Lateral roots often became rotten and were sloughed off. Fungal invasion also could have occurred through wounds caused by nematodes or insects (Chapter 2), or through the deep necrotic lesions caused by T. basicola and Cy. scoparium. Infection of the stele could have occurred by internal spread of fungal mycelium, from external necrotic lesions, or through wounds in stems or the crown caused by cutting or internal breakdown. Thus by one avenue or another, roots

of red clover plants eventually become infected by fungi which can cause root deterioration. This process appears to start shortly after emergence, progress gradually and increase in severity as plants age. Root symptoms associated with fungal infection were cortical rots, and stele browning. (Experiments 1; 2; 4; 5; 6; 7).

The most common cortical rot symptom seen on roots from 4 weeks onwards were superficial, localised, brown lesions. Fusarium spp., and some other soilborne fungi commonly isolated from roots in this study such as G. roseum, C. destructans etc., are frequently associated with this type of symptom (Fezer, 1961; Ylimäki, 1967; Leath et al., 1971; Ruflet, 1986; Skipp et al., 1986; R.A. Skipp and M.J. Christensen, pers. comm.). Sometimes the lesions covered the whole surface of the tap roots and were often accompanied by rootlet rotting (Experiments 1; 2; 4; 5; 6; 7).

Black sunken lesions caused by T. basicola or Cy. scoparium occurred to a lesser extent but were the most destructive. The lower part of the tap root of infected plants was often girdled or lost completely. The fungi have been reported to cause similar symptoms on other forage legumes (Hill, 1979; Ooka and Uchida, 1982; Lim and Cole, 1984).

Stele browning was seen in roots of pot-grown red clover plants from 8 weeks onwards, and field-grown plants from 10 - 12 weeks. A dark brown discolouration throughout the whole stele was attributed to infection by V. dahliae; similar symptoms were seen in pathogenicity tests with this fungus (Skipp et al., 1986; Experiment 6). Faint discolouration in the outer regions of the stele occurred in roots where Fusarium spp. but no V. dahliae could be isolated. A reddish brown, rather than dark brown discolouration of the whole stele was seen occasionally and was associated with F. solani. The finding that V. dahliae was the major cause of stele discolouration (present study and Skipp et al., 1986) contrasts with reports from other parts of the world where Fusarium spp. are thought to be the major cause of this symptom (Fezer, 1961; Ylimäki, 1967; Elliott et al., 1969; Ruflet, 1986).

Another type of symptom consistently seen was internal breakdown of crown tissue. This is regarded as a "physiogenic disease" (Graham et al., 1960),

resulting from a "malignant cytological disturbance" (Cressman, 1967). The relative importance of internal breakdown as a cause of plant death is not clear, but it can provide access for pathogenic fungi from the soil into the stele of the tap root, and contribute substantially to the deterioration of the tap root (Newton and Graham, 1960; R.A. Skipp and M.J. Christensen, pers. comm.).

Fungal invasion was affected by plant age, soil temperature and moisture (Experiment 2). The total fungal biomass in the root (expressed as numbers of colonies per root) increased gradually from the seedling stage onwards. However, the concentration of pathogens (expressed as numbers of colonies per gram root tissue) actually declined between 4 weeks and 8 weeks which meant that the concentration dynamics of propagules in the roots showed an increase - decrease - increase pattern. Further research is needed to monitor the dynamics at more frequent intervals during early stages of plant growth which appear critical in determining the course of root deterioration. Generally, more colonies were isolated from roots of plants grown at 20 and 25°C than at 10 and 15°C, and from 60 and 80% WHC than at 40% WHC.

8.3 SIGNIFICANCE OF ROOT-INVADING FUNGI IN PASTORAL AGRICULTURE

There was considerable evidence from the present study that soilborne fungal pathogens can adversely affect survival and productivity of red clover in the field.

The first effect of fungal invasion on red clover plants was lower seedling emergence (Experiments 2; 4; 5; 7). Seedling emergence in the field microplots containing inoculum of F. solani or T. basicola was 20-25% lower, and in those containing Cy. scoparium was 83-92% lower, than in control microplots (Experiment 7). The application of prochloraz or benomyl drenches to field plots in the plant breeder's evaluation block was accompanied by 25-50% increases in seedling emergence (Experiment 5). This also suggested that fungi affected seedling emergence. No attempt was made in this study to test the effects of C. destructans, G. roseum, and V. dahliae on seedling establishment, however, G.

roseum has been reported to reduce emergence of red clover seedlings (Kilpatrick et al., 1954; Fulton and Hanson, 1960).

The emerged seedlings faced a continuous struggle with root-invading fungi for survival. Twenty weeks after sowing, populations of plants grown in the field microplots had been reduced nearly 20% by T. basicola, and 71% to 100% (depending on inoculum level used) by Cy. scoparium. By 76 weeks, the percent of plants remaining in microplots inoculated with T. basicola, Cy. scoparium, or F. solani, were only 60, 24, and 67% respectively, of those in the controls, (Experiment 7). Plants infected with V. dahliae in field plots (Experiment 6), and with F. oxysporum in microplots (Experiment 7), also survived less well than control plants.

Infected plants which survived in the microplots often produced less dry matter yield than those in the control treatment. Over a period of 76 weeks, the percent reduction in cumulative yield per plant caused by the individual fungal species were: Cy. scoparium, 60-100; T. basicola, 52-55; F. solani, 30, and F. oxysporum, 20 (Experiment 7). A 42% yield reduction was recorded for V. dahliae in field plots (Experiment 6) 8 weeks after transplanting into the field.

Increases in yield in field plots treated with prochloraz also gave some indication of the potential of soilborne fungi to reduce productivity of red clover (Experiment 5). Six weeks after application of prochloraz drench at 3.46 g/m^2, shoot and root weights were 95 and 40%, respectively, greater than in untreated control plots. Forty five weeks after the drench treatment, the respective weights were 29 and 35% higher than in control plots.

The root rot complex, as shown in the present study, can adversely affect seedling establishment, plant survival, and productivity of red clover. It seems that the problem will continue to plague red clover growers for some time because of the variety of fungi associated, and the complexity of interactions of environmental and biological factors. However, information obtained in the present study should aid understanding of root deterioration in red clover, and provide a sound methodological basis for further research.

APPENDICES

Appendix 4-3-1. Mean numbers of colonies ($x10^3$) per gram fresh roots in total, and of the major fungal species isolated from tap and lateral roots of red clover plants grown in pots, as affected by soil temperatures (A) and moistures (B) 8 and 12 weeks after sowing.

A. Temperatures (°C)

Temp.	Total	Vd[2]	Fs	Fo	Tb	Cs	Other
			Tap roots -- 8 weeks				
10	57.75 a[1]	0.37 a	42.09 a	0.48 a	0.00 b	0.10 c	14.71 a
15	13.31 a	0.42 a	1.62 b	0.34 b	3.90 a	2.09 b	4.93 b
20	29.64 a	0.98 a	12.75 b	1.70 a	5.18 a	5.34 a	3.69 b
25	28.87 a	2.07 a	13.25 b	2.83 a	7.01 a	2.13 b	1.58 b
			Tap roots -- 12 weeks				
10	46.58 bc	0.00 b	35.78 b	0.14 b	0.00 b	0.00 c	10.66 a
15	8.97 c	0.14 b	4.55 b	0.23 b	0.72 b	0.82 b	2.51 b
20	59.03 ab	2.18 a	27.31 b	3.33 a	3.32 b	12.47 a	10.45 a
25	85.90 a	1.99 a	57.89 a	4.51 a	8.22 a	2.00 b	11.29 a
			Lateral roots -- 8 weeks				
10	3.73 a	0.03 a	0.10 c	0.62 a	0.00 b	0.00 d	2.98 a
15	1.56 a	0.02 a	0.11 c	0.38 a	0.26 a	0.08 c	0.71 b
20	1.94 a	0.05 a	0.47 b	0.29 a	0.18 a	0.55 a	0.40 b
25	2.36 a	0.06 a	1.04 a	0.36 a	0.35 a	0.23 b	0.32 b
			Lateral roots -- 12 weeks				
10	1.76 b	0.02 a	0.40 b	0.07 b	0.07 a	0.00 b	1.20 b
15	1.86 b	0.03 a	0.16 b	0.12 b	0.11 a	0.12 b	1.32 b
20	42.00 a	0.02 a	20.01 a	4.44 a	0.44 a	8.96 a	8.13 a
25	33.97 a	0.01 a	21.08 a	2.69 a	0.47 a	1.82 b	7.89 a

Appendix 4-3-1 continued

B. Moistures (% WHC)

Mois.	Total	Vd	Fs	Fo	Tb	Cs	Other
			Tap roots -- 8 weeks				
40	53.97 a	0.88 a	38.86 a	1.74 a	4.58 a	1.44 b	6.29 a
60	17.70 a	1.75 a	5.43 b	0.81 a	1.15 a	0.53 b	8.02 a
80	25.70 a	0.25 a	7.99 b	1.46 a	6.34 a	5.27 a	4.37 a
			Tap roots -- 12 weeks				
40	26.39 b	1.06 a	9.31 b	1.63 a	2.79 a	1.43 b	10.17 a
60	20.79 b	2.07 a	9.42 b	1.33 a	2.05 a	0.98 b	4.94 b
80	103.18 a	0.10 a	75.42 a	3.19 a	4.35 a	9.07 a	11.05 a
			Lateral roots -- 8 weeks				
40	2.57 a	0.06 a	0.80 a	0.73 a	0.35 a	0.17 a	0.46 a
60	2.43 a	0.04 a	0.21 b	0.16 a	0.11 b	0.17 a	1.74 a
80	2.19 a	0.02 a	0.28 b	0.33 a	0.13 b	0.31 a	1.22 a
			Lateral roots -- 12 weeks				
40	9.63 b	0.03 a	4.77 b	1.18 a	0.48 a	0.78 b	2.39 b
60	6.42 b	0.02 a	3.86 b	0.95 a	0.14 a	0.51 b	1.02 b
80	43.65 a	0.01 a	22.62 a	3.36 a	0.20 a	5.89 a	10.57 a

[1]: Data for one factor were averaged over all levels of the other factor. Means followed by the same letters for each fungus at each harvest date were not significantly different at 5% probability level in an analysis of transformed values (sq.rt.).

[2]: Vd: V. dahliae, Fs: F. solani, Fo: F. oxysporum Tb: T. basicola, Cs: Cy. scoparium.

Appendix 4-3-2. Mean numbers of colonies ($x10^3$) **per root** in total, and of the major fungal species isolated from **tap** and **lateral** roots of red clover plants grown in pots as affected by soil temperatures (A) and moistures (B) 8 and 12 weeks after sowing.

A. Temperatures (°C)

Temp.	Total	Vd[2]	Fs	Fo	Tb	Cs	Other
			Tap roots -- 8 weeks				
10	0.74 b[1]	0.01 b	0.52 ab	0.01 b	0.00 a	0.01 c	0.21 a
15	0.63 b	0.02 b	0.07 b	0.02 b	0.16 a	0.01 c	0.26 a
20	2.51 a	0.11 ab	1.09 a	0.14 a	0.43 a	0.39 a	0.34 a
25	2.52 a	0.21 a	1.10 a	0.23 a	0.64 a	0.19 b	0.14 a
			Tap roots -- 12 weeks				
10	1.73 b	0.00 a	1.19 b	0.01 b	0.00 a	0.00 b	0.53 b
15	1.47 b	0.03 a	0.69 b	0.04 b	0.13 a	0.14 b	0.45 b
20	10.41 a	0.44 a	4.70 a	0.63 a	0.59 a	2.25 a	1.79 a
25	12.51 a	0.34 a	8.11 a	0.61 a	1.67 a	0.25 b	1.53 a
			Lateral roots -- 8 weeks				
10	0.15 c	0.01 a	0.01 c	0.04 b	0.00 b	0.00 b	0.11 a
15	0.39 b	0.01 a	0.04 c	0.09 ab	0.07 ab	0.02 b	0.17 a
20	0.78 a	0.03 a	0.19 b	0.13 a	0.08 ab	0.20 a	0.18 a
25	0.97 a	0.02 a	0.36 a	0.15 a	0.16 a	0.11 a	0.16 a
			Lateral roots -- 12 weeks				
10	0.36 b	0.01 a	0.07 b	0.01 b	0.02 a	0.00 c	0.46 b
15	1.75 b	0.03 a	0.21 b	0.13 b	0.10 a	0.14 bc	1.16 a
20	11.33 a	0.02 a	5.73 a	1.17 a	0.15 a	2.02 a	2.24 a
25	10.68 a	0.01 a	6.75 a	1.31 a	0.28 a	0.46 b	1.86 a

B. Moistures (% WHC)

Mois.	Total	Vd	Fs	Fo	Tb	Cs	Other
			Tap roots -- 8 weeks				
40	1.38 a	0.04 b	0.79 a	0.08 a	0.22 a	0.07 b	0.18 b
60	1.53 a	0.21 a	0.64 a	0.10 a	0.15 a	0.06 b	0.36 a
80	1.88 a	0.01 b	0.66 a	0.12 a	0.55 a	0.38 a	0.16 b
			Tap roots -- 12 weeks				
40	3.35 b	0.13 b	1.25 b	0.21 a	0.48 a	0.23 b	1.05 a
60	5.43 b	0.47 a	2.83 b	0.30 a	0.72 a	0.24 b	0.88 a
80	10.80 a	0.01 b	6.94 a	0.46 a	0.59 a	1.50 a	1.29 a
			Lateral roots -- 8 weeks				
40	0.66 a	0.02 a	0.24 a	0.12 a	0.11 a	0.06 a	0.12 a
60	0.57 a	0.03 a	0.13 b	0.10 a	0.08 a	0.11 a	0.13 a
80	0.48 a	0.01 a	0.08 b	0.07 a	0.05 a	0.08 a	0.21 a
			Lateral roots -- 12 weeks				
40	5.39 a	0.02 a	2.57 a	0.64 ab	0.26 a	0.50 a	1.41 a
60	5.72 a	0.02 a	3.35 a	0.93 a	0.14 a	0.53 a	0.93 a
80	6.98 a	0.01 a	3.66 a	0.40 b	0.02 a	0.94 a	1.95 a

[1], [2]: See Appendix 4-3-1 for explanation.

Appendix 5-2-1. Mean percentage inhibition of spore germination of seven fungal species on corn meal agar containing four concentrations (μg/ml) of different fungicides 20 h after inoculation.

Fungicides	Concen.	Tb	Cd	Cs	Fs	Fo	Gr	Vd
EBI								
Fenarimol	0.1	0.3	2.0	0	2.0	0.3	1.9	1.6
	1	0.3	2.7	0	3.7	0.3	1.7	24.1
	10	3.0	5.2	0	8.7	1.7	9.0	71.8
	100	7.0	34.8	0	13.3	0	72.5	67.8
Flutriafol	0.1	2.8	1.4	0	1.0	1.0	2.0	0
	1	1.4	1.7	0	0	1.7	1.0	0
	10	1.4	1.4	0	3.7	0.7	2.0	56.4
	100	19.3	4.0	0	6.4	33.1	100.0	66.0
Prochloraz	0.1	3.1	2.0	0	3.7	20.2	85.9	39.4
	1	2.1	4.3	0	7.0	20.7	97.0	55.8
	10	3.1	4.4	0	6.7	23.1	100.0	70.4
	100	3.8	4.0	29.2	5.4	40.1	100.0	72.5
Propiconazol	0.1	4.5	2.7	0	1.7	1.3	11.8	10.0
	1	7.3	2.7	0	3.7	2.7	84.8	49.7
	10	5.9	5.0	0	8.7	13.9	93.3	52.2
	100	5.5	3.7	0	9.7	26.7	91.3	45.5
Triadimefon	0.1	7.6	2.7	0	1.0	1.7	6.4	5.7
	1	5.9	1.4	0	1.3	0.7	8.4	3.8
	10	4.4	3.4	0	2.6	1.3	5.8	4.7
	100	6.9	1.7	0	11.7	2.0	42.5	6.8
Triadimenol	0.1	5.9	1.4	0	2.0	1.0	3.0	3.0
	1	3.1	0.7	0	3.7	1.4	3.7	10.8
	10	3.8	2.7	0	3.4	1.7	1.7	53.2
	100	4.5	1.3	0	12.3	1.7	22.4	52.0
Other systemics								
Benomyl	0.1	1.1	2.0	0	4.4	1.3	5.2	3.6
	1	3.1	1.4	0	3.7	0	9.9	2.7
	10	0.4	2.4	0	3.7	0	8.7	4.3
	100	1.4	1.3	0	3.4	0.3	9.5	4.3
Iprodione	0.1	4.4	2.3	0	1.0	0	2.4	0.2
	1	2.1	1.0	0	1.7	0	3.7	0
	10	2.0	1.0	0	1.4	0	17.1	71.2
	100	1.4	0.7	0	1.0	0	24.7	32.7
Metalaxyl	0.1	0	0.7	0	2.0	0.3	4.3	1.1
	1	0	1.0	0	1.7	0	1.7	2.2
	10	0.7	2.0	0	2.0	0.7	4.2	1.1
	100	1.1	1.0	0	2.4	0	2.5	8.4
Protectants								
Captafol	0.1	1.3	76.6	0	16.0	48.2	80.8	25.7
	1	100.0	100.0	100.0	100.0	100.0	100.0	93.1
	10	100.0	100.0	100.0	100.0	100.0	100.0	100.0
	100	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Thiram	0.1	27.3	1.3	0	1.0	0	4.1	3.2
	1	50.0	3.0	0	16.0	39.4	100.0	4.3
	10	100.0	100.0	100.0	100.0	100.0	100.0	100.0
	100	100.0	100.0	100.0	100.0	100.0	100.0	100.0

Appendix 5-2-2. Mean germination percentage of red clover 7 days after sowing 10 seeds in each petri dish[1] on filter papers soaked with four concentrations of different fungicides.

Fungicides	1 µg/ml		10 µg/ml		100 µg/ml		1000 µg/ml	
	25°C	15°C	25°C	15°C	25°C	15°C	25°C	15°C
Control	83.3	90.0	83.3	90.0	83.3	90.0	83.3	90.0
EBI								
Fenarimol	96.7	93.3	93.3	93.3	86.7	86.7	80.8	83.3
Flutriafol	76.7	86.7	80.0	90.0	83.3	73.3*	73.3	66.7*[2]
Prochloraz	93.3	90.0	96.7	93.3	86.7	80.0	63.3	86.7
Propiconazol	80.0	80.0	76.7	86.7	50.0*	86.7	80.0	80.0
Triadimefon	96.7	90.0	83.3	93.3	43.3*	90.0	50.0*	63.3*
Triadimenol	90.0	86.7	86.7	86.7	73.3	83.3	76.7	83.3
Other systemics								
Benomyl	86.7	90.0	86.7	90.0	86.7	90.0	90.0	90.0
Iprodione	90.0	93.3	83.3	90.0	76.6	93.3	83.3	83.3
Metalaxyl	90.0	93.3	93.3	93.3	70.0	90.0	53.3*	73.3
Protecants								
Captafol	86.7	93.3	76.7	83.3	90.0	86.7	80.8	93.3
Thiram	80.0	100.0	83.3	90.0	93.3	90.0	100.0	86.7

[1]. The petri dishes were incubated in the dark at 15 or 25°C.

[2]. The symbol * indicated that means were significantly different from control at 5% probability level in an analysis of transformed values (arscine).

Appendix 6-2-1. Mean daily soil temperatures at 10 and 20 cm depth, and rainfall over five periods between harvests and of 30 year average.

Weeks after planting	Date	Soil temperatures (°C) 10 cm	20 cm	Rainfall (mm)
0--8	Currently	18.1	19.5	2.3
	30 years	18.0	18.8	2.4
9--16	Currently	14.8	16.0	3.4
	30 years	15.9	17.0	2.3
17--39	Currently	9.3	10.3	2.0
	30 years	8.7	10.4	2.8
40--49	Currently	13.6	14.4	2.3
	30 years	13.2	13.8	2.5
50--62	Currently	17.6	18.8	2.8
	30 years	17.8	18.6	2.6

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