Some Interactions Between the Clover Cyst Nematode *Heterodera trifolii* Goffart and Resistant and Susceptible White Clover *Trifolium repens* L.

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ABSTRACT

Although white clover Trifolium repens L. contributes significantly to the New Zealand economy through nitrogen fixation and its nutritive value to stock, its productivity and persistance are restricted by many diseases and pests. A plant breeding programme has resulted in white clover genotypes of widely differing resistance to the clover cyst nematode Heterodera trifolii Goffart. The most important resistance mechanism operates after nematodes have penetrated roots: they are unable to progress beyond the J2 stage. The few nematodes that do succeed in reproducing on resistant plants are smaller and produce fewer eggs than their counterparts on susceptible genotypes. Progeny of these successful individuals are more likely to succeed in parasitising resistant genotypes than is the broader H. trifolii population. Since considerable damage to seedlings may occur without establishment of a feeding site, resistant seedlings are not necessarily more tolerant of H. trifolii than are susceptible seedlings. Nevertheless, some seedlines exhibit high levels of both resistance and tolerance. Selection of an appropriate fertiliser regime was important to the experiments in this thesis and in resistance screening. Inappropriate regimes restricted the number of cysts on a susceptible genotype.

A number of people have kindly helped in the production of this thesis. Chris Mercer of AgResearch Grasslands has allowed me the use of work time and research facilities, co-supervised, and generally instilled in me a zeal for nematology over many years. Terry Stewart of the Department of Plant Science has been an encouraging and efficient supervisor. Special acknowledgement is due my wife Janine, who took on the lion's share of domestic responsibility over the months of thesis preparation. Her parents, Keith and Beryl Malkin, have let me use their office facilities. Thank you all.

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CHAPTER 1 THE ROLE OF WHITE CLOVER IN NEW ZEALAND AGRICULTURE

White clover (Trifolium repens L.) has been said to contribute more to the New Zealand economy than any other plant. It was estimated by Mercer (1994) to be worth over $1.8 billion annually and so to outstrip kiwifruit and pipfruit at $495 million and $286 million respectively. Only a fraction of this $1.8 billion figure - the $19 million value of white clover seed exports - is generated directly; the balance is contributed by way of nitrogen fixation ($1.16 billion) and value as animal feed ($700 million) and may be calculated by the value of fertiliser nitrogen and animal products.

Legumes are sown in pasture primarily in order to convert atmospheric nitrogen (N) into compounds of use to plants. This is achieved by bacteria living in symbiotic relationship with the plant; in white clover's case the bacterium Rhizobium trifolii is to be found in nodules on the roots. Within these nodules, atmospheric nitrogen is reduced to ammonia via the nitrogenase enzyme system, then incorporated into amino acid compounds in other plant organs (Crush 1987). By decay of these organs, and via grazing by animals, N is cycled to pasture grasses. Since N is the element most limiting production of developed pasture (Watson & Barker 1993), traditional fertiliser programmes have aimed at growing legumes well and in this way enhancing N availablity to grasses. It has been estimated (Steele 1982) that 1.1 million tonnes of N are made available each year by this mechanism operating in New Zealand's white clover.

White clover contributes directly to animal production through its high digestibility (Thomson 1984). Compared with pasture grasses, white clover herbage is less fibrous and has a higher ratio of soluble to insoluble

carbohydrate; these attributes result in rapid growth of stock on clover-dominant pastures. In dairy farming, white clover's high complement of calcium and magnesium help combat metabolic disorders, while its high protein content contributes to the non-fat milk solids for which the farmer is currently paid.

The other attributes of white clover that make it the legume of choice in many situations have been thoroughly reviewed by Watson & Barker (1993). The species' great phenotypic plasticity allows it to adapt to a wide range of environments in continental, mediterranean, oceanic and subalpine regions; this adaptability extends to the micro-environments found across a single pasture. Such adaptability is maintained by outcrossing - the plant's near-obligate mode of pollination - and the wide spreading of pollen by bees.

White clover's prostrate habit allows it to persist under grazing. This was clearly shown in an experiment by Brougham (1960) in which herbage production of pasture components was measured under lax or hard rotational grazing by cows. When grazed down to 7.5 cm the prostrate white clover cultivar Huia produced similarly to the erect red clover cvr Turoa; grazing to a 2.5 cm residual resulted in Huia outproducing Turoa nearly tenfold. The prostrate habit is made possible by the production of creeping stems, or stolons. About four weeks after seedling emergence, these stolons begin to grow horizontally from the seedling crown. Leaves and adventitious roots arise from the nodes of the stolons, which may also branch to produce secondary and higher order stolons. Since the nodes possess tissue able to form all the necessary organs, they each have the potential to become a new plant in their own right. Indeed, this is what happens in pasture; the original taproot and older stolons senesce and, over the seasons,

a patch of clonal individuals forms. Under hill country conditions, nearly all stolons are renewed annually; fewer than 10% survive more than a year (Chapman 1983). In addition to allowing white clover to colonise new soil areas and thus compete in the sward, the stoloniferous habit reduces the effects of crown and root pathogens that limit persistance of tap-rooted legumes (Watson & Barker 1993).

The density of nodes and growing points determines white clover's ability to recover from drought, and is itself somewhat determined by the plant's order of stolon branching. In situations of severe environmental stress, the plant's ability to produce seed begins to outweigh stolonation as the factor most influencing persistance. The cultivar "Prop" has been selected for early, profuse flowering and is therefore claimed to be suited to areas of summer dryness. An important feature of white clover seeding is the production of dormant seed. This so-called " hard" seed is impermeable to water and represents a soil seed reservoir able to germinate at a later date.

Despite these attractive features, limitations to white clover's usefulness are apparent. Watson & Barker (1993) thought that principal among these are plant death when soils of <30% w/w moisture are associated with high temperatures, and displacement by other legumes in situations where annual regeneration is required. Displacement by grasses will also occur where grazing is too lax or infrequent; although stolons may "float" in the sward to some extent, shading by long grass is generally detrimental. Animal production can be adversely affected by T. repens. Most seriously, cattle feeding on the species can develop the condition, known as "bloat", when a build-up of foam in the rumen prevents the normal expulsion of fermentation gases.

White clover in New Zealand agriculture.

Some 60% of all dairy herds are affected by bloat each year; 40% suffer stock loss through the disease (Holmes & Wilson 1994). Of more minor importance are interference with cattle reproduction due to oestrogens from fungi on clover leaves and occasional feed taint in dairy products (Williams 1987).

The species is host to a wide range of pest and disease-causing organisms. Larvae of the Lepidopteran porina (Wiseana spp.) and the beetle Tasmanian grass grub (Aphodius tasmaniae Hope) feed on aerial plant parts and are fairly well known to farmers. Similarly the larvae of the native Grass grub (Costelytra zelandica White) are a justifiably acknowledged root-feeding pest with a preference for white clover over grasses (Kain et al. 1979). Damage caused by slugs and the springtail known as "lucerne flea" (Sminthurius viridus L.) is sufficiently recognised that farmers attempt chemical control. The former are particularly known as pests of clover in establishing swards; metaldehyde pellets may be broadcast to limit damage. The springtail is sometimes controlled by the use of organophosphates in spring or by juvenile hormones in autumn. In the Waikato it is more commonly known by the name "clover flea", reflecting the current importance of two legumes in that region.

Many genera of fungi are associated with white clover. Among the leaf diseases, Sclerotinia trifoliorum Erikks is considered important and has been recorded in most countries, including New Zealand, where clover is grown (Latch & Skipp 1987). Resistance screening is undertaken in New Zealand selections targeting the European market. Sooty blotch - caused by Mycosphaerella fillianii Petrak - can produce very severe symptoms in autumn (Latch & Skipp 1987). Plant yield is reduced by stunting and partial defoliation, and toxins and oestrogens in

infected herbage may affect fertility in sheep.

In addition to these up-front pests white clover is attacked by some lower profile organisms that Watson & Barker (1993) believed may have even more deleterious consequences upon productive sustainability of pasture. Among these are viruses, nematodes and perhaps root-infecting fungi. The former have been well reviewed by Latch & Skipp (1987) who describe 23 virus species detected internationally; in New Zealand, Alfalfa mosaic virus (AMV) and White clover mosaic virus (WCMV) are among the more important. Both of these have been reported to induce a yield reduction exceeding 25% in white clover grown in the glasshouse; the number of nodules may also be much lower on plants infected with either virus. Additive effects occur in plants infected by more than one virus.

Among root-infecting fungi, Fusarium oxysporum Schlect seems particularly prevalent although vigorously growing plants may host the fungus without showing symptoms (Latch & Skipp 1987). Interactions between root rots and other organisms are suspected, but the complexity of these relationships makes them difficult to quantify.

Two species of root-feeding nematode are regarded as primary causes of poor clover growth and N fixation (Watson et al. 1985). A species of root-knot nematode - of the genus Meloidogyne - causes swelling and division of cells of the root cortex as it forms a specialised feeding site. There are two implications. The swellings, or galls, disrupt the normal rooting pattern and thus reduce the plant's ability to compete with other pasture plants and especially to recover from drought. Secondly, the specialised feeding site acts as a sink for the plant's nutients and so chronically debilitates it. The nematode species is widely distributed throughout New

Zealand, although not present in the coolest areas. Formerly thought to be the Northern root-knot nematode (M. hapla Chitwood), this has recently been shown not to be the case (Mercer unpub. data) in spite of similar cytology (Mercer & Grant 1993b) and morphology. The other important root parasite - and subject of this thesis- is the clover cyst nematode (Heterodera trifolii Goffart). Almost ubiquitous in distribution - Yeates (1975) found H. trifolii in 76 of 77 sites - this animal owes its great tolerance of heat, cold, and dessication to the production of a resistant egg-filled cyst. It is particularly considered a pest of seedlings (Wilson 1978) and perhaps small plants regenerating from excised stolons. Other root-parasitic nematodes, including species of the genera Pratylenchus, Helicotylenchus, Paratylenchus and Paratrichodorus have been recorded in association with white clover, but their pest status remains unquantified. Above the ground, the stem nematode Ditylenchus dipsaci (Kuhn) Filipjev can be damaging to establishing seedlings (Williams & Barclay 1972). Screening of seedlings quickly identified resistance to stem nematode, but the most resistant genotypes were of the Ladino type; that is, they were large-leaved and erect and so not of wide agronomic suitability. Nevertheless the cultivar Grasslands Kopu - suitable for rotational grazing by dairy cows - has a degree of resistance (DSIR Grasslands 1989). D. dipsaci does not seem to be a serious pest of established plants (Grandison 1965) although symptoms are sometimes observed in Winter.

The extent of herbage yield loss due to nematodes was investigated by Watson et al.(1985). During a series of 16 field trials, plots receiving nematicide produced an average of 13% more herbage than untreated control plots;this was due mainly to a 40% increase in clover herbage. N fixation was increased by 57%. The fact that

so much of the yield response occured in the clover, rather than the grass componant, is evidence that these results reflect a genuine response to a decreased pest burden, rather than merely to a nitrogen flush which, as others have suggested, would follow invertebrate death from the nematicide. In similar trials Watson et al.(1993) have reported increased total pasture dry matter yields of up to 1600 kg/ha between spring and mid-autumn. The largest response was, again, from the white clover componant. Particularly noteworthy was the fact that, during a summer drought, twice as many clover growing points were to be found in the treated compared with the control plots; this promoted clover recovery when growing conditions were restored in April, and prompted a huge response in autumn N fixation.

While I have attempted to identify individual pest and pathogenic agents, and to quantify the damage caused by some, clearly to do so is to dramatically oversimplify the situation in nature. Several tiers of factors influence the plant's persistance and productivity. Beyond the host/agent relationship are interactions among these agents and with other organisms. Some root pathogenic fungi, for instance, may be aided in invading roots by the action of nematodes, but may also parasitise the nematodes' eggs (Watson & Barker 1993). Beyond this sort of relationship again are the environmental influences including temperature and moisture, the role of competing species of pasture plant and management practices. Thus, for example, the full picture of white clover's recovery from drought may depend not only upon density of nodes, but also the effects of nematodes and fungi, fertility, and grazing management following rain.

Plant breeders have sought to improve white clover for about 80 years. In spite of this relatively short time

frame, selection for an extensive list of characters has occured. The history has been reviewed by Williams (1987), and characters include plant morphology, tolerance of cold, drought, and low phosphorus soils, aspects of flowering and seeding, nitrogen fixation and pest and disease resistance. The latter has not seen many successes, perhaps because resistance is frequently polygenic and so many cycles of selection and crossing are necessary to stabilise the character (Mercer et al. 1992). In recent times the crossing of white clover with other Trifolium species and insertion of foreign DNA have come to complement traditional breeding techniques. Breeding approaches to the nematode problems will be outlined in the next chapter.

White clover in New Zealand agriculture.

CHAPTER 2 THE CLOVER CYST NEMATODE.

The so-called cyst nematodes are included in the 19 genera (Stone 1985) of the family Heteroderidae. Most, including all agronomically important species, are characterised by the formation of a toughened sac, or cyst, by oxidation of the cuticle of the female nematode's body after death. The typical spherical,sub-spherical and lemon shapes of these cysts result from the swelling of the female's body to accommodate the enormous development of coiled, paired ovaries (Stone 1985). Eggs may be exuded into a gelatinous matrix, but, in many species, they are mostly retained within the female's body and so are later enclosed in the protective cyst.

Those species of cyst nematode regarded as serious pests tend to parasitise crops of temperate, rather than tropical, regions. Within the genus Globodera are the two pests of potato, G. rostochiensis (Stone) Mulvey & Stone and G. pallida (Wollenweber) Mulvey & Stone. Pest species of the genus Heterodera include the beet cyst nematode (H. schachtii Schmidt) - a pest of brassicas as well as sugar beet -the soybean cyst nematode (H. glycines Ichinohe), the cereal cyst nematode (H. avenae Wollenweber) and the clover cyst nematode (H. trifolii Goffart).

The Lifecycle of Heterodera trifolii. (Plates 2.2-2.6)

H. trifolii passes through four juvenile stages. First stage juveniles (J1) develop and moult within the egg. The nematode may remain within the egg for several years (Yeates & Visser 1979) as a second stage juvenile (J2) before emerging as the infective stage.In many species of Heterodera, the mechanism of stimulation of emergence differs according to whether the egg is within the cyst or in the gelatinous matrix (Ibrahim et al. 1993). Often, eggs in the cyst require stimulation by root exudates before hatching while those outside the cyst depend mostly on temperature. The ratio of eggs in these locations varies between species; it does not seem to have been studied

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extensively in H. trifolii, although Shepherd (1962) thought the degree of stimulation by root exudates only slight in this species.

Following emergence, the J2 are able to propel themselves through moist soil and along root surfaces (Mankau & Linford 1960). By persistant thrusting of the stylet, the nematodes are able to weaken cell walls of the root epidermis and thus penetrate the root. Penetration is facilitated by wounds - including those caused by the entry of other J2 - and the disturbance of tissue at the site of eruption of secondary roots. J2 moving along the root exterior will increase the frequency of stylet thrusting as they approach a wound (Mankau & Linford 1960), suggesting sensitivity to chemicals near these sites.

Suitable hosts in pasture appear to be restricted to T. repens and red clover (T. pratense), Lotus pedunculatus, and weed species of the genera Rumex and Chenopodium (Skipp & Gaynor 1987; Yeates et al. 1972).

Once inside the root, the J2 may migrate directly toward the stele, or tunnel along the cortex. If tunnelling continues through a root's apical meristem, severe disruption and even root death may occur; apparently more cells die than are actually touched by the nematodes (Mankau & Linford 1960).

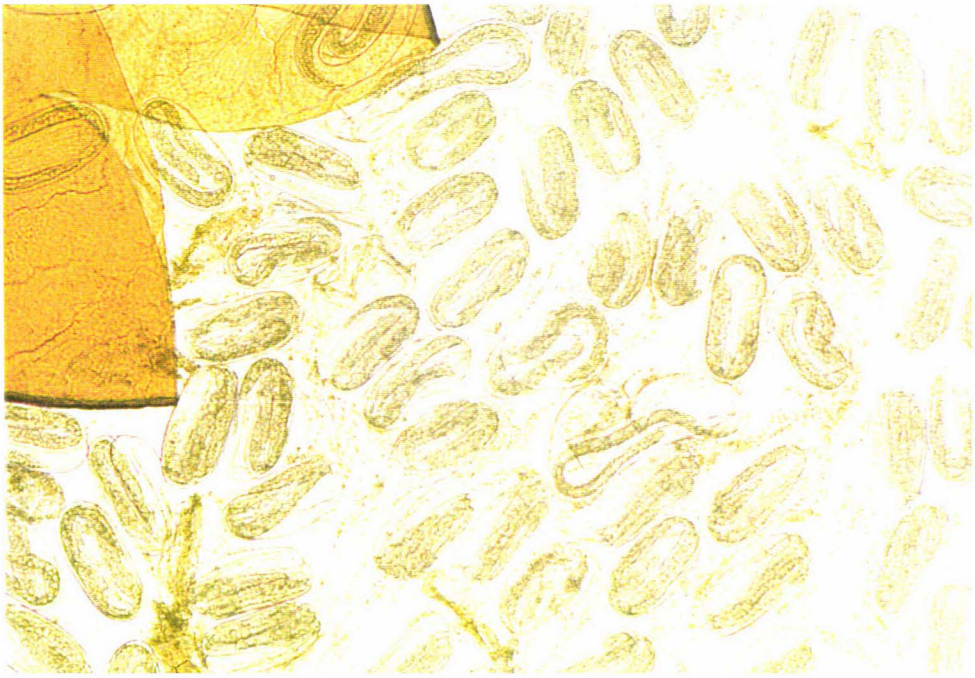
To continue its lifecycle, the nematode must form a permanent feeding site. Typically, the body is aligned along the root axis close to the stele, with the head "dipped" into an endodermal cell (plate 2.4). A dense, multinucleate mass - the syncitium - is formed by lysis of walls of cells within the stele. From this stage the nematode is sedentary, feeding from the syncitium. Three more moults occur as the nematode swells toward the characteristic lemon-like shape of the mature female and cyst. First production of the next generation of eggs has been observed 20-30 days after hatching

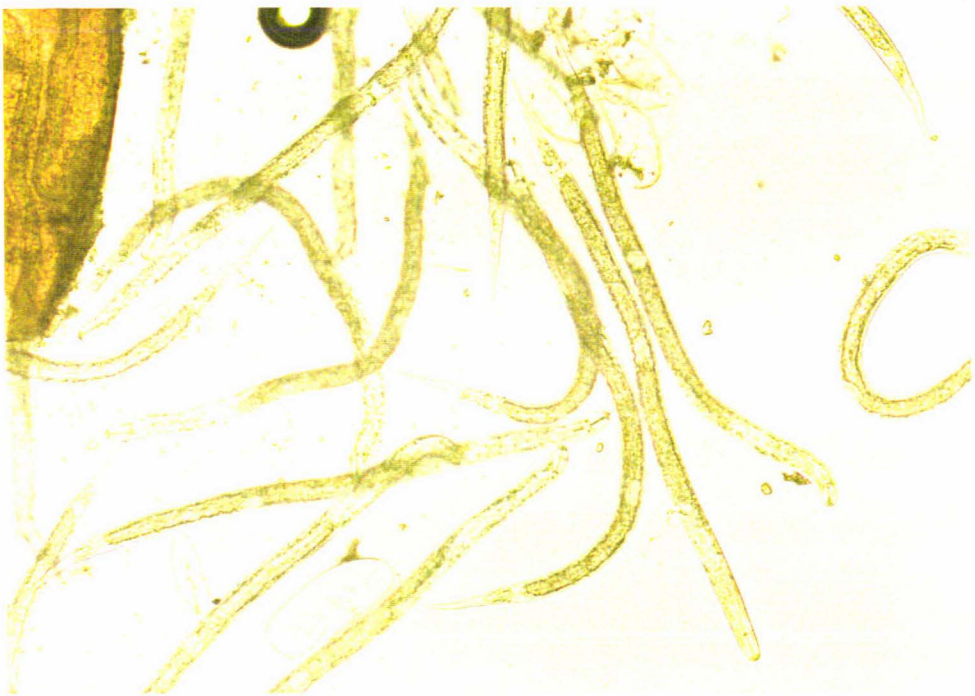
The *H.trifolii* lifecycle.

Plate 2.1. Eggs from a ruptured brown cyst. These would make excellent experimental inoculum, as they are nearly all embryonated and free of fungal and bacterial pathogens. (100x).

Plate 2.2. Second stage juveniles (J2).

Plate 2.3 Second stage juveniles within a root tip. (Stained after the technique of Byrd *et al*. (1983); 100x)





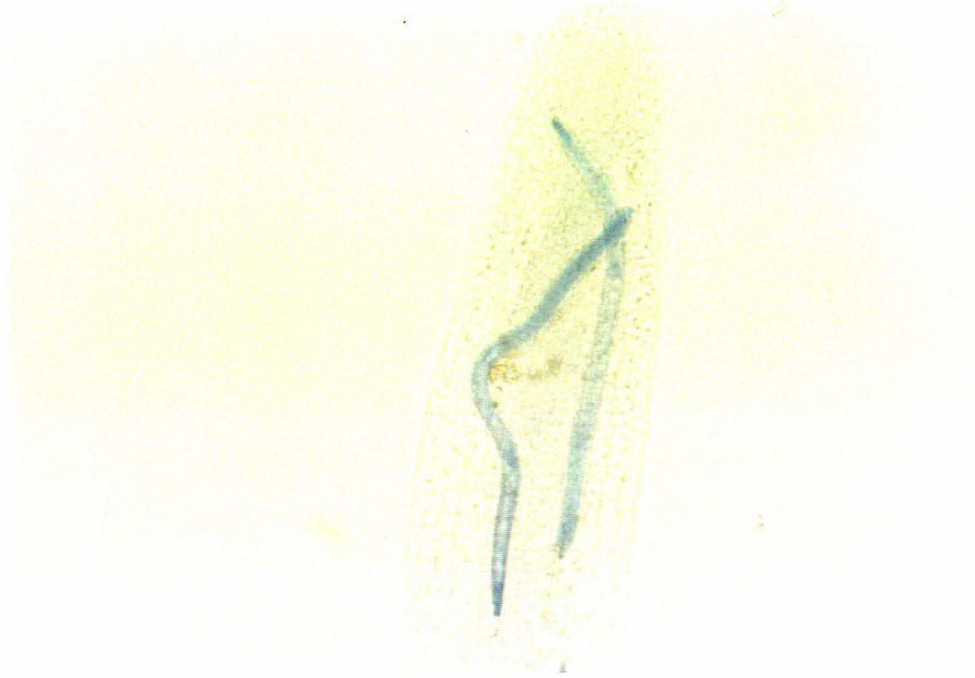
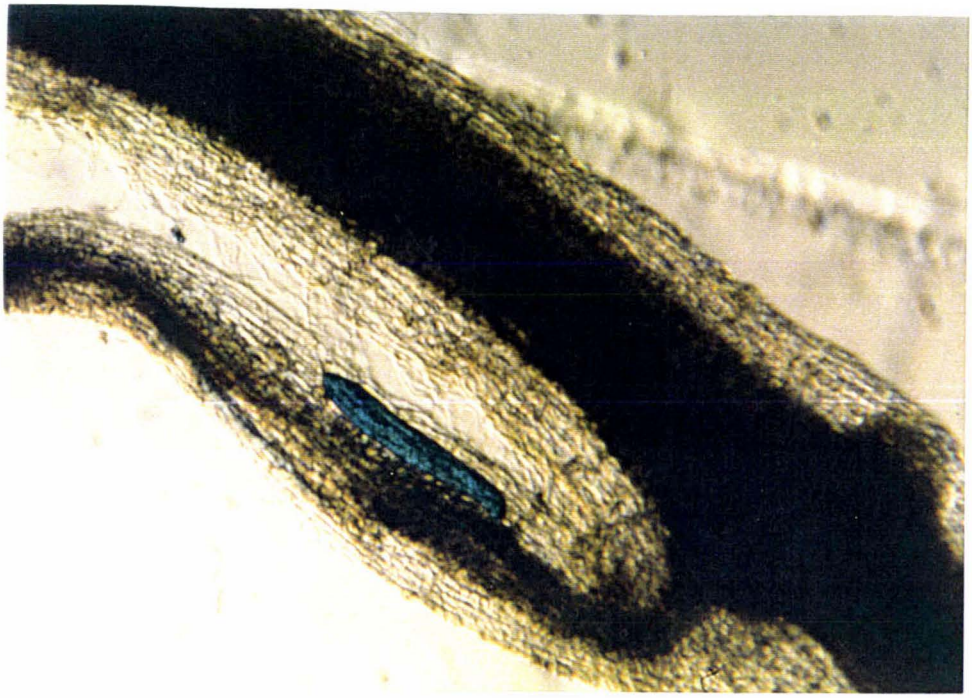
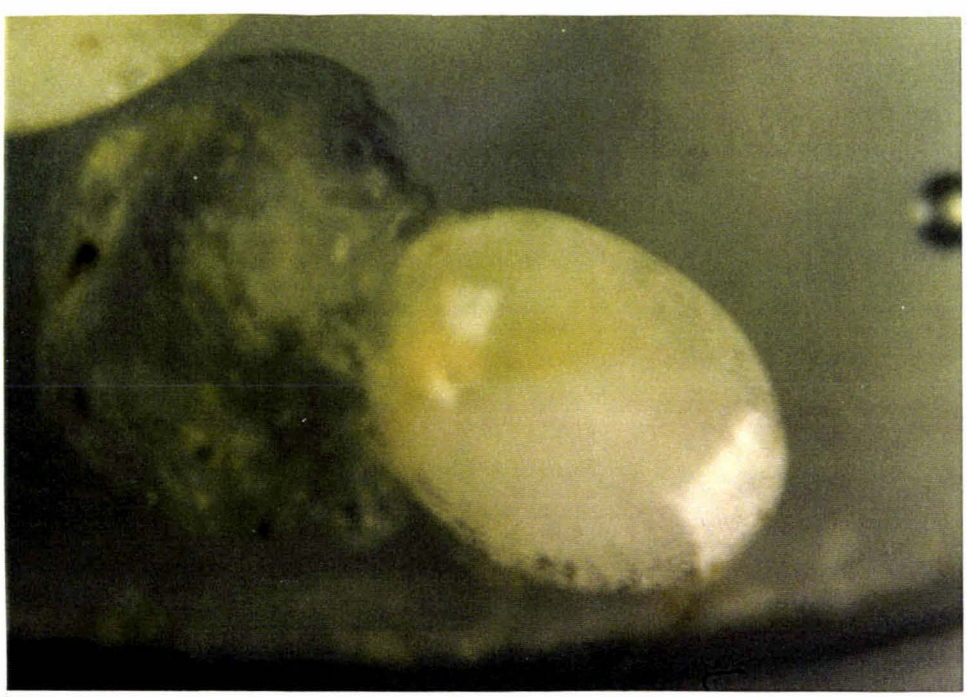


Plate 2.4. A second stage juvenile has turned its head toward the stele, initiated a feeding site, and begun to increase in diameter. In the terminology of chapter 5, this is a swollen juvenile (sJ2). The now-sedentary nematode will remain at his site, although the head may move. (Stained after the technique of Byrd *et al*. (1983); 100x)

Plate 2.5. A third stage juvenile (J3). (Stained after the technique of Byrd *et al*. (1983); 100x)

Plate 2.6. The lemon-shaped adult nematode. This individual has exuded some eggs into a large egg sac. After the adult's death the body will become a resistant, egg-filled cyst.





(Mercer 1990). Oogenesis is by obligatory mitotic parthenogenesis. In contrast to most species of Heterodera, all individuals of H. trifolii are apparently female; Wouts (1978) regarded the isolated reports of males as misidentifications.

The mature female nematode protrudes from the root surface. Following death of the female, eggs within the cyst may remain viable for at least 180 weeks (Yeates & Visser 1979).

The first report of H.trifolii's occurence in New Zealand, by Grandison (1963), coincided with considerable international interest in the species, particularly in the Netherlands. Dutch nematologists grappled with the nematode's pest status (Hidding et al. 1963; Seinhorst & Sen 1966); they also grappled with one another a little, as will be described in Chapter 6.

In New Zealand, the 1970's saw a flourish of research interest in pests, as the banning of DDT saw them coming into new prominence. H. trifolii came in for close scrutiny; studies ranged from those defining the annual cycle of the nematode on different soils, through glasshouse trials to study its effect on legume growth and nitrogen fixation, to multifactorial investigations of the effects of agronomic practices and nematicides.

A series of investigations revealed some variations in the nematode's lifecycle. Most notably, two periods of root invasion were observed on a yellow-grey earth at Masterton (Yeates 1973a) and on a yellow-brown earth near Invercargill (Yeates & Risk 1976), but only one on a yellow-brown loam at Hawera (Yeates 1973b). In each case invasion - measured by counting nematodes in stained root - coincided with periods of peak pasture production, but the invasion period could not be

adequately predicted by either soil moisture, rainfall or temperature data in isolation. Only at the Hawera site was the root-knot nematode present; perhaps this somehow correlates with conditions suitable for autumn H.trifolii invasion. At all sites a period of summer dormancy occured, during which cyst numbers were high but juveniles did not emerge or invade roots; the dormancy period lasted eight weeks in Southland but 14 weeks at Masterton and coincided with dry periods at both sites.

Yeates et al. (1973) studied the effect of H. trifolii on top yield of five legume species. They used methyl bromide to eliminate pests and pathogens from pots of sieved soil and so provide controls with which to compare plant production in pots of soil naturally containing H. trifolii. Red clover (T.pratense) and subteanean clover (T. subteraneum) were slightly affected and lucerne (Medicago sativa) rather more so; the most severely affected species were Lotus pedunculatus and white clover. After 33 days yields of parasitised plants of seven white clover seedlines were only 25-47% those of uninfested plants. Similarly, field trials revealed up to 46% more yield in fumigated white clover pure sward than in plots from which H.trifolii had not been removed (Yeates & al. 1975). Of particular note was the superior performance of unparasitised plants in a dry November, when it was thought that more intact root systems were better able to utilise the declining moisture supply. The view that these data might result from a nitrogen flush following fumigation, rather than from removal of nematodes, was refuted by Ross & McNeilly (1975). The work of Watson et al. (1985) again underlined the important pest status of clover nematodes. Nematicidal rates of insecticides were shown to result in major increases in N fixation and herbage yield at 16 North Island field sites.

With the view to developing pasture management techniques that would reduce the incidence of H. trifolii, Yeates & Visser (1979) investigated the survival of encysted eggs and juveniles under a range of environmental conditions. Although temperature and moisture effects on viability were noted, live juveniles were found after 180 weeks in all temperature/moisture combinations. In another experiment, Yeates (1978) noted rapid reinvasion of nematode-free sites when cysts are transported by stock. These data suggested it unlikely that management options to boost clover production via nematode removal would be found. Nevertheless, careful reading of the more recent work of Yeates (1992) in conjunction with Mackay et al. (1991) leads to the inference that such a goal may be achievable. These papers describe aspects of experiments in which a so-called pastoral fallow was used to return large quantities of organic material to the soil as a means of improving winter and spring production. Fallow plots were closed to grazing between September and April/May, then mob stocked in order to trample the accumulated plant matter. From the agronomic viewpoint, Mackay et al. (1991) reported that "In late winter and early spring after the fallow white clover stolons invaded the bare ground left by the decomposing plant litter." and that legume biomass was higher in fallowed than control plots in late summer. Yeates (1992) reported on the effects of the fallow on many nematode genera. He found the juveniles of Heterodera and Meloidogyne spp. to be "...significantly less abundant in the fallowed plot...". Since the samples were taken in November, when Mackay et al. (1991) found no difference in legume biomass between fallow and control plots, we are left to deduce that something has happened to the parasite population. Perhaps, as Yeates (1993) suggests, their populations were reduced during the fallow as tall grass shaded the legumes. As stolons invaded the open sites after trampling, they may have effectively "grown away" from the nematodes. Another possibility is that populations of bacteria antagonistic to the parasitic nematode were enhanced by the organic matter;

such a view is supported by the enormous rise in numbers of the bacterial-feeding rhabditid nematodes. If the latter were the case, superior legume production could be the result of suppression of parasitic nematodes. Notwithstanding these interesting results the benefits of fallowing lasted only a year in Mackay et. al's experiment; the cost of land out of grazing suggests this will be a little-used method of boosting production.

It might be said that the investigative work of the 1970's and 80's initiated parallel arms of research which, following reforms in Government-funded research, have now coalesced. Efforts based at Ruakura Research Centre - until recently under the Ministry of Agriculture and Fisheries - have concentrated on further investigation of nematode biology and pest status and a vigorous selection programme to identify white clover seedlines that produce well in the presence of pests. The programme involves growing seedlings in boxed soil naturally infested with clover cyst and root-knot nematodes. A small percentage of seedlings - those growing most vigorously - are selected for replanting in the field. By transplanting through a black polythene mulch temperature and moisture conditions can be manipulated so as to magnify the parasitic nematode population by as much as seven times (R N Watson pers. comm.) The genotypes are categorised in small, medium and large leaf size classes and scored for production; thus there also exists a degree of automatic selection for ability to produce in the presence of other pest and disease agents. The best material is retained for breeding. At a fairly early stage in the programme, some selections are already outyielding established cultivars (R N Watson pers. comm); this superiority seems to result from factors other than a particularly high level of resistance to nematodes (Grant & Mercer unpub. data).

At the Research Centre in Palmerston North - formerly

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belonging to DSIR and currently to AgResearch Grasslands - a glasshouse programme designed specifically to select plant genotypes for resistance to H. trifolii is in operation. Initially, seed sourced from a wide variety of New Zealand and overseas sources was germinated and the seedlings challenged with a known number of nematodes. The number of cysts resulting from this inoculation provided a measure of the level of resistance of individual seedlings and of seedlines. Parts of this screening technique have been basic to some of the work described in this thesis, so further technical description will be given in Chapter 3. The most resistant material from the initial screening was retained and crossed by caging plants with bumblebees. In order to maintain susceptible genotypes for comparison, crossing of a few susceptible plants was also perfomed. Selected progeny were then subjected to a similar selection and breeding programme. Four of these breeding cycles have now been completed and extreme seperation of types achieved; in the most recent screening the resistant genotypes hosted only 4% the cyst number of the susceptible and many individuals were completely free of cysts.

Other possible avenues toward nematode control are being explored by AgResearch Grasslands. With the view to interspecific hybridisation, other legume species have been tested for H. trifolii resistance (Mercer 1989). Some crossing work has been undertaken, and a hybrid with resistance from parents of two species is a future possibility. With the view to achieving biological control, the fungal community associated with the cyst is being investigated (Hay 1993). Some species found in clover roots have been isolated from within cysts and shown to parasitise the eggs. Manipulation of these potential control agents may be a way of improving clover production; such approaches, via an understanding of rhizosphere ecology, have been promoted by Watson & Barker (1993).

Availability of white clover genotypes of widely differing susceptibility to H.trifolii has opened the door to more

in-depth study of the host/parasite relationship. It is against this background that the experiments described in this thesis have been designed. In Chapter 5 an answer is sought to that favorite question of seminar and conferance delegates: "...but what is the mechanism of resistance?". This is a practical as well as an academic issue; for instance, crossing plants manifesting different mechanisms could result in a type with a suite of resistance. Chapter 6 examines the issue: will resistance actually help a plant be more persistant or productive in infested soil? It also looks at some of the models available in assessing nematode damage and considers what relevance these might have to the goal of improving white clover. The experiment of Chapter 7 is more nematode-centred than plant-centred; the goal is to determine whether consistant exposure of nematodes to resistant plants might alter the parasite population's ability to reproduce on them. Chapter 4 is a little different in that it is backwards - as well as forwards - looking. As a necessary prerequisate to the thesis, the experiment aims to assess the suitability of fertiliser regimes used in experiments and screenings. Desirable features of fertilisers are that they allow a true expression of the plants' resistance status while being quick to prepare and apply. The compound fertiliser used in early screenings did not meet the second of these requirements; Chapter 4 describes the testing of possible alternatives.

CHAPTER 3 GENERAL METHODS AND MATERIALS

In order to provide inoculum for experiments and for screening plants for resistance, a glasshouse colony of *H. trifolii* is maintained at Grasslands. Colony maintenance involves growing white clover plants from seed and inoculating them with nematode eggs. Scarified seed is scattered over a sand/topsoil medium in 10 cm diameter pots placed directly on a heated wet mat. This mat consists of a layer of capilliary matting (Ralta, Palmerston North) over which is a permeable plastic sheet is laid. A 4 x 2 cm wick cut from the capilliary matting material protrudes from the bottom of each pot and is the route by which daily automatic watering maintains the soil medium at field capacity. The system is maintained at a minimum of 10 °C by means of an electric blanket under the mat. Probes embedded in the soil allow temperature to be recorded every six minutes; customised software graphs this information and can use it to calculate accumulated temperature-time.

Three weeks after germination the seedlings are inoculated with eggs of *H. trifolii.*
Two generations of the resulting nematodes are usually allowed; frequently populations of half a million eggs per pot are achieved after 16 weeks. Mature cysts may be stored at 10°C for several weeks until required; it appears that more eggs are produced during this period of storage and that the incidence of fungal disease infecting the eggs is lower at 10° C than on the wet mat. A chill is also thought to encourage hatching in *H. trifolii* (Shepherd 1962). The inoculum for a new colony is usually obtained from an earlier glasshouse colony, but periodically fresh collections of cysts from the field are made so as to avoid selection of a nematode type specific to the glasshouse conditions.

Inoculum for experiments described in this thesis was extracted by sieving and centrifugation. A pot of infested plants was emptied over nested 200 mm diameter sieves

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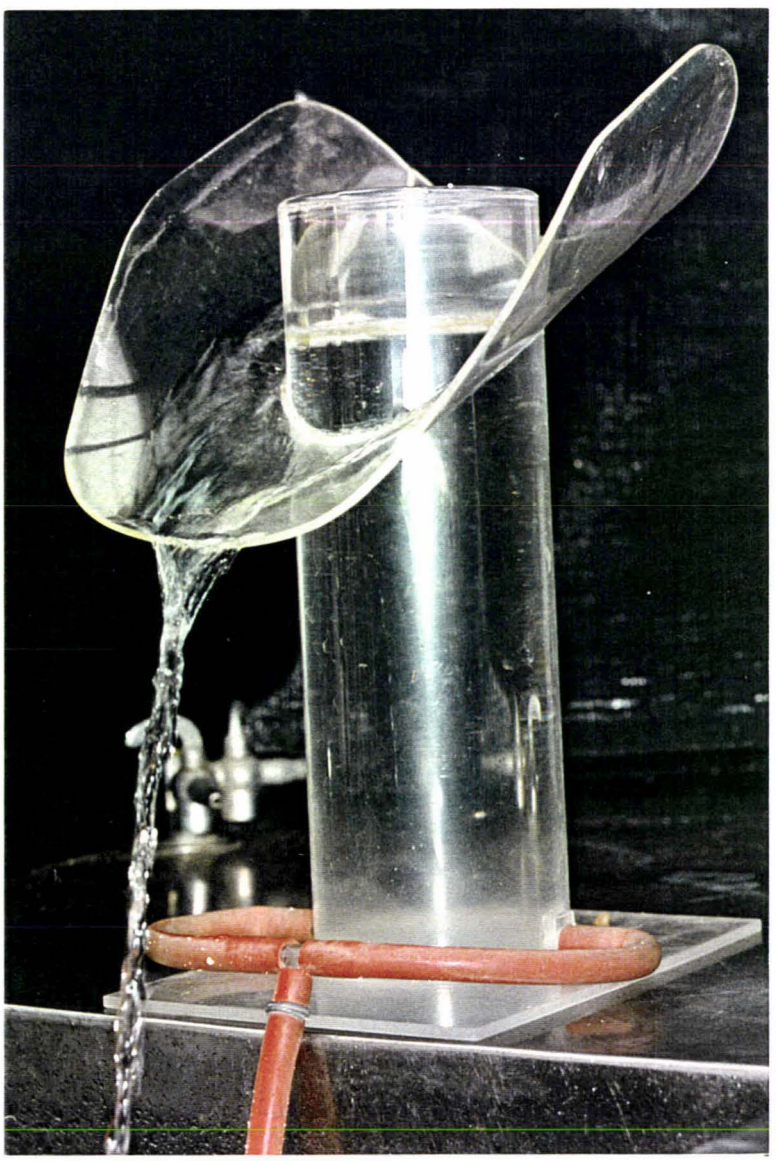
Plate 3.1. Individually-potted white clover plants grown in the glasshouse. Steel trays facilitate application of water and nutrients and are rotated about the glasshouse to minimise the effects of temperature and light gradients.

Plate 3.2. The elutriation tower. When the contents of plant pots are lowered into the top of the tower, the flow of water carries organic matter over the rim to be caught in sieves. Heavier mineral matter sinks to the bottom of the tower.



(Endecotts, London) of 3 mm and 600 and 180 micron aperture. Tap water was used to wash the soil through the sieves, then the water flow was increased and used to dislodge cysts from the roots. The contents of the 180 micron seive were dried from below with a sponge before being transferred to four centrifuge buckets. A quantity of sugar solution (specific gravity = 1.25, made by dissolving 1200 g of white sugar in one litre of water), was added to the buckets, which were then centrifuged to a maximum of 2000 rpm. The effect of this was to compress most of the mineral matter into a pellet while leaving the organic matter suspended in the viscous supernatant. The suspension was poured through a 180 micron seive. The cysts, caught again in the seive, were rinsed free of sugar before being ground with a plastic kitchen spatula to free the eggs. A stream of water carried the eggs through the seive and into a collection vessel.

The number of eggs extracted was estimated by means of a Doncaster dish (plate 3.1). These dishes are engineered in such a way that each of the concentric rings contains a known proportion of the total contents; ring 1 holds 1%, ring 2 holds 3% and so on. To test the dish's accuracy, a 10 ml aliquot of egg suspension was pipetted into a dish and the eggs in each ring counted; this was done three times. Each ring seemed quite accurate, with the exception of the overestimating ring 9 (fig 3.1). In spite of the attempt to achieve a random distribution of the 10 ml over the dish, it seems that moving the emptying pipette backwards and forwards resulted in it spending slightly more time over ring 9 compared with other rings. The problem can be overcome by first pipetting the aliquot into a pottle, which is then emptied into the dish.

The convention in the Grasslands laboratory is to count 9% of the dish's eggs by examining the fifth ring. By setting a dissecting microscope to a 40x magnification it was possible to distinguish between embryonated and younger or diseased

Plate 3.3. The Doncaster dish. Each of the concentric rings contains a known proportion of the dish's total contents.

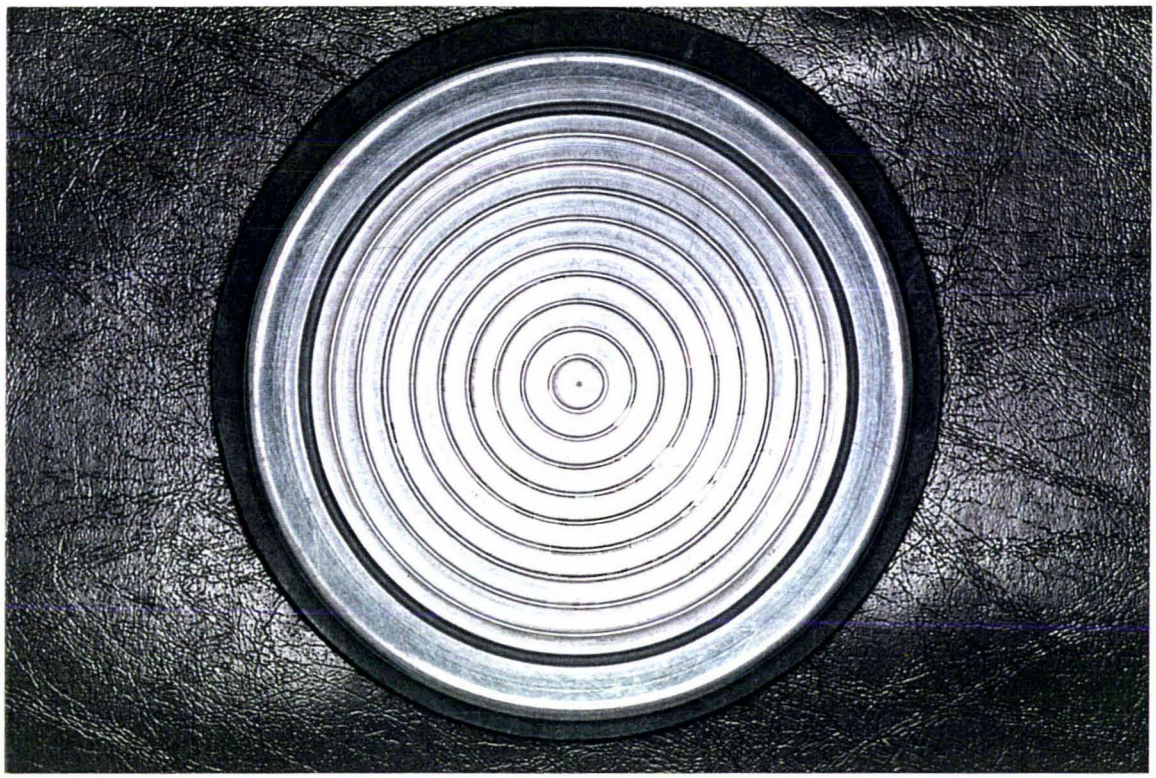
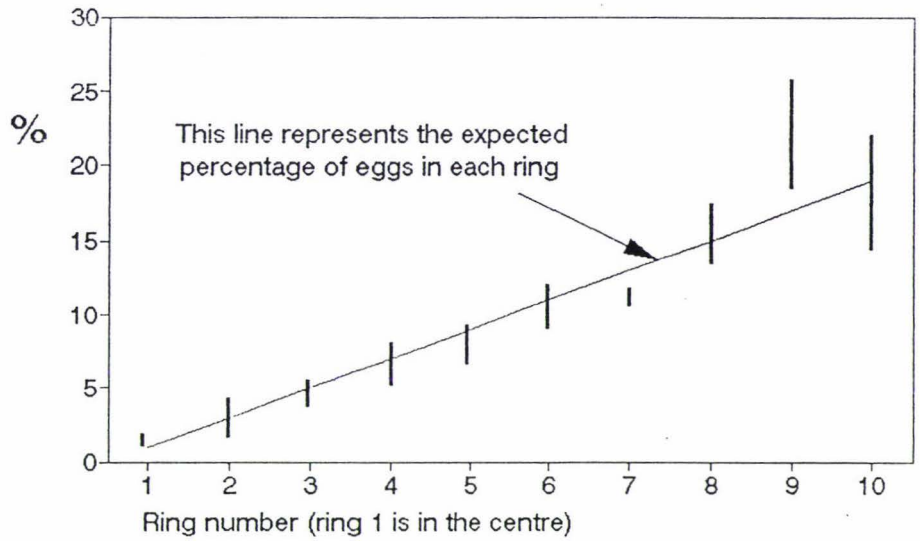


Figure 3.1. Percentages of the total number of H. trifolii eggs counted in each ring of a Doncaster dish. Vertical bars represent +ve and -ve standard errors of means of three replications of the experiment.


30
25
%
20
15
10
5
0
This line represents the expected
percentage of eggs in each ring
1
2
3
4
5
6
7
8
9
10
Ring number (ring 1 is in the centre)

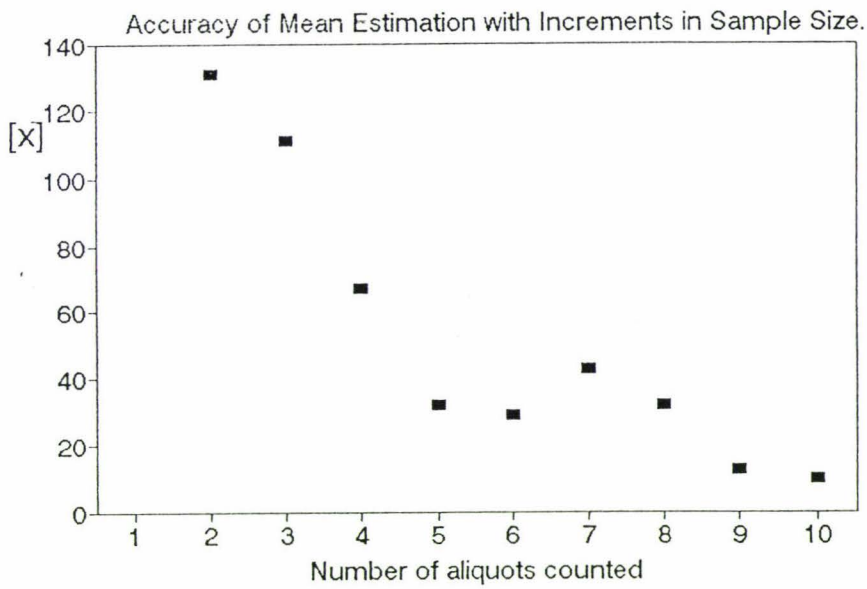
eggs, and so to gain some idea of the inoculum's quality.

A repeating syringe was used to inject inoculum under plants. Usually 3 ml of water was injected; accuracy was within a range of 0.02 ml either side of this. In order to determine how many assessments were necessary to accurately predict the number of eggs in 3 ml, aliquots were injected into nine 100 ml pottles each containing 7 ml of water. These were tipped into a Doncaster dish and the eggs in ring 5 counted. Each count was added to the mean of the previous counts so that a marginal change in the mean's absolute value could be found. A plot of these marginal changes against the number of counts suggested that four counts gave an acceptable balance between accuracy and speed (Fig. 3.2)

To inoculate a seedling or cutting, a pencil was first used to make a hole in the medium directly under the plant. Inoculum suspension was injected into the hole, which was then refilled with soil to prevent drying.

The soil medium was prepared from topsoil dug from pasture. The topsoil was spread on a covered concrete pad and turned several times each day to speed drying. When dry enough that fist-sized clods would shatter, peds of less than 6 mm diameter were collected by passing the soil through a mechanically-vibrated sieve. The resulting material was blended with sand (grain diameter < 3mm) by loading alternate shovelfulls into a concrete mixer; the mix was then held for 12 hours at 85-95° C in a steriliser (Sterisoil, Auckland.) This was effective in killing nematodes, but it was also necessary to store the soil in a deep-freeze to prevent reinvasion by saprophytic fungi that could later damage seedlings. Sometimes the medium would dry excessively during this process; it was then necessary to soak the potted soil overnight before transplanting germinated seed. This could be achieved by half-filling trays (layout as in Plate 3.1) and covering the pots with clear plastic sheeting to prevent

Figure 3.2. Marginal change in the estimate of the number of *H. trifolii* eggs in a sample, as more subsamples are taken and counted. Four subsamples give a satisfactory estimate.


Accuracy of Mean Estimation with Increments in Sample Size.
[X]
140
120
100
80
60
40
20
0
1
2
3
4
5
6
7
8
9
10
Number of aliquots counted

access of fungal spores.

White clover is readily propagated by cuttings. A challenge in this work, however, has been to produce material free of nematodes and of fungi that might interfere with nematode growth. In the latter category, vesicular arbuscular mycchorrizae are particularly important (Yeates 1987). When preparing a nodal cutting it was important to select those that had not come into contact with soil. The fresh cutting was soaked briefly in a fungicide suspension (active ingredient (a.i.) 1.6g/l captan), then pressed into a plant tray of moist sand which had been sterilised by heating 2 kg lots for 8 minutes in a microwave. The sand was kept wet with 1.6g/l a.i. captan and covered with plastic sheeting. Cuttings treated in this way would sometimes have a root a centimetre long within four days.

Plants raised from seed were germinated before transplanting. The seed was scarified by rubbing it briefly between sheets of sandpaper; the step is necessary to allow water to permeate the otherwise dormant seed. Double sheets of filter paper in a 10cm Petri dish were moistened with 80 mg/l a.i captan. Seed was sprinkled over the moistened filter paper and, as a further dormancy breaking technique, the Petri dishes were stored for 24 hours in 8° C refrigeration. Next they were transferred to a 20° C cabinet, where much of the seed germinated within 12 hours.

My attitude to statistical design and presentation has been challenged and moulded by Maindonald (1992) and especially by the book "The Visual Display of Quantitative Information" (Tufte 1983). The former was particularly helpful in stimulating consideration of what was wanted out of the experiment and how to efficiently obtain valid information. The latter taught me to consider how a graph might be designed to fulfill its basic function of communicating information. The book encourages the readers to consider whether they are

---

unwittingly sending extra, possibly untrue messages. Examples of these are the use of the wrong type of graph or the use of area or volume to represent what could be shown with fewer dimensions. This book was useful helpful in using graphical packages designed for the more flamboyant business world rather than sober scientific applications.

Quattro-Pro® (Borland, Scotts Valley) was used for data entry, manipulation and transformations. Most graphs were produced using the Quattro-Pro annotator. The non-linear functions of Chapter 6 were produced using CoPlot® (CoHort Software, Berkeley). The package CS-Statistica® (Statsoft, Tulsa) was used for analyses. It allowed the testing of the non-linear models of Chapter 6 as well as linear regression and comparison of treatments by t-tests and analysis of variance (ANOVA). The conservative Tukey's HSD test was used to separate means. A strength of this package is its ability to allow the ANOVA assumptions, such as the distribution of residuals, to be tested, along with the validity of transformations.

Raw data have been stored electronically.

The technique of Byrd *et al.* (1983) was used to stain nematodes within roots prior to microscopic examination. Four stages are involved in this technique:-

> the washed roots are decolourised by being immersed for 4 minutes in a 1.25% NaOCl solution,
>
> after a brief rinse, the NaOCL is removed from tissue by soaking the roots in tap water for 14 minutes,

paper towels are used to dry the roots, which are then boiled for 1 minute in the stain (1.25g aniline blue in 833 ml of each of glycerol, lactic acid and tap water),

storage in glycerol for at least 24 hours draws the blue stain out of the root tissue but not the nematode cuticle (see Plate 2.3). Adding 6 drops of 11 N HCL to 500 ml of this glycerol lengthens the roots' storage life.

CHAPTER 4 FERTILISERS FOR EXPERIMENTS AND SCREENINGS

Assessment of a plant's resistance to H. trifolii involves growing seedlings, inoculating them with the nematode's eggs and later counting the number of cysts and adult nematodes recovered by elutriation. Susceptible plants are included in the screenings to provide a benchmark from which to assess improvement in resistance over breeding cycles. It is therefore important that these plants host cysts in numbers large enough to allow contrast with resistant selections.

A challenge for the researchers has been to develop the optimum fertiliser regime for screenings. Nutrient additions are necessary for adequate plant and nematode growth, but excess nutrients have been linked with nematode suppression (Orion et al. 1980). Such suppression is strongly suspected to have occurred in Grasslands' screenings.

The objective of this experiment was to identify a nutrient regime that would allow a large number of cysts to develop on susceptible plants while producing plants of sufficient size to maximise the juvenile nematodes' chances of finding a penetration site.

MATERIALS AND METHODS

Data from a resistance screening were analysed in order to select a seedline that combined susceptibility with narrow variance among individual plants in terms of numbers of nematodes hosted.

The seeds were germinated on moist filter paper in a Petri dish. A seedling was then transplanted into a 6 cm diameter pot containing pasteurised sieved topsoil/sand medium. Pots were assigned at random to

(9.7.41)

one of fifteen steel trays (460 x 210 x 50mm), to give seven pots per tray. Trays were housed in a glasshouse (soil temperature range 18-26°C) and were rotated weekly. Each tray received a unique fertiliser regime; this was applied by pouring one litre of the nutrient solution into the tray. Immediately after application, a 200 x 20mm synthetic sponge (Wettex, Sweden) wick was introduced to slowly remove surplus solution. Codes for the fertiliser treatments are shown in Table 4.1. The concentrations used were partly suggested by results of a preliminary experiment in which double strength Thrive® caused severe root burning and the use of double-strength Phostrogen® resulted in small plants.

Table 4.1 Codes and nutrient ratios for fertiliser/frequency treatments applied to white clover seedlings inoculated with 2000 *H. trifolii* eggs. Normal concentration is the label rate. N,P and K values refer to the parts per million of each element in the diluted product.

Fertiliser	N:P:K	Conc.	Weekly	Fortnightly
Ruakura	20:40:238	normal	RW	RF
Thrive	480:80:160	normal 1.8g/l	TIW	TIF
		half	THW	THF
Phostrogen	44:44:121	normal .44g/l	PIW	PIF
		triple	P3W	P3F
Nitrosol		normal 5 ml/l	NIW	NIF
		double	N2W	N2F

Fertilisers for experiments and screenings.

"Weekly" treatments received nutrients once every seven days for the duration of the experiment. "Fortnightly" treatments received nutrients every 14 days and one litre of water of the alternate week. "Control" treatments received water, with no added nutrients, each seventh day. When extra watering was necessary, one litre was applied to each tray in the same way.

Phostrogen® (Watkins) and Thrivé® (Yates) are made for the home gardener and must be dissolved in water before application. The makers of Nitrosol® (Rural Research) describe it as a "natural organic liquid blood and bone fertiliser" to which they have added "trace elements and the growth promoters gibberellin acid and triacontanol". It is a suspension and needs to be diluted before application. "Ruakura" is a recipe of inorganic salts developed for white clover (C F Mercer, pers. comm).

Thirty five days after sowing, nematode inoculum was applied. A 3 ml suspension containing 2000 eggs was injected into a hole in the soil close to each plant.On the same day, plants were scored for size. The smallest plant scored one and the largest, five. (Plate 4.2)

Fifty six days after inoculation, cysts were recovered by elutriation. They were caught on a 180 µm sieve and counted in a Doncaster dish under a dissecting microscope.

The plants' tops and roots were separated and oven dried for 15 hours at 80°C before weighing. Growth scores, dry weights and cyst counts were subjected to square root transformation, then treatments compared by Tukey's test. The effect of the amount of fertiliser applied over the entire experiment was further investigated by plotting cyst numbers and root weight data against total additions.

®ertilisers for experiments and screenings.

Plate 4.1. Experimental plants in the small steel trays used to allow one litre fertiliser treatments. The blue Wettex wicks allowed rapid drainage of surplus nutrient solution.

Plate 4.2. A very large (treatment N2W) plant and a very small control T.repens plant await separation of root and top for drying and weighing. Cysts from these plants have been collected in the pottles and will be counted under a dissecting microscope.

RESULTS

Each of the fertilisers Thrive, Phostrogen and Ruakura, could be used in at least one regime that would allow plants to host more cysts than the nutrient-free control (Fig 4.1). Nitrosol treatments, on the other hand, never hosted more cysts than the control and the N2W treatment resulted in very few cysts. Fortnightly application of any particular fertiliser concentration resulted in at least as many cysts as the corresponding weekly application; in five of the seven possible comparisons cyst recovery was in fact higher from fortnightly than the corresponding weekly treatment.

Growth scores at inoculation reveal differences in plant top size four weeks after germination (Fig. 4.2). The largest tops resulted from three of the Nitrosol regimes and P3W. Plants receiving these four treatments were also among the largest top dry weights at the end of the experiment, along with RW and TIW (Fig 4.3). Only PIF was no larger than the control at inoculation, and by the end of the experiment all treatments yielded heavier tops than the control.

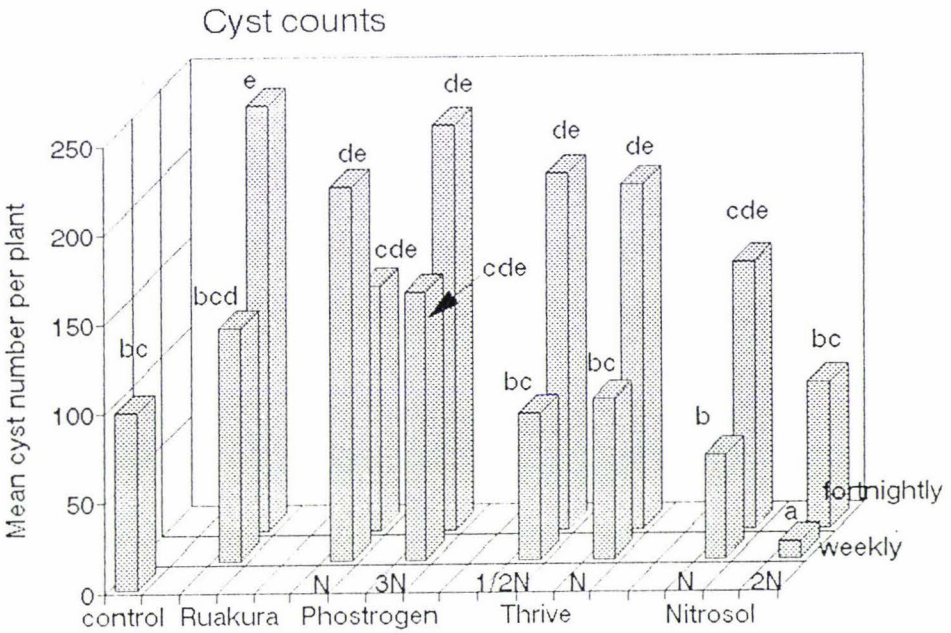
The largest root dry weights were produced by maximum applications of either Ruakura, Phostrogen or Thrive and by three treatments involving Nitrosol.

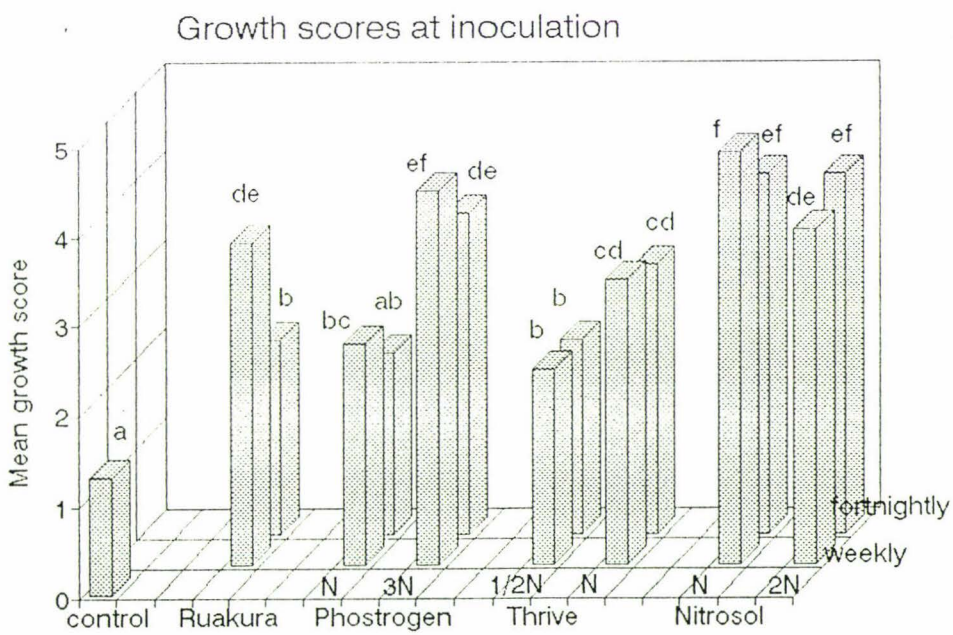
The fertiliser regimes that combined the largest root size with the highest cyst numbers were P3W and N1F. Several treatments resulted in cyst counts and root dry weights which were adequate for screening purposes; these included RF, the fortnightly Thrive treatments, and the remaining three Phostrogen regimes.

Fertilisers for experiments and screenings.

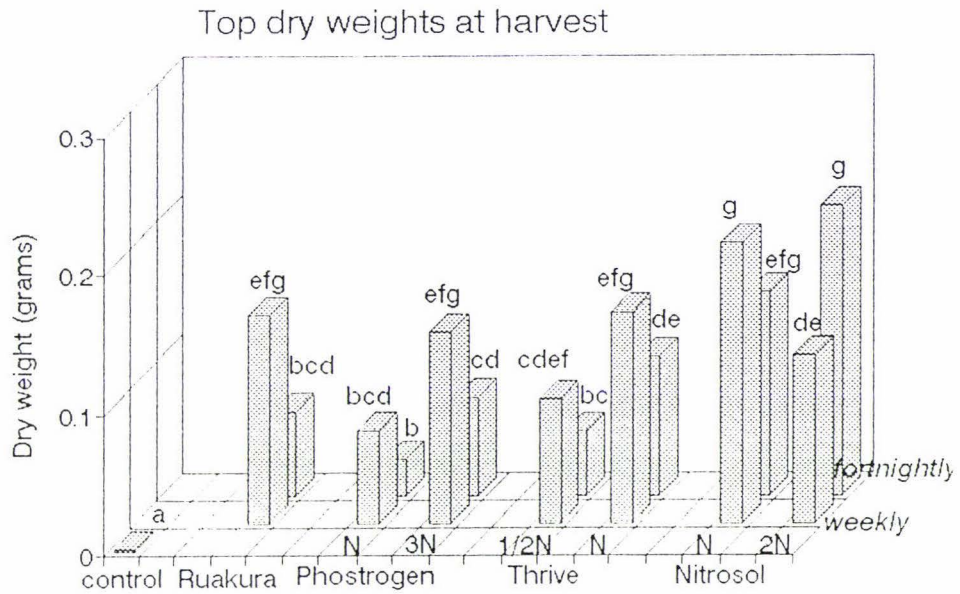
Figure 4.1. Mean numbers of cysts recovered from white clover plants 56 days after inoculation with 2000 *H. trifolii* eggs and treatment with one of 15 fertiliser regimes.

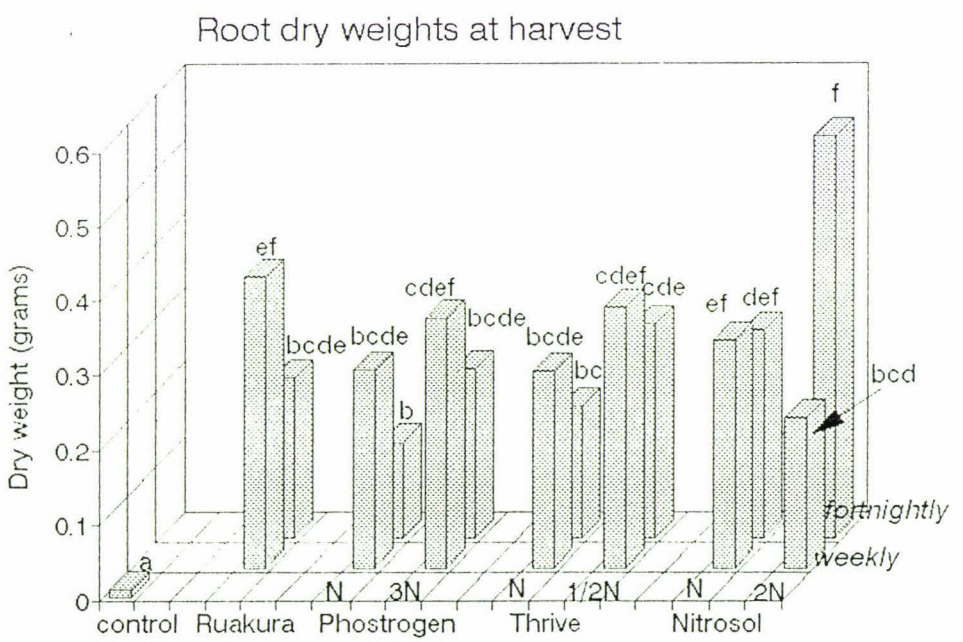
Figure 4.2. Mean growth scores of plants 35 days after germination, following treatment with one of 15 fertiliser regimes.


Cyst counts
Mean cyst number per plant
250
200
150
100
50
0
bc
e
bcd
de
cde
de
cde
de
bc
de
bc
de
b
cde
a
bc
fortnightly
weekly
control
Ruakura
N
3N
Phostrogen
1/2N
N
Thrive
N
2N
Nitrosol


Growth scores at inoculation
Mean growth score
5
4
3
2
1
0
a
de
b
bc
ab
ef
de
b
b
cd
cd
f
ef
de
ef
fortnightly
weekly
control
Ruakura
N
3N
Phostrogen
1/2N
N
Thrive
N
2N
Nitrosol

Figures 4.3 and 4.4. Mean top and root dry weights of plants 56 days after inoculation with 2000 *H. trifolii* eggs and treatment with one of 15 fertiliser regimes.


Top dry weights at harvest
Dry weight (grams)
0.3
0.2
0.1
0
a
efg
bcd
bcd
b
efg
cd
cdef
bc
efg
de
g
efg
de
g
fortnightly
weekly
N
3N
1/2N
N
N
2N
control
Ruakura
Phostrogen
Thrive
Nitrosol


Root dry weights at harvest
Dry weight (grams)
0.6
0.5
0.4
0.3
0.2
0.1
0
a
ef
bcde
bcde
b
cdef
bcde
bcde
bc
cdef
cde
ef
def
f
bcd
fortnightly
weekly
N
3N
N
1/2N
N
2N
control
Ruakura
Phostrogen
Thrive
Nitrosol

Plotting cyst counts against total fertiliser additions resulted, in each case, in a non-linear response (fig 4.5). When two-tailed t-tests were used to compare cyst numbers from the treatment resulting in the most cysts with those receiving the most and least fertiliser, a difference was noted for each product.

DISCUSSION

The control plants' small size and low cyst yield underline the importance of supplying nutrients. On the other hand, nematode suppression following weekly applications of Nitrosol®, Thrive® and Ruakura urges a conservative approach. Weekly application of the Ruakura recipe was the practice at Grasslands until 1992, when it was abandoned in favour of a fortnightly Thrive treatment. This change was implemented in order to reduce the time needed to prepare the fertiliser but in retrospect it also seems likely to have contributed to a large improvement in resistance identified since 1992 (J van den Bosch unpub data).

The current experiment related specifically to the resistance screening technique; it did not seek to identify which nematode lifestages were affected by fertilisers, nor which components of the fertilisers were responsible. The suppressive effect of frequent application could have reduced egg hatch - Oteifa (1955) thought ammonium ions had this effect on Meloidogyne incognita - or restricted J2 movement, penetration, or feeding site initiation. Effects on the feeding nematode are also possible. Nutrient deficiency, resulting when fertilisers are withheld, might affect the J2's chance of finding a penetration site, or affect it post-penetration. A further intriguing thought is

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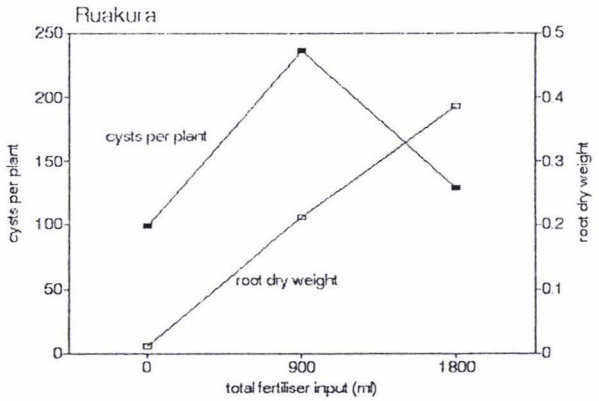
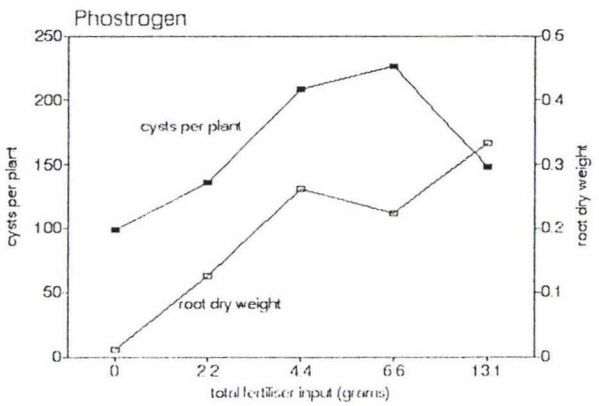
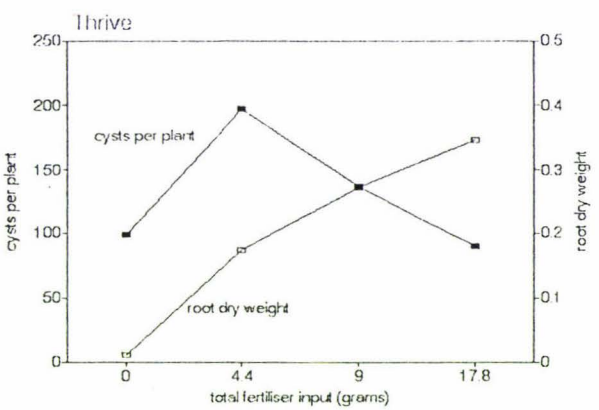
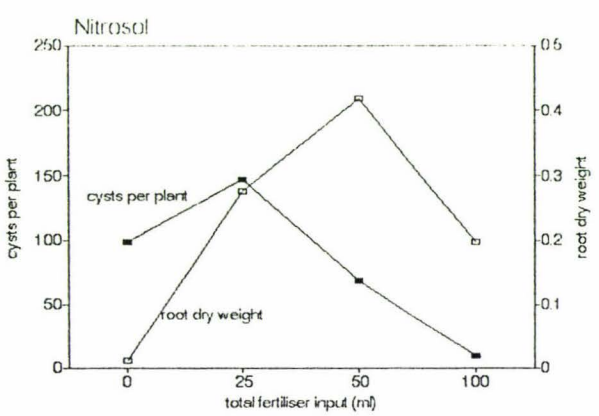
Fertilisers for experiments and screenings.

Figure 4.5. Responses in root dry weight and cyst numbers to fertiliser additions over nine weeks. Note that x-axes are specific to each fertiliser. Number of data points on graphs depends on concentrations used for each fertiliser.


Ruakura
cysts per plant
root dry weight
cysts per plant
root dry weight
250
200
150
100
50
0
0.5
0.4
0.3
0.2
0.1
0
0
900
1800
total fertiliser input (ml)


Phostrogen
cysts per plant
root dry weight
cysts per plant
root dry weight
250
200
150
100
50
0
0.5
0.4
0.3
0.2
0.1
0
0
22
44
66
131
total fertiliser input (grams)


Thrive
cysts per plant
root dry weight
cysts per plant
root dry weight
250
200
150
100
50
0
0.5
0.4
0.3
0.2
0.1
0
0
44
9
17.8
total fertiliser input (grams)


Nitrosol
cysts per plant
root dry weight
cysts per plant
root dry weight
250
200
150
100
50
0
0.5
0.4
0.3
0.2
0.1
0
0
25
50
100
total fertiliser input (ml)

that suppression caused by the organic Nitrosol[2] might arise via a different mechanism from that of the inorganic fertilisers. In support of this is the report of Heald & Burton (1968) who found fewer parasitic nematodes in turf treated with an organic material than was the case in plots receiving inorganic fertiliser; the effect could not be explained by NPK compliments alone. The current study, however, neither supports nor refutes inference of an organic amendment action.

Although beyond the scope of the current study, clarification of the suppression-causing mechanism could be sought using the root staining techniques to be described in Chapter five. Many researchers have investigated effects on nematodes when the host is grown in conditions of nutrient excess or deficiency. Oteifa (1953) studied the effect of potassium nutrition on the growth rate of M. incognita parasitising lima bean. He reported that, as K concentrations increased, nematode development was accelerated. This finding has this implication for Grasslands' resistance screenings: given that the technique involving dislodging and recovering cysts and that cysts from resistant plants tend to be smaller (see Chapter 5) it is important to ensure that cysts have reached sufficient size to be captured on the sieve. Standardisation of fertiliser regime will be important in determining whether this critical size is reached.

Nitrogenous fertilisers have been shown both to enhance and suppress nematode development. It might be considered that changing a host's N status from deficiency to adequacy would result in increased nutrient availability for the parasite and perhaps larger roots and thus additional penetration sites; we would then expect more nematodes to be hosted by the N-rich plant. Mahmood & Saxena (1980)

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[2] Fertilisers for experiments and screenings.

and Shands & Crittenden (1957) identified such an effect while working, respectively, with Rotylenchus reniformis on eggplant and M. incognita acrita on soybean.

Conversely it might be anticipated that adequate N nutrition would lead to a more fit plant which the nematodes consequently find a poorer host. Such a situation might result from an enhanced resistance mechanism or a less favourable set of metabolites in the normal plant compared with the deficient (Bird 1960). Orion et al. (1980) found that a high concentration (1650 mg/l) of ammonium nitrate in an agar medium inhibited feeding site development and growth of M. incognita feeding on excised tomato roots. Mahmood & Saxena (1980) noted an increase in phenol production in eggplant following N addition. While these authors reported no corresponding decline in nematode numbers, Singh & Choundry (1973) have linked phenols with resistance to root-knot nematodes in tomato. Ammonia, generated by break down of nitrogenous fertilisers, may also be toxic to nematodes in the soil.

In the current experiment, plotting cyst counts against total fertiliser additions resulted in non-linear responses in each case (Fig 4.5). Moderate applications of fertiliser enhanced cyst yield, while excessive fertiliser had a suppressive effect. Each graph also includes a range over which root weight continues to rise, while cyst numbers decline; this represents a range in which fertility status is beneficial to the plant but adverse toward the nematode.

The range of effects of fertilisers on pest nematodes underlines the importance of studying resistance under field conditions as well as in pot trails. In supplying enough N to maximise root weight, for instance, we might inadvertently select lines resistant only under high-N

Fertilisers for experiments and screenings.

conditions. This might occur if the lines produced N-rich compounds with deterrent action. Given that a major role of legumes in pasture is to be the supplier of nitrogen, a cultivar that required large inputs of nitrogenous fertilisers to grow successfully would be of little value.

Fertilisers for experiments and screenings.

CHAPTER 5 RESISTANCE MECHANISMS

Plant species are known to express resistance to nematodes at a number of stages in the organisms' interaction (Huang 1985). Pre-infectional resistance may occur some distance into the rhizosphere or at the root surface. Certain plants produce exudates thought to be toxic to nematode parasites; conversely some plants may be more chemically attractive than others. Physically impenetrable roots have been postulated as a resistance mechanism (Dropkin & Nelson 1960). Post-infectional mechanisms may deter nematodes from feeding, stop or slow development or inhibit reproduction (Huang 1985). Work reported in this chapter seeks to identify mechanisms by which white clover resists *H. trifolii*.

Part 1: Effects on the growing nematode

At Grasslands, individual white clover plants are screened for resistance by inoculating them with nematode eggs and, six weeks later, extracting cysts by means of an elutriation tower. Crossing of selected lines has resulted in plant genotypes which seem very resistant (van den Bosch *et al.* 1993), but it has not been shown which mechanisms have resulted in the numbers of cysts recovered. Low cyst counts may be the result of a single mechanism or a combination, while resistant genotypes may differ one from another. The primary objective of this study was to identify the nematode life stages affected by resistant genotypes.

It is also unknown whether resistant plants yield few cysts in screenings because the nematodes are not present, or because they are too immature to be recovered by elutriation. A further objective was to compare the rate of

development of those parasites successful on resistant and susceptible genotypes.

METHODS AND MATERIALS

Plants that had supported very high or very low numbers of cysts in a resistance screening were chosen for this study. Five plants from each category were selected on the basis of similar root weight. They were propagated by means of apical and nodal cuttings which were pressed into heat-sterilised sand and kept moist with a 100 ppm solution of Captan fungicide. When they had taken root, five cuttings from each plant were repotted into a pasteurised 50:50 sand/topsoil medium in a 180 ml plastic plant pot. A 3 ml suspension containing 2000 *H. trifolii* eggs was injected into a hole in the soil alongside the plant. Pots were rearranged to a random order in trays and the trays rotated each week. They received a weak (2g/l) solution of Thrive fertiliser fortnightly and were watered as necessary between fertiliser applications. Forty two days from inoculation cysts were extracted by means of an elutriation tower. On the basis of counts of cysts recovered, two resistant and two susceptible genotypes were selected for further study.

The four genotypes were propagated by the techniques described above. Two weeks after potting into the sandy/topsoil medium, all plants were washed free of soil and their roots trimmed to approximately equal volume. They were repotted in sterile medium, grown on for a further two weeks, then inoculated as described above.

The pots were randomised, maintained in a glasshouse at soil temperature 18-25℃ and received nutrients as above. Soil temperature was recorded every six minutes by a computer-controlled transducer. The computer stored this information as accumulated degree days over 10℃ (DDA).

Eight days after inoculation, the remaining plants were carefully washed and repotted into fresh soil mix. Thus, a ceiling was placed upon the time available for nematodes to hatch and penetrate roots. This step was necessary in order to better understand the pattern of penetration and development; J2 hatching later than day eight would have confused graphs and calculations.

On eight occasions over the 37 days following inoculation, four plants of each genotype were selected at random. Soil was removed under a gentle flow of water and the roots stained after the method of Byrd et al. (1983). It was considered that, by day 30, a few nematodes were likely to be dislodged by the washing process. To check this, the four plants of each genotype were washed over a 106 µm sieve. In fact the number dislodged from susceptible plants was surprisingly high and they outnumbered those still in the roots. On day 37, an elutriation tower and sieves were used to recover nematodes dislodged from individual plants.

On the day after each staining, a dissecting microscope was used to count nematodes in the roots. To facilitate counting, roots were spread in glycerol in the lid of a petri dish and the base of the dish inverted on top. Sandwiching the roots in this way was found to minimise the need for refocusing as the roots were examined. Nematodes were assigned to life stage categories according to the descriptions of Mulvey (1959). Modifications were made in the following two ways: it was noted whether second stage juveniles were vermiform (vJ2) or had started to swell (sJ2), and third and fourth stage juveniles were recorded as a single category (J3,4). Mulvey (1959) noted that "a gelatinous matrix is formed around the female posterior protuberance" and that this occurs "soon after the (fourth) moult". In light of this, the presence of the matrix was used to distinguish adult nematodes from advanced fourth stage juveniles. For each harvest, counts of stages on each

genotype were compared using analysis of variance (ANOVA). Means were separated by Tukey's test. Because the counts covered a wide range and included some very low counts, data were transformed using $\log_{10}\sqrt{(x+1)}$ before analysis (Steel & Torrie, 1981). As cysts recovered on day 30 were not assigned to individual clones, ANOVA was not possible for this harvest.

In order to investigate the development of nematodes successfully parasitising resistant plants, counts of life stages were also expressed as a percentage of total nematodes at each harvest date. These percentages were analyzed as above, except that the transformation used was arcsine $\sqrt{\text{(decimal fraction)}}$ (Steel and Torrie, 1981).

RESULTS

Replication using clones confirmed the resistance status of the four T. repens genotypes (Table 5.1).

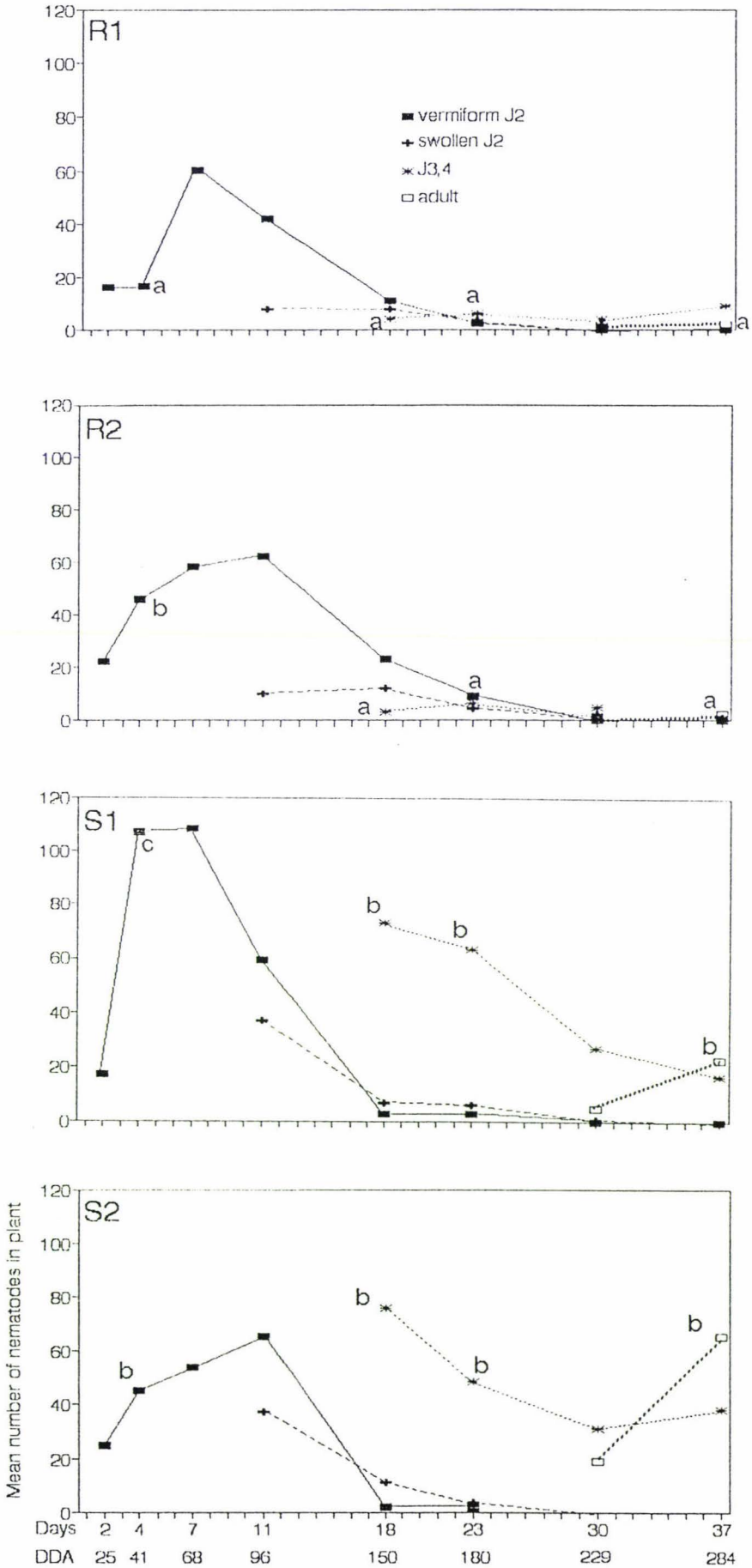
Table 5.1 Mean numbers of H. trifolii adults and cysts recovered in a screening of five clones of four T. repens genotypes 42 days after inoculation and four clones of the same genotypes 37 days after inoculation. In both experiments, plants were inoculated with 2000 eggs.

Genotype	Nominal Resistance Status	Rescreen using clones	Development experiment
R1	resistant	18a	2a
R2	resistant	4a	2a
S1	susceptible	364b	23b
S2	susceptible	517c	65b

A common letter within a column indicates the means do not differ ($P \leq 0.05$).

Second stage juveniles (J2) penetrated genotype S1 more rapidly, and genotype R1 more slowly, than they did the other genotypes (Fig. 5.1). Four days after inoculation J2 numbers ranged from a mean of only 17 in R1 to 109 in S1 with the other two genotypes intermediate. After a further three days, however significant differences in the counts were not found. J2 had started to swell by day 11 (96 DDA); the percentage of swollen J2 did not differ between genotypes. By day 18 (150 DDA) J3,4 were present. On the susceptible genotypes these greatly outnumbered the vermiform nematodes while, on the resistant genotypes the opposite state of affairs was the case. This pattern was

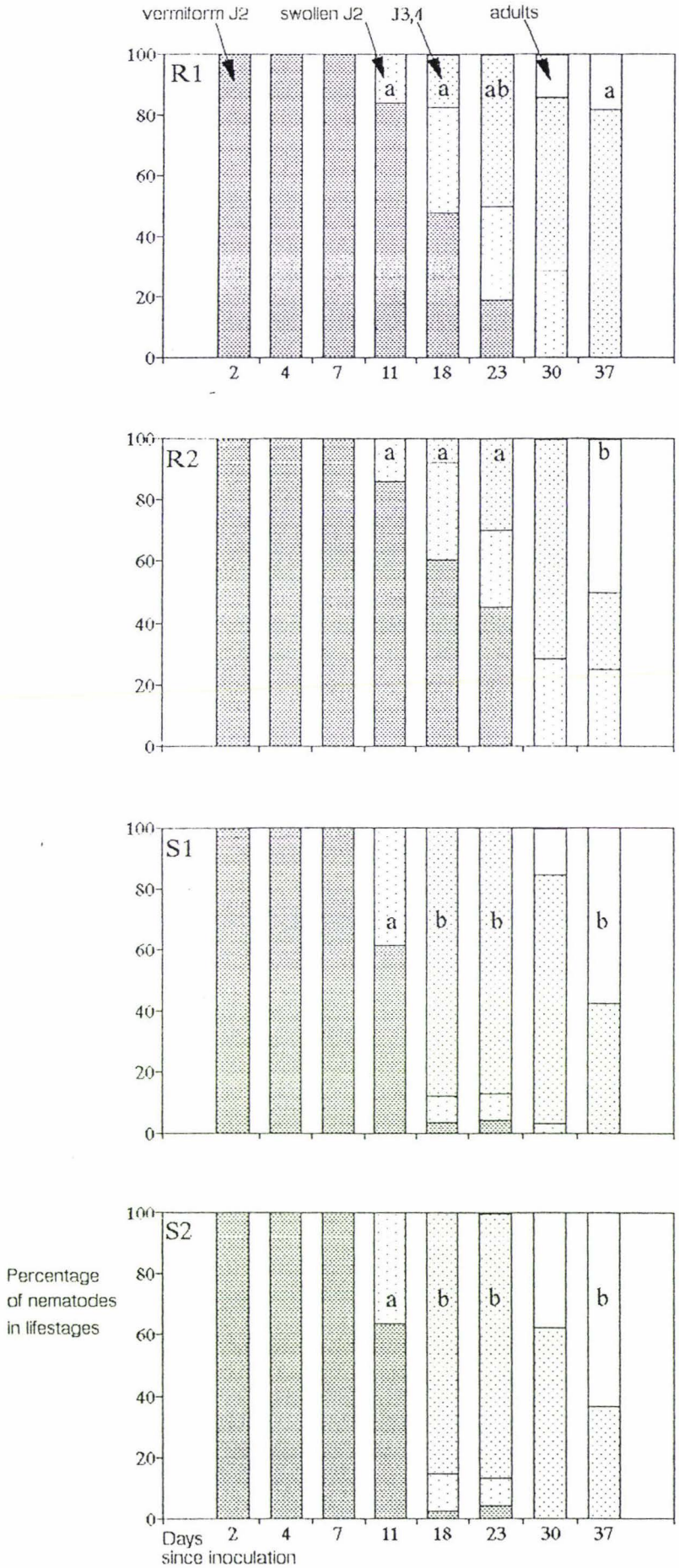
Figure 5.1. Mean numbers of *H. trifolii* lifestages in roots of four clones of *T. repens* genotypes on eight occasions following inoculation with 2000 eggs. letters indicate points of comparison between genotypes on a particular day. Comparisons with a letter in common do not differ ($P \leq 0.05$)


R1
vermiform J2
swollen J2
J3,4
adult
R2
S1
S2
Mean number of nematodes in plant
Days 2 4 7 11 18 23 30 37
DDA 25 41 68 96 150 180 229 284

repeated at the day 23 harvest. When expressed as a percentage of total nematodes, the J3,4 population on the susceptible genotypes on day 18 was significantly higher than on R1 and R2 (Fig. 5.2).

By day 30, the first adults could be seen on genotypes R1, S1 and S2. Juveniles were still more abundant than adults on all genotypes. By day 37, adult nematodes had become common on the susceptible genotypes although the late juvenile stages were still present. On genotypes S1 and S2 the percentages of adults were 58 and 63, respectively. Few nematodes remained on the resistant genotypes. On R1 the small population of successful parasites consisted mostly of juveniles; only some 15% had reached adulthood (Fig. 5.2, day 37). The four clones of genotype R2 hosted only ten nematodes between them.

Figure 5.2. Percentage of *H. trifolii* individuals in each lifestage on eight occasions following inoculation of each of four *T. repens* genotypes with 2000 eggs. Letters indicate points of comparison between the four genotypes as follows: swollen J2 (day 11), J3,4 (days 18 & 23), adults (day 37). Comparisons with a letter in common do not differ ($P \leq 0.05$).


vermiform J2
swollen J2
J3,4
adults
R1
R2
S1
S2
a
a
ab
a
a
a
a
b
a
b
b
b
a
b
b
b
100
80
60
40
20
0
2
4
7
11
18
23
30
37
Percentage of nematodes in lifestages
Days since inoculation

DISCUSSION

The differing rates at which four plant genotypes were penetrated suggest the existence of some sort of pre-infectional resistance. The effect of this resistance was to delay, rather than prevent, penetration. Such an attribute may be an advantage to a plant in competition with others of its own species, in which case the delay might be sufficient to encourage nematodes to penetrate another plant in preference to the resistant one. In agronomic terms, however, pre-infectional resistance is unlikely to be of major value because the pool of J2s in the soil would survive longer than the few days delay achieved. Many plants in field conditions might follow the pattern of genotype S2, which displayed a measure of pre-infectional resistance but eventually hosted large numbers of cysts (Fig. 5.1).

It may have been imagined that root size determined the different J2 numbers on day 4; we might expect larger roots to provide more penetration sites. The evidence, however, is against this. The only difference in root weight on day 4 was between genotypes R1 and S1; R1 had significantly larger roots (mean fresh weight 1.78 versus 0.53 g) but only about one fifth as many nematodes as S1 (Fig. 5.1). Root weight was not therefore, a factor in determining nematode numbers.

Because eggs, rather than J2, were used as inoculum, this experiment did not distinguish resistance mechanisms affecting hatching from those affecting J2 locomotion or root penetration. Shepherd (1962) considered hatching of *H. trifolii* to be only slightly stimulated by white clover root exudates, but comparison of genotypes would be a useful first step in further describing the pre-infectional resistance mechanism. Such a study might involve leaching a sand medium in which plants are growing, then immersing eggs

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in the leachate in microtitre plates. The percentage of eggs hatching could then be assessed under a dissecting microscope.

Final counts of nematodes were, for the most part, determined by post-infectional resistance. Differences between genotypes, in terms of numbers of J3 and later stages, were maintained from day 18 (150 DDA) until the end of the experiment. Such resistance, identified when nematodes in roots fail to develop to the second moult, has been observed in several plants resistant to the Heteroderidae. Interactions reported include alyce clover (Alysicarpus spp.) with Meloidogyne spp. (Powers et al. 1993) and Kenya white clover (Trifolium semipilosum) with M. hapla (Mercer & Grant, 1993).

Several possible mechanisms that would inhibit development to the J3,4 stage may be postulated. Inhibition might result from the J2s' inability to initiate syncitium development; alternatively such a feeding site may develop but later disintegrate. Forrest et al. (1993) found the latter to be the case in potato resistant to Globodera rostochiensis. They observed that initial syncitial cell walls broke down close to the feeding site and thought that, even when a syncitium continued to enlarge, such breakdown close to the nematode would eventually lead to complete syncitial destruction. Nematode emigration or death would result. Forrest et al. (1993) also thought feeding tube development might be abnormal in resistant potato, though Endo (1991) did not find this to be true of resistant soybean hosting Heterodera glycines. In the current study, similar numbers of J2 were observed to swell - and thus, it is assumed, to feed - on each genotype. This would suggest that syncitia were at least initiated. However, this inference should not be pressed too far, because at a significance level of $P \leq 0.07$ differences in sJ2 numbers become apparent. Even at $P \leq 0.05$, analysis by Fisher's

Protected LSD, rather than the more conservative Tukey's test, suggests differences. Expertise in the study of syncitial ultrastructure is now available at Grasslands and electron microscopy will be used to further define the resistance mechanism.

Even if a normal syncitium is initiated and maintained, the blend of materials available to the nematode may not be conducive to its growth. Powers et al. (1992) reviewed the action of some of the chemical agents that might inhibit the growth of nematodes initially feeding successfully. They thought that even in the absence of nematodes some resistant plants might maintain a pool of compounds that would act to localise infection when plants were penetrated. Brueske (1980) thought this to be the case in tomato resistant to M. incognita. Other plant species were thought to produce, after being infected, compounds that interfere with nematode metabolic pathways. Soybean for instance produces the phytoalexin glyceollin in response to M. incognita (Huang 1985). A further possibility is that resistant genotypes may not actively oppose nematode parasites but provide a blend of metabolites unsuitable for their growth. Nutrient deficiencies have been linked with the emigration of J2 out of root systems (Huang 1985); deficiencies are also a plausible explanation of the slow rate of development and low fecundity (see chapter five, part two) observed in the current experiment.

CHAPTER 5 RESISTANCE MECHANISMS

Part 2: Effects on Fecundity

In describing a plant's efficiency as a host of parasitic nematodes, it is considered desirable to say something about its effect on nematode reproduction (Canto-Saénz 1985). Ideally then, evaluation of the plant will take into account the number of healthy eggs produced by nematodes developing in it.

In practice, nematode reproduction is frequently estimated by measurement of other parameters; these measurements may take the form of some symptom of parasitism or counts of other nematode life stages. At Grasslands, for example, resistance to the root-knot nematode is assessed by counting galls on roots while, in the case of the clover cyst nematode, the number of cysts is the usual screening parameter. Thus, a compromise is reached between the ideal measure of nematode reproduction and the need to process large numbers of samples to maximise plant genetic diversity in the breeding programme.

The current experiment was initiated with two questions in mind. From the biological standpoint, I wished to test for a further parameter of resistance operating in addition to those identified in the earlier experiment. From the plant breeding angle, it was important to consider whether screening by cyst counts either overestimated or underestimated the plants' true effect on nematode reproduction. Yeates (1987) noted that data on nematode fecundity are "relatively few". His comment appears valid even in the case of the Heteroderidae in which family it might have been imagined that the enclosure of eggs in a cyst and/or egg sac would greatly facilitate counting. Yeates (1987) also pointed out the difficulties of summarising the available data, because of differences in experimental technique. Again, his broad observation seems to hold true for the Heteroderidae; reports range from there being more eggs per female on resistant compared with susceptible plant genotypes (Powers et al. 1992) through no effect of

resistance upon fecundity (Balhadere & Person-Dedryver 1991) to fewer eggs per female on resistant plants (Person-Dedryver 1988). While these reports all refer to Meloidogyne spp., Dijkstra (1971) reported studies of white clover's resistance to H. trifolii. He estimated the size of cysts from resistant and susceptible hosts and supposed the former to be smaller. This observation was not supported by statistical evidence, however, and egg-per-cyst counts were not presented.

Methods and Materials

Clones of each of genotypes R1, R2, S1 and S2 were raised from nodal cuttings. After taking root the cuttings were transferred to sand/soil mix in 6 cm diameter pots. Two weeks later five clones of each genotype were selected for even top size. These plants were washed free of soil and their roots trimmed to similar volume. The pots were randomised and after a further two weeks 2200 eggs were injected under each plant. Half-strength Thrive fertiliser was applied four times at approximately fortnightly intervals.

After 42 days, cysts were recovered by elutriation and those from each plant counted using a dissecting microscope. In order to select five cysts at random, each plant's cyst count was divided by five to give a value 'n'. As a path was traced through the Doncaster dish's concentric rings, every nth cyst was picked out with forceps until five had been taken. Two clones of genotype R2 hosted fewer than five cysts, so four extra cysts were taken from another clone to bring the total for the genotype to 25. The five were transferred to a drop of water on a microscope slide and their length and breadth measured under the stereomicroscope. Next, each set of five cysts was crushed between two microscope slides along with a small volume of water. Offsetting the slides by about 2 mm separated the eggs in each cyst sufficiently for them to be counted, at 50 x magnification, under a compound microscope. Empty eggshells were occasionally seen; these were included in

the egg count. Juvenile nematodes, on the other hand, were not included.

Data were pooled across the five clones of each genotype; differences between clones were not included in the design. An expression of cyst size was generated by multiplying the cyst's length by its breadth (Shetty & Reddy 1985). The products were compared by ANOVA and Tukey's HSD test. Egg numbers per cyst were compared using the Mann-Whitney U test. This non-parametric test was used because non-normal distributions (Fig. 5.3) rendered ANOVA invalid.

Efficiency of the genotypes as nematode hosts was expressed by calculating Pf/Pi values. These were computed by dividing the final number of eggs on a plant by 2200, the number of eggs in the inoculum.

Results

Cysts hosted by susceptible genotypes were significantly larger than those on resistant genotypes; those from genotype S2 were larger than all other genotypes (Table 5.2)

Table 5.2 Parameters of cysts raised on resistant (R1, R2) or susceptible (S1, S2) genotypes, 42 days after inoculation with 2200 eggs. Cyst/plant data are the average of five replicates, while cyst size and eggs/cyst figures are means of 25 cysts from each genotype.

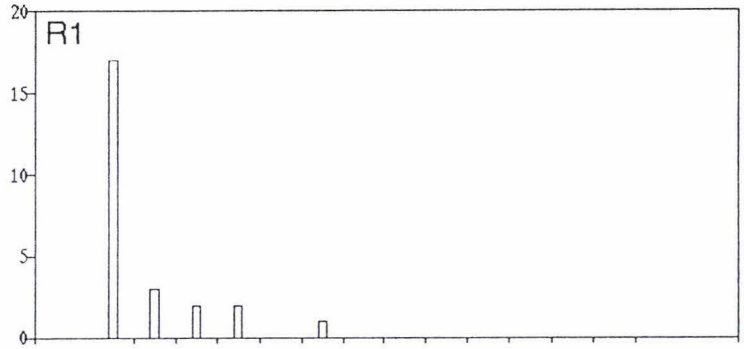
Genotype	cysts/plant	cyst size (mm^2)	eggs/cyst	eggs/plant	P_f/P_i
R1	14 b	0.14 a	17 a	238	0.11
R2	5 a	0.16 a	24 ab	120	0.06
S1	34 bc	0.25 b	48 b	1632	0.74
S2	73 c	0.31 c	85 c	6205	2.8

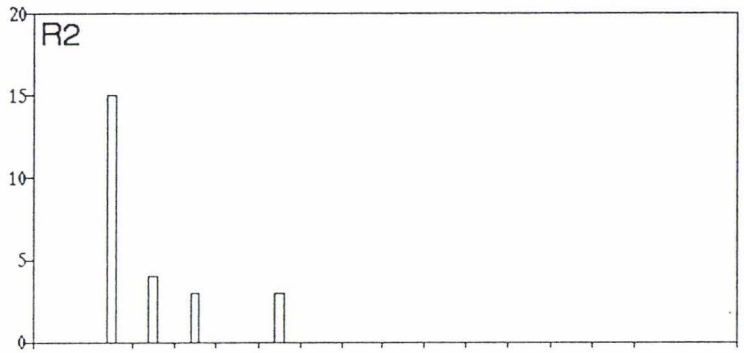
Within a column, means followed by the same letter do not differ at $P \leq 0.05$.

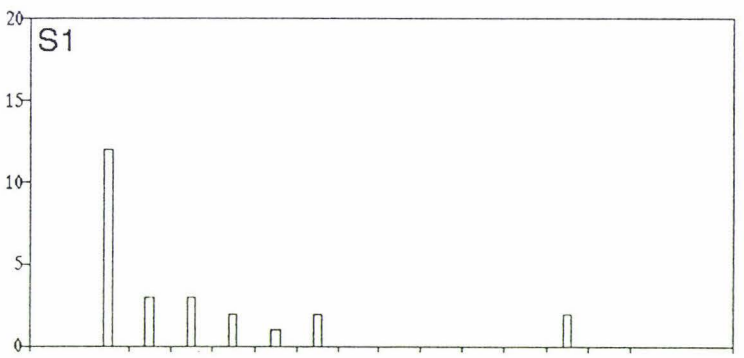
A similar pattern was observed in the eggs-per-cyst counts, except that genotypes R2 and S1 did not differ in this respect. Figure 5.3 shows that a large proportion of the cysts hosted by S1 and the resistant genotypes had produced fewer than twenty eggs. Only five cysts of the 75 from these genotypes contained more than 100 eggs. Genotype S2, on the other hand, hosted cysts with egg counts more evenly distributed in categories up to 200 and a highest count of 250.

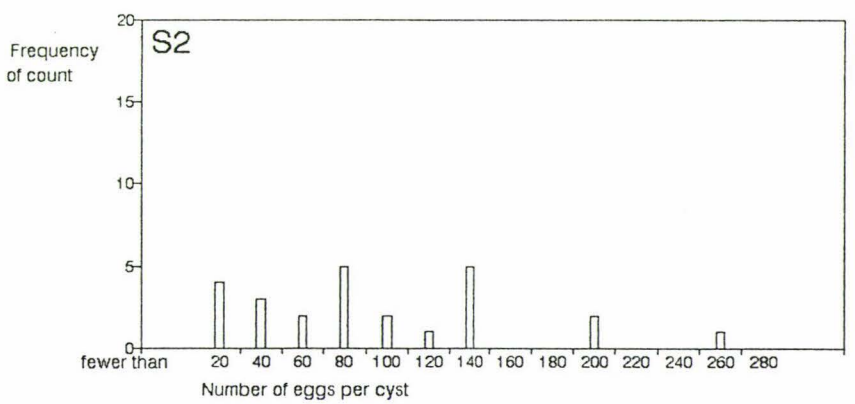
Resistance mechanisms: effects on fecundity

Figure 5.3. Distribution of numbers of eggs per cyst, 42 days (about one generation time) after clones of four *T. repens* genotypes had been inoculated with 2200 *H. trifolii* eggs. Egg/cyst number categories are of the progression 0-19, 20-39, 40-59 etc. Note the non-normal nature of the distributions.


R1
20
15
10
5
0


R2
20
15
10
5
0


S1
20
15
10
5
0


S2
Frequency of count
20
15
10
5
0
fewer than
20
40
60
80
100
120
140
160
180
200
220
240
260
280
Number of eggs per cyst

Cyst size and egg content were correlated positively (R = 0.63) and very significantly ($P < 10^{-4}$).

Discussion

The results of this experiment identify a further mechanism by which white clover is able to resist the clover cyst nematode. If we choose to distinguish between nematode production (measured by cyst size) and reproduction (egg count) it might even be said that two further mechanisms have been shown. Use of a simple length x breadth calculation to express cyst size effectively treats the cyst as a two-dimensional rectangle; in fact it is a three dimensional, lemon-shaped entity. The most valid measurement of size would, therefore, be in terms of volume and comparing areas will tend to underestimate true differences in size. The fact that significant differences were identified using the more conservative approach strongly indicates the relatively small size of cysts on resistant genotypes.

To the encouragement of the breeding programme, it seems that the excellent progress made by screening on cyst count in fact underestimates the true effect on nematode reproduction. Table 5.2 indicates that a 14-fold difference between R2 and S2 in terms of cysts/plant translated to a 52-fold difference in egg production. Pf/Pi values covered a correspondingly wide range. The low values for the resistant genotypes would categorise them as non-efficient hosts ($P_f/P_i < 1$) in the usage of Jones (1956). Genotype S2 is an efficient host, while we might regard S1 as slightly inefficient or neutral.

More than one hypothesis might be advanced to explain the subnormal size and fecundity of cysts on resistant genotypes. Perhaps, as discussed in part 1, direct antagonism from the host, or an unsuitable mix of food constituents, restrain the development of the few nematodes that survive to maturity. In this case, whatever mechanism is responsible for halting

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Plate 5.1. A dozen cysts and female *H. trifolii* picked at random from a resistant (above) and a susceptible (below) *T.repens* host. In spite of apparently being at a similar stage of maturity, as indicted by the proportion of white to brown individuals, those from the susceptible host are larger and have secreted larger gelatinous egg sacs.

development of the majority of J2s would be seen as also suppressing development of those nematodes able to break resistance. Another premise, however, is that nematodes carrying genes that allow them to break resistance are inherently smaller and less fecund than others of their species. If the latter is the case, the cost of carrying genes to break resistance is low vigour.

The performance of genotype S1 deserves consideration. While cysts from this genotype were larger than those from R1 and R2, they did not contain more eggs than cysts from R2. S1's distribution of eggs/cyst (Fig. 5.3) more closely resembled those of R1 and R2 than that of S2. What explanation might be given for this apparent anomaly? Jones (1980) divided the plant-parasitic nematode genera by their population strategies. His "r-strategists" or "exploiters" seek to quickly exploit opportunities for population increase and tend to have unstable populations. The "K-strategists" are "persisters" and have relatively stable populations. Heterodera spp. tend toward the K end of the r-K continuum, and Yeates (1987) showed that this means they will increase resource utilization efficiency when resources are limited. The practical implication is this: that Heterodera spp. will tend to increase body size and decrease reproductive effort when resources are restricted. If genotype S1 provided less resource to the nematodes than other genotypes, we would expect the attributes of the K-strategist to be more expressed in cysts on S1. Roots were not weighed in this experiment but evidence from other experiments suggests that S1 is a small genotype. On day 37 of the first experiment, for instance, the mean S1 root weight was below half that of the other genotypes. Although hosting fewer nematodes than S2 in absolute terms, S1 hosted nearly 50% more cysts per gram root weight on day 37. It seems likely that inter-specific competition between nematodes has come strongly into play on genotype S1, and that they have partitioned the limited available resources towards body development rather than egg production.

Resistance mechanisms: effects on fecundity

The tension between plant susceptibility and nematode density in determining a female's fecundity would explain a surprising result reported by Powers et al. (1992). Two lines of alyce clover hosted fewer M. arenaria females than susceptible lines, yet allowed more eggs in each egg mass. It seems likely that the effects of low intra-specific competition on the resistant lines overwhelmed any suppressive effects on fecundity. Such was not the case for Person-Dedryver (1988). This author found that, as well as hosting fewer M. naasi females Grasslands Ruanui allowed fewer eggs per female than the susceptible Réveillé perennial ryegrass cultivar; these results are comparable with the main finding of the current experiment.

CHAPTER 5 RESISTANCE MECHANISMS

Part 3 Conclusions and Implications for Plant Breeding

Resistant white clover genotypes affect *H. trifolii* in a number of ways. Firstly slight resistance is offered by some plants as they delay penetration. Far more importantly, most nematodes are prevented from developing to the second moult and presumably either die or emigrate from the root. The few nematodes that attain later juvenile stages are restrained in their development; the rate with which they pass through life stages may be slowed, as was the case for parasites of genotype R1. If and when adulthood is attained, cysts from resistant plants are in some cases smaller than those hosted by susceptible plants; they also produce fewer eggs.

This suite of resistance effects carries important implications for the likely performance of resistant cultivars. Useful cultivars need a high percentage of survival as seedlings; it is at this stage in the plant's life that *H. trifolii* is a particularly significant pest (Dijkstra 1971). Presumably this pest status extends to the small plants that result from rooting of excised stolons; survival of these small plants is critical to the perennial survival of the species in a pasture. A strong seasonal shift from large plant size to a preponderance of small plants occurs in spring and it is at this time that white clover is most vulnerable to adverse conditions (Hay *et al.* 1988). It is during spring that large numbers of *H. trifolii* emerge and penetrate roots (Yeates 1973a) and so this is when protection of the white clover is most important. Because of this, plant genotypes that delay cyst maturation or suppress egg production, as well as expressing other resistance mechanisms, should be seen as doubly valuable. These would lower the amount of inoculum carried over between seasons.

While a pre-infection resistance mechanism has been identified, it is not of sufficient importance to justify special attention as a selection factor. Post-infection resistance is of much greater importance and is adequately identified by the existing screening technique. The technique involves elutriation at around 320 DDA; we can have confidence in it because washing the roots after only 284 DDA in the first experiment succeeded in dislodging nearly all nematodes.

Screening plants for their ability to restrict nematode development and egg production seems likely to be fruitful. A method of screening large numbers of plants for this trait might involve counting cysts after allowing several generations of nematode reproduction on each plant.

CHAPTER 6 DAMAGE FUNCTIONS OF RESISTANT AND SUSCEPTIBLE SEEDLINES.

Considerable research effort has been invested in quantifying the yield losses that plant parasitic nematodes cause to agriculturally important plants. Indeed, Oostenbrink (1966) attributed much of the development of the science of nematology to the seeking of this kind of information. An understanding of the damage associated with varying levels of pest density, together with economic considerations, is critical to the planning of any action to try to ameliorate yield loss.

In annual crops, prediction of loss is in some respects more simple for nematodes than for other pests (Ferris 1978). This is largely due to the relatively slow motility of nematodes. Since little immigration is likely, a crop's yield is often well related to the size of the nematode population before planting. This pre-plant population (Pi) is the independant variable of most models that aim to serve as yield predicters; frequently a plot of yield against some logarithmic transformation of Pi is used to allow interpolation of the effect of varying nematode density. Inherent in the use of such transformation is the notion that damage, on a per nematode basis, is greater at low values of Pi compared with high. As damage increases, so does competition for feeding sites; thus the proportion of the soil nematode population that succeeds in parasitising the plant falls as Pi increases.

Oostenbrink (1966) sought to summarise the relationship between nematode density and plant production and found a model similar to the dose-response curves of De Jongh (1964) to be useful. Most of the curve (Fig 6.1) could be

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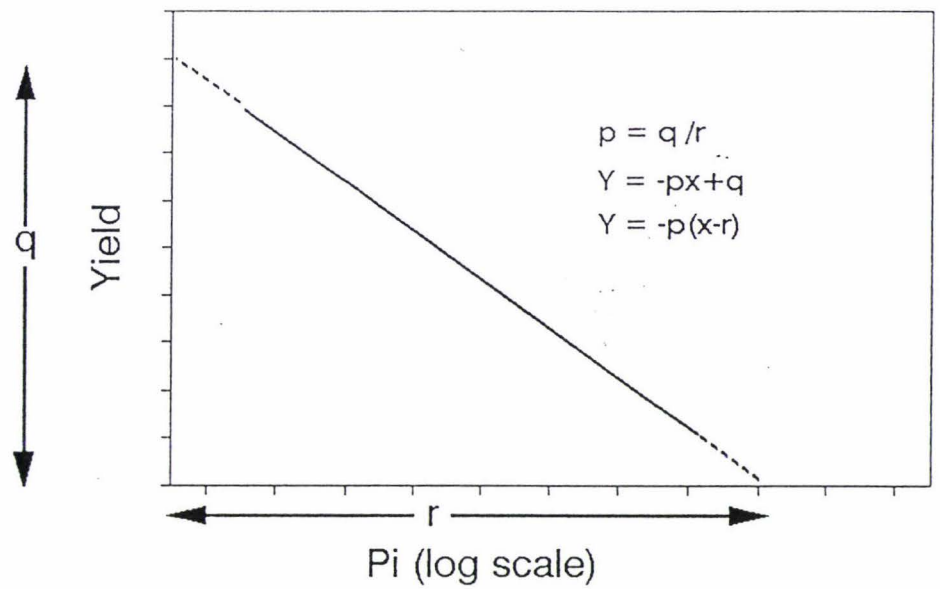
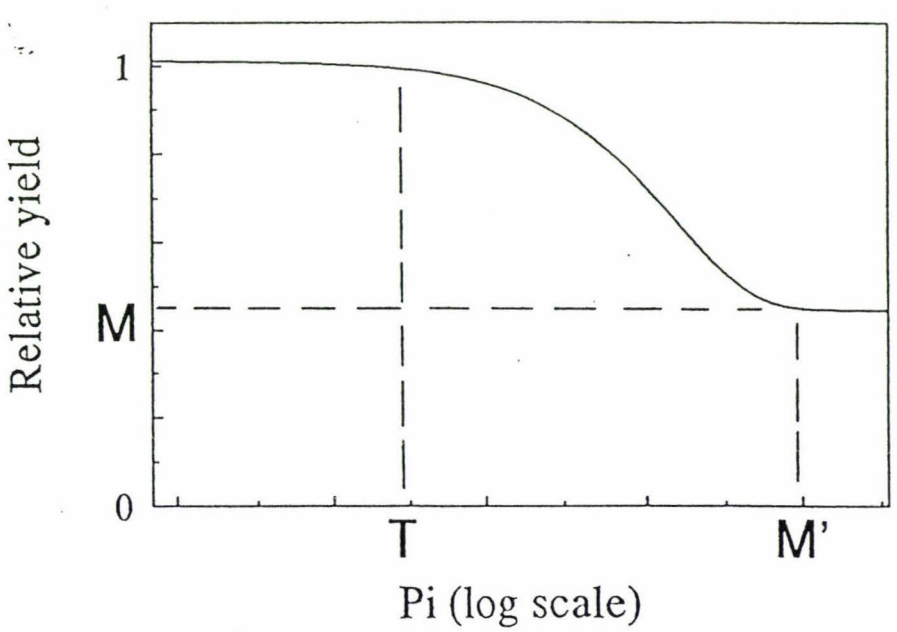
Damage functions for resistant and susceptible seedlines.

Figure 6.1. The model of Oostenbrink (1966) relating crop yield to the initial population density (Pi) of a parasitic nematode species.

Figure 6.2. The model of Seinhorst (1965) relating crop yield to the initial population density (Pi) of a parasitic nematode species.


q
Yield
p = q /r
Y = -px+q
Y = -p(x-r)
r
Pi (log scale)


Relative yield
1
M
0
T
M'
Pi (log scale)

described by rectilinear regression of plant yield upon logarithmically transformed nematode densities, although the author discussed possible variations on this at very high or low densities.The ratio of the values q and r in Oostenbrink's model gives the value p; thus p is the slope of the regression and a measure of the effect of each nematode on plant yield. Data from several studies were presented in support of this model; one of these was the study of Lownsbery & Peters (1955) who reported the effects of H. tabacum upon some growth parameters of tobacco. Their work is significant in that their attempt to relate tobacco height and weight to Pi involved the fitting of models of differing polynomial order. Starting with the linear term, they investigated whether residual deviations of yield from the fitted line could be reasonably ascribed to random variation. If such was not the case, the next order of term (quadratic, cubic etc.) was fitted. Their conclusion was that higher order terms were trivial and that the linear model was adequate.

Seinhorst (1965) was wary of the fitting of linear regression models to such data. In his view, there existed no theoretical basis for the fitting of straight lines and to do so begged invalid extrapolation; his main concern was that damage to plants would be overestimated at low densities of nematodes. Seinhorst proposed a model (Fig.6.2) based on Nicholson's competition curve (Nicholson 1933). The logic behind this model is shown in Table 6.1, in which an individual nematode is said to attack a given proportion, d, of a root system.

Damage functions for resistant and susceptible seedlines.

Table 6.1 Formulae describing proportion of a root system destroyed or left by increments in nematode population. (Adapted from Seinhorst (1965)).

	Proportion of root destroyed	Proportion of root not destroyed
1 nematode	d	$(1-d)$
2 nematodes	$d+d(d-1)$	$1-d-d(1-d) =(1-d)^2$
3 nematodes	$d+d(d-1)+d(d-1)^2$	$(1-d)^3$
P nematodes	$d+d(d-P-1)$	$(1-d)P =zp$

Thus, $y=zp$ describes the expected yield at a given population density, while values of y, the relative yield, lie between 0 and 1. The value z reflects the damage inflicted by a single nematode and was said by Plowright (1985) to range between 0.97 and 0.99 in most plant/nematode interactions.

Both Seinhorst (1965) and Oostenbrink (1966) considered the implications of their models at high or low nematode densities. Seinhorst argued that the yield of plants was unaffected at densities below a certain threshhold which he called the tolerance limit, T. Biologically, the existance of T was said reflect the plant's ability to replace lost roots, or sometimes that plants have more roots than they need to support a given top size. He also noted that it was wrong to assume that yield at high density must necessarily be very low; sometimes aerial parts of plants could be productive in the absence of much root and sometimes soil conditions might limit nematodes' pathogenicity. Thus, a value for the relative minimum yield, M, was included in his model; this appears

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Damage functions for resistant and susceptible seedlines.

graphically as a plateau when nematode density is high. In its full form, Seinhorst's model is represented by the formula

$$Y = M + (1-M)z^{(P-T)}$$

The reply of Oostenbrink (1966) was that he was not convinced of the existance of tolerance limits. While he acknowledged that they were apparent in a few experiments, he denied that such was the case under field conditions; he reported experiments in which extrapolation of the regression to the y-axis seemed valid as well as situations in which of plant yield was seemingly enhanced by low numbers of nematodes. He was less sceptical of Seinhorst's notion of relative minimum yield, but cited an example in which death of the host plants meant continuation of the regression to the x-axis was valid. To Oostenbrink's way of thinking, Seinhorst's curve was not well supported by data and the combining into a single mathematical formula of observations resulting from a number of different biological phenomena was unsound. Some of his views were shared by Ferris (1978) who thought "...error incurred by the assumption of linearity is minimal relative to the inherent variability of field data." Oostenbrink's tacit view was that linear regression should be applied to the middle portion of the function and that curve fitting at the extremes was a matter for the experimenter's discretion. This approach would now be regarded as the fitting of linear regression with breakpoints dictated by data and biological understanding. Such an approach has been taken by Schmitt et al. (1987), whose complete model provides different formulae depending whether P is greater or less than T.

Damage functions for resistant and susceptible seedlines.

Recent work has acknowledged that changes in nematode population over the course of an annual crop's growing season could substantially influence yield. Noe et al. (1991) and Noe (1993) included midseason and at-harvest values of P in models describing damage functions brought about by Hoplolaimus columbus. The complexity of these functions introduce a whole new tier of variability to the description of the crop/nematode interaction, even given the relatively simple case of the annual crop. The situation with perennial crops is presumably yet more complex because of ongoing response and counter-response between host and pathogen, and environmental fluctuations. Ferris and McKenry (1975), for instance, made several thousand measurements of grapevine yield and growth and nematode populations yet found "...relatively few clear relationships..."

Damage functions for resistant and susceptible seedlines.

The concept of tolerance

Nematologists continue to grapple with terms and concepts used to describe relationships between plants and parasitic nematodes. The system of Trudgill (1986) is useful in describing effects on plant yield and nematode reproduction :-

		Host yield	
		high	low
Nematode reproduction	high	tolerant/ non-resistant	non-tolerant/ non-resistant
	low	tolerant/ resistant	non-tolerant/ resistant

The terms in this chart describe extreme cases. Commonly, plants do not express absolute resistance or tolerance to cyst nematodes so the degree to which they have these attributes is described in relative terms (Trudgill 1986). The notion of tolerance is sometimes not well defined and authors use it in different ways. Strictly, tolerance describes "the extent to which a plant is able to to withstand infection without undue damage" (Robinson 1969); thus a very tolerant plant is able to host large numbers of parasites without appreciable yield loss. Confusion arises when tolerance is described in terms of numbers of nematodes that could potentially be supported, rather than numbers actually supported. A plant growing well in the presence of much nematode inoculum may be doing so for two different reasons: it may be tolerant in the above strict sense, or it may resist the nematodes and thus be little

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Damage functions for resistant and susceptible seedlines.

affected by them. Although inconsistantly with Robinson's definition, the latter case is sometimes referred to as "tolerance conferred by resistance" (eg. Trudgill 1986). It seems important to explain whether the term is being used of a plant with regard to nematode numbers actually hosted, or numbers in the surrounding soil (eg. in terms of Pi). In this thesis I shall refer to these phenomena as, respectively, "strict-sense tolerance" and "conferred tolerance". It will be noted that Seinhorst's "tolerance limit" does not necessarily have anything to do with tolerance, and the concept might be better communicated under another name.

T. repens and H.trifolii

Seinhorst & Sen (1966) graphed white clover seedling dry weights against a log scale of H. trifolii inoculum densities and used the information to estimate a value of T in a pot experiment. He did not present a regression so did not discuss likely values of the parameters z or m from his equation. Plowright (1985) and Mercer (1990) both performed similar pot experiments in glasshouses and fitted Seinhorst's model to the resulting data. Plowright's work involved two cultivars of white clover but he did not use the regressions to make comparison between them.

Tolerance of a number of seedlines was compared by Mercer et al. (1992) who derived tolerance indices for each seedline by comparing parasitised with nematode-free plants. These authors concluded that, compared with the calculation of tolerance indices, the sort of regression analysis described above would be a superior method of assessing tolerance.

Damage functions for resistant and susceptible seedlines.

Although seedlines expressing a high degree of resistance to *H. trifolii* are now available, it has remained to be investigated what effect this resistance might have on the plants' top yield in the presence of nematodes. The attractive assumption that improved yield must follow selection for resistance is rather dampened by the findings of Trudgill *et al.* (1985) who reported that a potato selection resistant to both potato cyst nematode species was tolerant of neither. Similarly, tolerance was not conferred to oats resistant to *H. avenae* (Trudgill 1986).

The aim of this experiment was to investigate whether resistance in *T. repens* seedlings confers tolerance to *H. trifolii*.

Damage functions for resistant and susceptible seedlines.

Methods and Materials.

Six white clover seedlines were selected for use in this study. They were chosen because of their similar root weights and extreme resistance status (Table 6.2)

Table 6.2 Mean root dry weights and cyst counts (n=10) of seedlines in a resistance screening. Figures in brackets are standard errors of the means.

Line	Nominal status	Root DWT(g)	Cyst count
R1	resistant	.232 (.086)	21 (15)
R2	"	.313 (.110)	16 (13)
R3	"	.256 (.107)	22 (24)
S1	susceptible	.256 (.073)	215 (157)
S2	"	.243 (.060)	195 (152)
S3	"	.240 (.075)	221 (82)

Germinated seeds were planted singly into 6 cm diameter plant pots; about 135 seeds of each line were sown. The pots were placed in two steel trays to facilitate watering,and the trays were rotated about the glasshouse once every seven days for three weeks. Temperatures were maintained between 18 and 24 °C. After 21 days the pots were randomised as follows:-

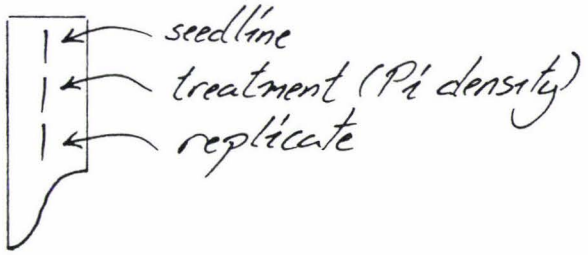
1/ R1 pots were lined up in descending order of top size.

Damage functions for resistant and susceptible seedlines.

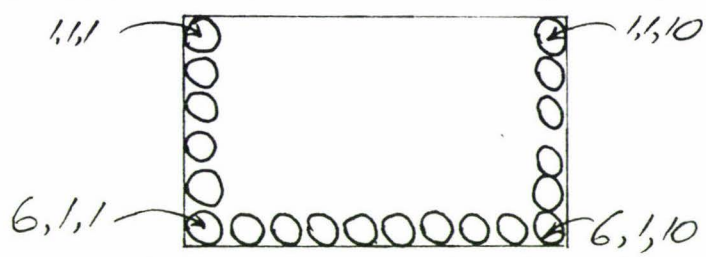
2/ They were divided into ranks of 10, such that the first rank comprised the 10 largest plants, the second rank the next largest 10, and so on. The procedure was repeated for the other seedlines.

3/ Labels were made up in the following form:

Label 1,1,1.



The labels 1,1,1, to 1,1,10 were placed in a carton. This was shaken to rearrange the labels to a random order so that they could be used to randomly assign the pots in the first rank to a treatment. After this had been repeated for every rank, pots were arranged so that all to receive each treatment were together, thus:



This meant that contamination from pots of higher inoculum rate was minimised.

4/ The six plants across each row were rearranged to an order determined by using the computer programme Stats Perrnd to permutate the numbers one to six, 100 times.

The aim of this multi-stage procedure was to achieve an unbiased distribution of plants of all sizes across the treatments.

Damage functions for resistant and susceptible seedlines.

Obtaining the large quantity of inoculum required for this experiment called for careful planning. Twenty weeks before the planned inoculation date, scarified seed of white clover (cv. Huia) was scattered over sterilised sand/soil medium in a dozen 10 cm diameter pots. The resulting seedlings were inoculated with 20,000 H. trifolii eggs from another colony and the nematodes were allowed to develop through two generations. Four weeks before the intended inoculation date, many large cysts were visible so the pots were transferred to a 10° C cabinet. This procedure resulted in more than 6,000,000 eggs being available.

The number and size of Pi treatments were established after consideration of the experiment's aims. Estimation of tolerance limits (T) would best be achieved by having a number of low Pi treatments; the 0.63 eggs per gram of dry soil value of Mercer (1990) was a guide. Accurate estimation of relative minimum yield (M), and the value of Pi at which it first occured, would require at least one treatment of high density (Seinhorst 1965). Further analysis of the data of Mercer (1990) showed Seinhorst's model predicted that M would first occur at a Pi of 148 eggs per gram of dry soil.This was beyond the range of densities applied by the researcher, but the abundance of inoculum available for the current study meant a highest density exceeding 148 could be used.

The values of Pi were designed to be evenly spaced along a logarithmic scale. To help identify suitable densities, a pencil line 798 mm long was drawn to represent the natural log values between e^{-2} (= 0.135) and e^{6} (= 403). The points representing the progression $e^{-1}, e^{-2} \ldots e^{5}, e^{6}$ were marked and the points $e^{-1.61}$ (= 0.200) and $e^{5.12}$ (= 167)

Damage functions for resistant and susceptible seedlines.

found by measurement. The distance between the latter two points was divided to give nine evenly-spaced Pi densities. Volumes were taken from the well-agitated stock suspension to give suspensions approximating these target densities. A single Doncaster dish estimation of each true density was made before adjustment by dilution or concentration using a 20 micron seive. Finally an accurate estimate was made by replicated counts of 3 mL aliquots; four counts were made, as was suggested by the experiment summarised in Fig 3.2. Pots were inoculated using the technique described previously. In order to allow assessment of invasion, eight additional seedlings were inoculated using density suspension number eight.

Table 6.3 Pi densities (eggs/g dry soil; n=4)
Figures in brackets are standard errors of means.

Treatment code	Target density	Actual density	
1	0.00	0.00	
2	0.20	0.30	(0.05)
3	0.47	0.72	(0.18)
4	1.09	1.21	(0.14)
5	2.51	2.38	(0.49)
6	6.30	7.94	(1.68)
7	13.5	14.5	(4.14)
8	32.2	28.3	(1.73)
9	68.7	72.3	(7.83)
10	167	203	(27.3)

After inoculation, trays were rotated each Monday, Wednesday and Friday for 28 days. One litre of half-

strength Thrive® solution was poured into each tray on two occasions; these dates were seven days after planting and 10 days after inoculation.

Assessment of damage symptoms began 14 days after inoculation, when the proportion of plants having yellowed cotyledons was noted for each seedline. Nine days later, a similar count was made of plants showing reddening of petioles, and each plant was assessed for the number of fully-opened trifoliate leaves and the length of the longest petiole.

Over the period 28 to 30 days after inoculation, each plant was washed free of soil and the top and root separated. Tops were dried to constant weight in an oven set at 80° C, then weighed to an accuracy of 0.5 mg on a balance (Mettler A30, Switzerland). Additional data were collected for inoculum densities 1,7 and 9 in that top fresh weights and root dry weights were recorded to allow study of the nematode's effect on moisture content and root:top ratio. Nematode counts were undertaken for Pi densities 6 and 10. The elutriation tower and sieves were used to capture nematodes dislodged by the washing process, and the roots were stained and examined by the technique outlined in Chapter 5. Nematodes were categorised as vermiform J2, swollen J2 and J3.

Top dry weight, trifoliate leaf number and petiole length were plotted against the natural logarithms of the Pi values. The nematode's effect on trifoliate leaf number was adequately described by linear regression; values of regression weights were calculated and compared using the Probability Calculator of the programme CS-Statistica®. Top dry weight and petiole length were approximately described by Seinhorst's model, so values for the parameters T and M and the slope of the regression were sought. Initially the equation was entered into the CS-Nonlinear Estimation

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Damage functions for resistant and susceptible seedlines.

module and the programme used to iteratively estimate means of T, Z and M for each seedline. The estimate of Z was then inserted into the equation; this allowed the programme to use the quasi-Newton algorithm to calculate standard errors of the T and M means. With this information in hand it was a small step to find s^2 -the weighted average of sample variances (Steel and Torrie 1981)- and thus the use of t-tests to compare lines was possible. Single-tailed tests were appropriate to test the hypothesis that T or M values were larger for resistant than for susceptible lines. With the values of Pi corresponding to T and M' known, investigation of the slope of the mid-portion of the function could proceed. Data concerning Pi values lower than T or greater than M' were first eliminated, then the remaining data subjected to linear regression. The regression coefficient of each resistant line was compared with that of each susceptible line.

Dry matter percentage and root:shoot ratio were plotted against three nematode densities on a log-linear scale. The CS-Linear Regression module was used to find regression coefficients for each seedline and the values compared using CS-Statistica's Probability Calculator.

To give an idea of the Pi density in pasture, four cores (4.5 cm diameter x 4.5 cm depth) were taken from a permanent sheep pasture near the Grasslands campus. Cysts were extracted by sugar centrifugation and the numbers of white and brown cysts per core recorded. Old, empty cysts were not included. The cysts were pooled, and the technique in Chapter 5 used to find the number of eggs and juveniles in a sample of 10 taken at random. Another four cores of the same volume were dried to constant weight and weighed.

Damage functions for resistant and susceptible seedlines.

Results.

Top Dry Weights.

The best-fitting Seinhorst functions for the six seedlines are shown (Fig. 6.3). Top Dry Weight of individual plants in a line was subject to considerable variation (Fig. 6.4). Values of the parameters M and T are given below (Table 6.4).

Table 6.4. Top dry weight parameters. Figures in brackets are standard errors of estimates of M and T. Regression weights are as follows: r^a refers to each line's Seinhorst function and the % variance for which the function accounts; r^b refers to linear regression of Pi values between T and M'; r^c refers to linear regression over the whole range of Pi values. All values of r are, of course, negative.

Line	M	T	r^a	r^b	r^c
R1	.42 (.07)	1.3 (3.3)	.54 29%	.29	.51
R2	.43 (.09)	1.6 (4.0)	.47 22%	.37	.41
R3	.13 (.07)	0.0 (2.8)	.53 29%	.61	.61
S1	.27 (.10)	0.0 (3.0)	.48 23%	.44	.44
S2	.20 (.08)	0.0 (2.8)	.59 35%	.55	.55
S3	.27 (.08)	0.9 (2.9)	.59 35%	.45	.56

Values of M ranged from 0.199 to 0.427 (Table 6.4). Resistant lines recorded the highest two, and the lowest, mean values of M. Statistically significant differences in M were found in the comparisons between S2 and the

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Damage functions for resistant and susceptible seedlines.

Figure 6.3. Best-fitting Seinhorst models of the effect of initial *H. trifolii* density upon relative top yield of three resistant and three susceptible *T. repens* seedlines, 28-30 days after inoculation.

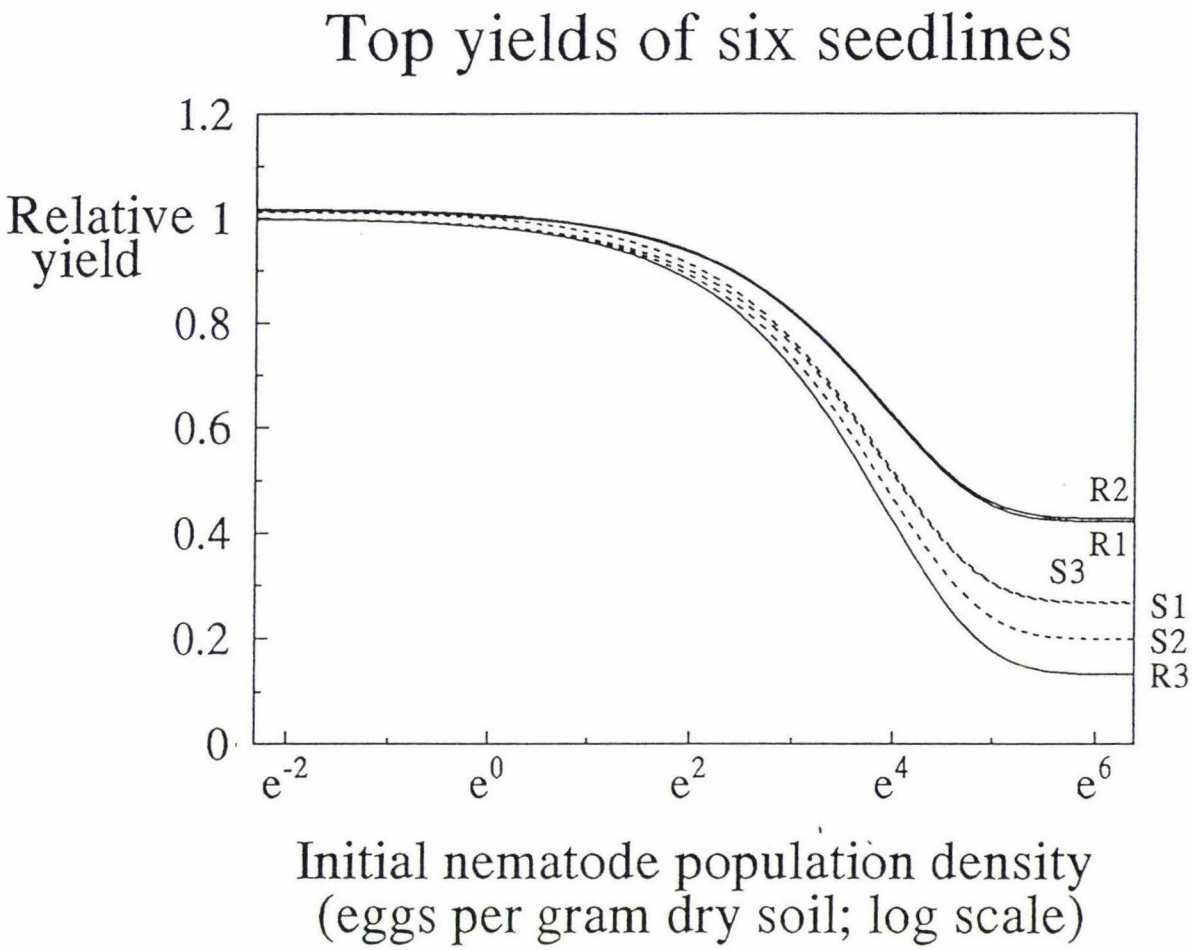
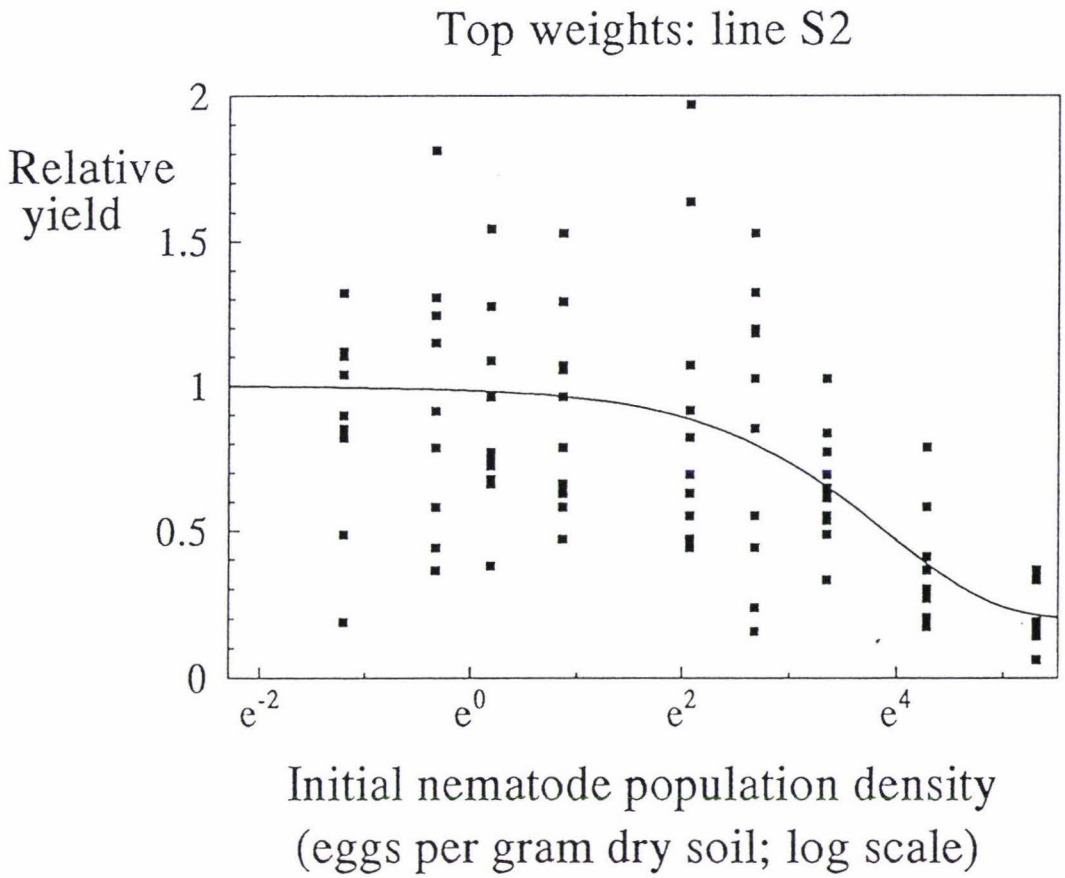

Top yields of six seedlines
Relative yield
1.2
1
0.8
0.6
0.4
0.2
0
R2
R1
S3
S1
S2
R3
e-2
e0
e2
e4
e6
Initial nematode population density
(eggs per gram dry soil; log scale)

Figure 6.4. Relative yields of individual seedlings of the *T. repens* seedline S2 challenged with a range of initial egg population densities of *H. trifolii*. The figure illustrates the variability in relative yield among seedlings challenged with the same number of nematodes, and a possible stimulation effect of Pi values in the range e^{-1} to e^{2}.


Top weights: line S2
Relative yield
2
1.5
1
0.5
0
e-2
e0
e2
e4
Initial nematode population density
(eggs per gram dry soil; log scale)

resistant R1 and R2. Values of T ranged between 0 and 1.6 eggs/gram. In each case the standard error greatly exceeded the mean value of T and no significant differences between lines could be detected. Coefficients of linear regression did not differ between lines; this was true whether the regression was performed over all values of Pi or only those between T and M'.

Petiole Length.

No relationship between petiole length and resistance status was discernable (Fig.6.5); for this reason significance testing of the parameters was not performed. Values of the parameters are given below (Table 6.5).

Table 6.5 Petiole length parameters. See Table 6.4 for definitions of parameters.

Line	M	T	ra	rb	rc
R1	.57 (.04)	0.0 (3.1)	.62 38%	.59	.59
R2	.70 (.05)	0.0 (3.8)	.44 19%	.43	.43
R3	.52 (.05)	2.4 (2.5)	.65 42%	.30	.62
S1	.73 (.07)	16 (5.2)	.39 15%	.08	.33
S2	.62 (.05)	7.6 (3.0)	.57 33%	.27	.53
S3	.54 (.05)	0.0 (2.7)	.62 39%	.60	.60

Values of M lay between 0.518 and 0.731; T ranged from 0 to 16.26 eggs/gram.

Trifoliate Leaf Number and Top Dry Weight.

Significant linear regression characterised the effect of nematode density on trifoliate leaf number (Fig 6.6). Leaf number fell with increasing inoculum density; regression

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Figure 6.5. Best fitting Seinhorst models of the effect of *H. trifolii* population density (Pi) upon the length of the longest petiole of three resistant and three susceptible *T. repens* seedlines, 23 days after inoculation.

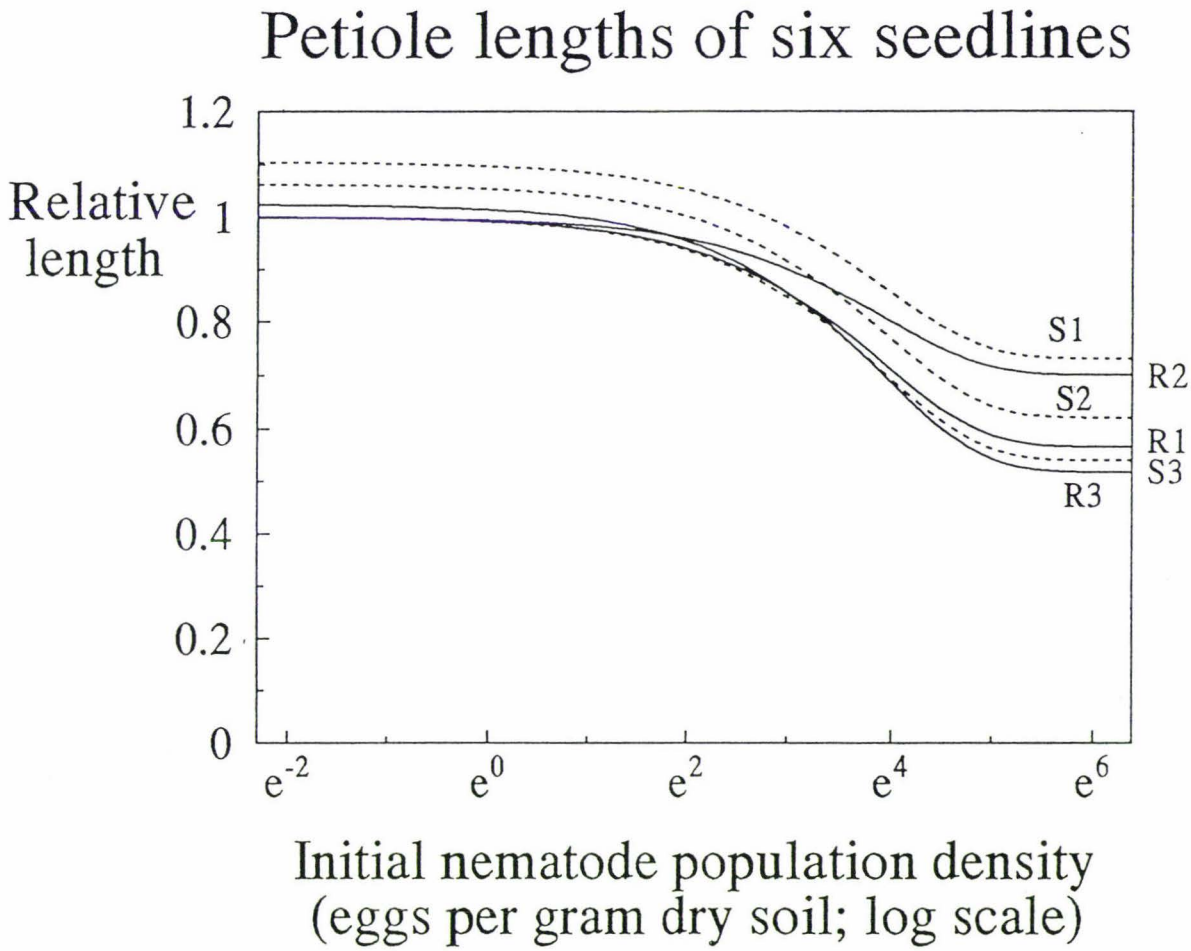
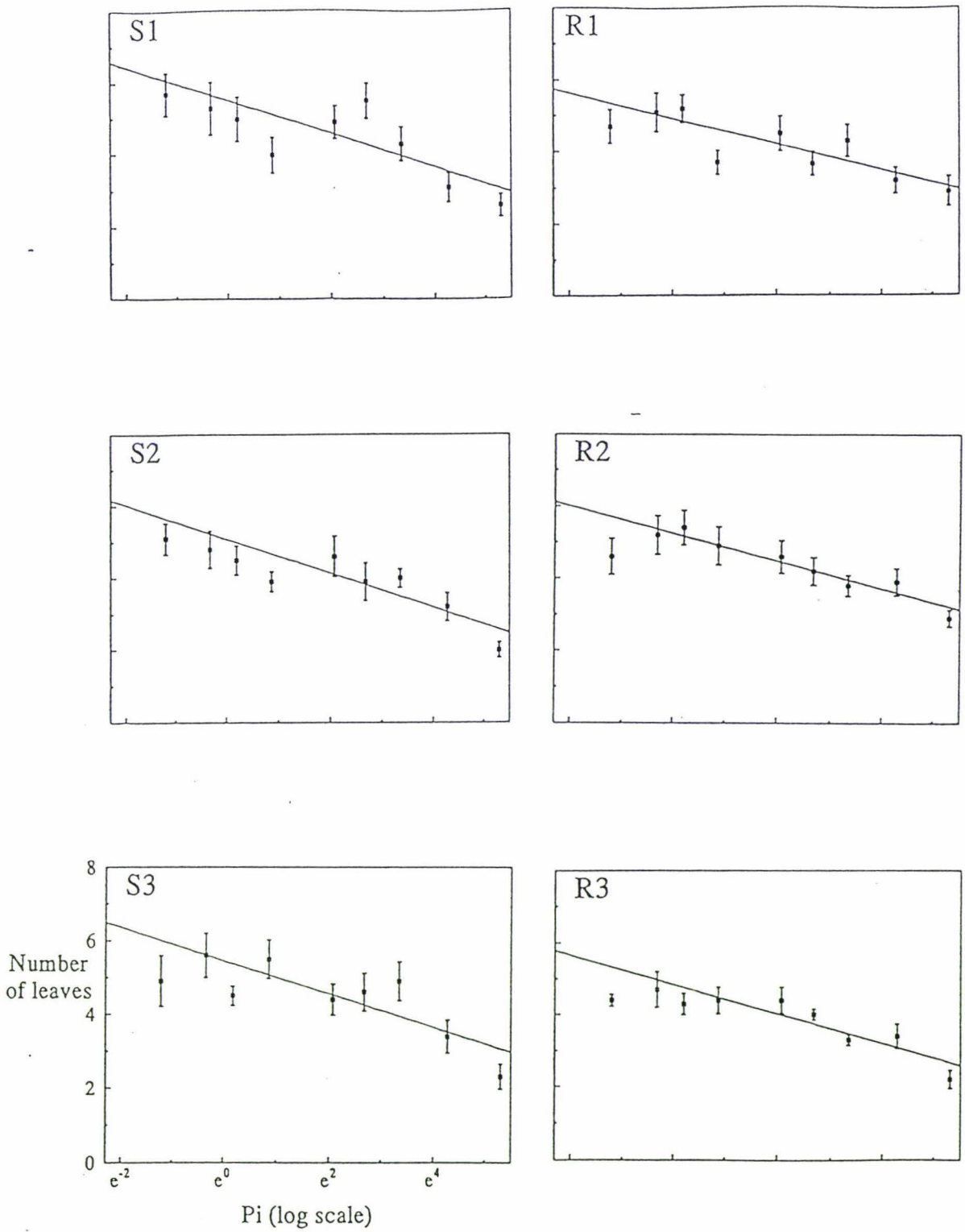

Petiole lengths of six seedlines
Relative length
1.2
1
0.8
0.6
0.4
0.2
0
S1
S2
R3
R2
R1
S3
e-2
e0
e2
e4
e6
Initial nematode population density
(eggs per gram dry soil; log scale)

Figure 6.6. Number of trifoliate leaves of seedlings of three resistant and three susceptible *T. repens* seedlines 23 days after inoculation with a range of densities of *H. trifolii* eggs.


S1
R1
S2
R2
S3
R3
8
6
4
2
0
Number of leaves
e^-2
e^0
e^2
e^4
Pi (log scale)

coefficients were in the range -0.416 to -0.587 and did not differ significantly between seedlines.

Dry matter content rose with increasing inoculum with coefficients between 0.528 and 0.873 but without significant differences between lines (Table 6.6).

Table 6.6. Regression coefficients of plant top dry matter percentage upon initial population density (Pi). Pi value were 0, 14.5 and 72.3 eggs per gram of dry soil. In each case $P < 0.05$.

Seedline	r
R1	0.873
R2	0.528
R3	0.769
S1	0.680
S2	0.681
S3	0.813

Root:top dry weight ratio was not changed by nematode infestation.

Symptoms of infestation were visible on all lines at high inoculum densities. Yellowing of cotyledons was almost universal at the maximum density but varied among seedlines at the next highest density. The number of plants showing red stippling of petioles at maximum density also varied from line to line. Expression of these symptoms was independent of a line's resistance status.

Damage functions for resistant and susceptible seedlines.

Plate 6.1. Symptoms of severe H. trifolii infestation. Compared with the nematode-free plant (left), the infested seedling (right) is smaller, has shrivelled cotyledons and stunted roots and red stippling of the petioles.

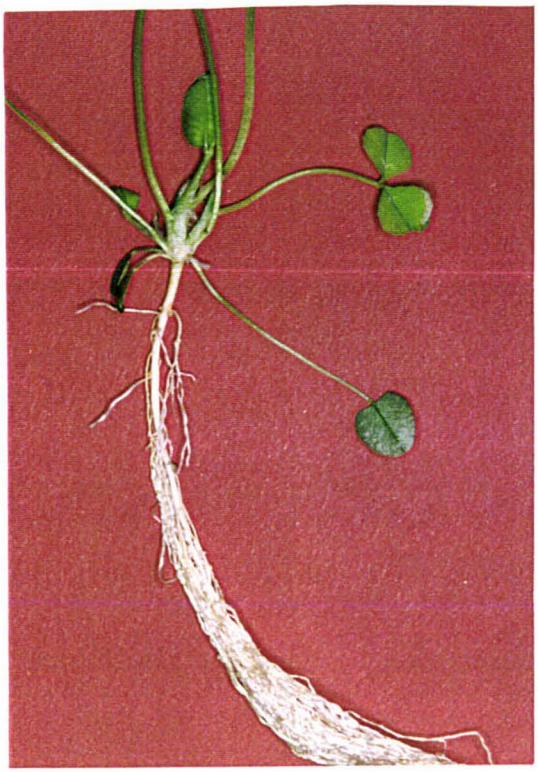


Plate 6.2. T.repens plants 27 days after inoculation with (left to right) 0, 7.9 and 203 H. trifolii eggs/g dry soil. Above is the relatively tolerant seedline R2, while below is the relatively intolerant S2.

Plate 6.3. *T.repens* plants of six seedlines: not parasitised (above) or inoculated at the rate of 203 *H.trifolii* eggs/g dry soil (below).

Numbers of nematodes in stained roots are given in Table 6.7.

Table 6.7. J3 and total *H. trifolii* numbers in stained roots 30 days after inoculation with 7.9 or 203 eggs per gram of dry soil.

Line	J3 (Pi=7.9)	J3 (Pi=203)	Total (Pi=203)
R1	14 ab	21 a	137 a
R2	4 a	12 a	207 a
R3	7 a	18 a	126 a
S1	37 b	48 a	324 a
S2	24 b	36 a	111 a
S3	42 b	61 a	154 a

Within a column, a common letter indicates means are not different ($P< 0.05$).

Differences in nematode populations were found only in terms of J3 numbers and only at the lower inoculation rate, where they reflected the nominal resistance status.

Table 6.8 summarises the results of field sampling.

Table 6.8. Cysts per core and eggs or juveniles per cyst extracted from soil cores from pasture.

	n	mean	std error
cysts/core	4	29	30
eggs or J2/cyst	10	108	34

The field Pi density of encysted eggs and juveniles was about 58 per gram of dry soil.

Damage functions for resistant and susceptible seedlines.

Discussion.

Only a little evidence was found that would support the view that resistant seedlings are necessarily tolerant of *H. trifolii.* Given the undistinguished M and T top yield values of line R3, even the high M value of line R2 can hardly be considered representative of resistant material.

Damage to seedlings seems to occur soon after infestation rather than being dependant upon establishment of syncitia. That this is so is shown by the yellowing of cotyledons soon after inoculation and the large numbers of J2 in the severely damaged plants of the highest Pi density. Bearing in mind a finding reported in Chapter 5 - that resistance status has little effect on initial infestation - perhaps it is not surprising that tolerance is little related to resistance status in young seedlings. The unfortunate implication is that resistant selections will be no more protected from nematode damage than any other seedline during the first weeks after sowing.
The question will arise: what,then,is the value of incorporating resistance into a white clover cultivar? It seems that the benefits of resistance are most likely to be seen in the longer term. By lowering the equilibrium population density, it is likely that protection will be afforded to plants growing from seed or excised stolons in subsequent seasons. Glasshouse investigations of this are feasible, but, especially given the long inoculum build-up period, are well outside the timeframe of this thesis. An appropriate approach might be to repeat the current experiment, but sow fresh seedlings of each line into the pots following completion of a nematode generation. The damage functions of this second plant generation would then suggest whether a line's resistance can confer tolerance - in yet a different sense - in the longer term. The performance of line R2 is encouraging because it confirms the existence of plant genotypes which are both

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Damage functions for resistant and susceptible seedlines.

resistant and tolerant as well as producing high yield in the unparasitised state. Such is not always true; Mercer & Grant (unpub. data) challenged T. semipilosum genotypes with eggs of a Meloidogyne species and noted that immune plants in this interaction were generally smaller than susceptible plants. Plants expressing both resistance and tolerance may well be a key to improving white clover survival and productivity in infested pasture, but some theoretical and practical barriers need to be overcome before identification of tolerance by pot trials can proceed.

One such barrier to the recognition of tolerance is the selection of an appropriate tolerance parameter. Seinhorst's tolerance limit (T) initially seems an attractive measure, but in this experiment models describing top dryweight generated values of T no higher than 2 eggs per gram of dry soil for each seedline. These low values look to be typical of cyst nematodes; in summarising models for five cyst nematode species parasitising six host species, Seinhorst (1986) reported each T value to be below 3 eggs per gram. Models derived for white clover seedlings attacked by H trifolii have given T values of " below 3" (Plowright 1985) and 0.9 (Mercer 1990). As well as being universally low, the current experiment's ' estimates of T were characteristically subject to great variation, and standard errors were frequently larger than their mean. This fact contributed to the finding of no differences between seedlines in terms of top yield tolerance limit. A further difficulty in defining tolerance limits arises when plants hosting a few nematodes outyield the nematode-free control; that is, when parasitism stimulates increase in yield. Such was the case ' in analysis of the effect of parasitism on petiole length and is the reason why some regressions in Fig 6.5 intercept the y-axis at a point well above 1.0. The issue was raised by Oostenbrink (1966)

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but does not yet seem to have been satisfactorily addressed. Ferris (1978) tried to get around the problem by defining maximum yield not as the yield of controls,but as the mean for all treatments up until where the researcher thought T might lie. As a technique for identifying T, however, this approach seems merely to initiate a cyclical argument. In summary, there exist strong reasons to doubt whether a parameter which is subject to a questionable assumption,liable to immense variability and consistantly of small value is at all useful in comparing seedlines. The assertion of Seinhorst & Sen (1966) that the tolerance limit of the T. repens/H. trifolii association exceeds 50 eggs/g is unconvincing in the light of three later sets of results and may be an artifact of the fact that a regression was not actually derived for their data. It is likely, judging by the field population density reported here, that white clover seedlings are commonly challenged by numbers of nematodes far surpassing any tolerance limit that may exist in nature.

In contrast to T, minimum relative yield (M) values were generally significant for regressions describing both top yield and petiole length and values of M could be meaningfully compared. The summaries of both Trudgill (1986) and Seinhorst (1986) support the view that consideration of M is the best measure of tolerance. With this in mind, it becomes possible to consider how an efficient screening technique for nematode tolerance might be designed. The approach of Mercer et al (1992) was to generate tolerance indices by dividing yields of parasitised plants by those of nematode-free plants. Although these authors concluded that identification of tolerance might be better performed by fitting of regressions, the tolerance index technique would have merit if used to estimate values of M. To achieve this, a high Pi treatment would have to be used; the 6 eggs/gram

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applied by Mercer et al. (1992) was too low a density and indeed it may have caused stimulation, rather than suppression, of top yield. Results from the current experiment suggest that densities approaching 70 eggs/gram dry soil would be appropriate. Expertise in the production of large quantities of inoculum is now available and concurrent screening of perhaps 200 seed lines is feasible.

Features observed in high Pi treatments - yellowing of cotyledons and increase in dry matter content- were considered diagnostic of nematode infestation by Seinhorst (1986). He saw these as the result of a separate mechanism from that causing yield reduction at lower densities and considered the model introduced above was strictly best used only for Pi values up to about 64T. Taking this approach with the current data set was not helpful, however, because the stimulation effect described above caused the model to account for considerably less than 10% of the variability of each seedline. Seinhorst (1986), too, acknowledged that his attempt to model for two separate mechanisms did not really describe the situation much more accurately than the more simple model.

Since white clover selection has tended to be by means of in-pasture trials, it may well be that selection for nematode tolerance is already more advanced in white clover than other plants since the latter have often been selected in the absence of disease (Wallace 1987). Nevertheless, seedlines that outperform the existing cultivars in the presence of nematodes continue to be developed (R.N. Watson pers. comm.) and appear to be tolerant of nematode damage rather than resistant (Grant & Mercer unpub. data). The use of field testing techniques to identify these lines seems likely to result in useful material especially since productive selections have almost certainly tolerated a broader range of stresses

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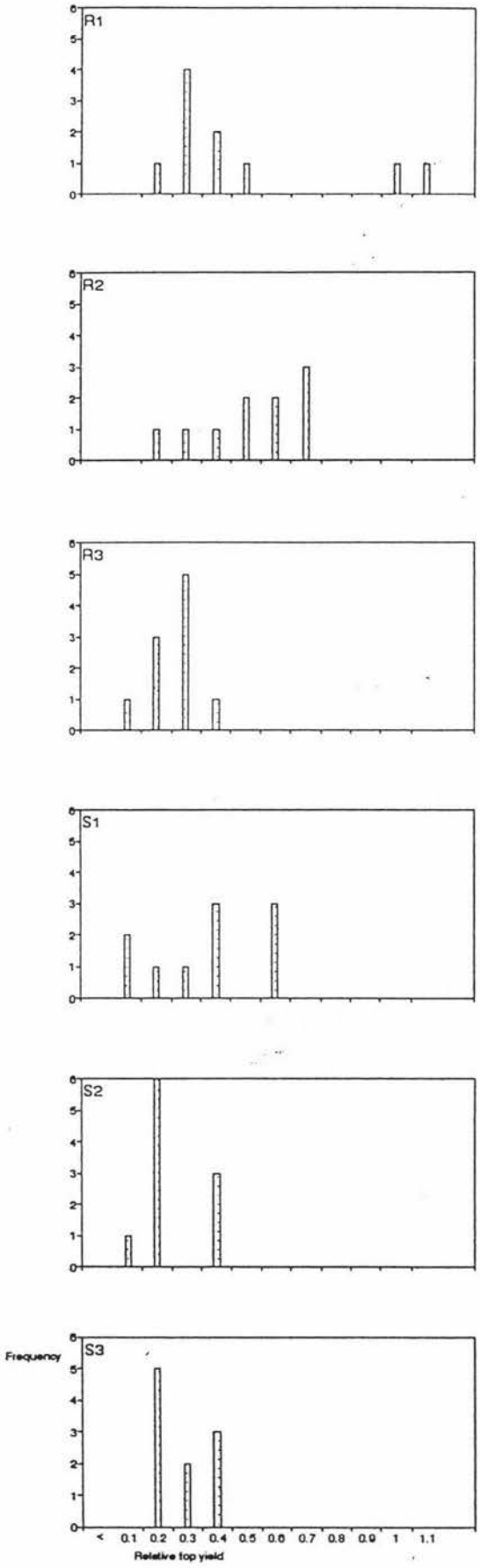
than nematode damage alone. Regarding the nature of stresses on plants, Wallace (1987) speculated that tolerance to nematodes may not be at all specific, but that so-called tolerant plants respond to the damage symptom, rather than to the nematodes themselves. He argued that a plant tolerant of water stress would respond identically whether the stress was caused by drought or root damage by nematodes, fungi or insects; that is to say, hosts do not distinguish causal agents. This thought suggests that plants may be screened for tolerance by simulating damage. Perhaps nematode damage might be mimicked by the standardised pruning of roots of potted plants and using yield data to find tolerance indices; difficulties induced by high inoculum requirements would then be avoided.

At the highest Pi density, relative yield varied considerably among individuals within a seed line (Fig 6.7). This is to be expected, since tolerance is probably controlled by many genes (Dalmasso _et al_. 1992) and, because white clover is an obligatorily cross-pollinating species, the range of possible combinations of these genes is probably wide. A refined screening technique might take this into account, perhaps by finding tolerance indices for only the largest 50% of individuals in the line. Bearing in mind that non-vigorous seedlings are unlikely to prosper in pasture (Mercer 1990) it is likely that such an approach would provide more useful data than would averaging indices for all plants.

Damage functions for resistant and susceptible seedlines.

Figure 6.7. Frequencies of relative top dry weight yields, of 10 individuals of each of three resistant and three susceptible T.repens seedlines. Yield was found 28-30 days after inoculating these seedlings with 203 H. trifolii eggs/g dry soil, and expressing their top yield as a fraction of that of unparasitised seedlings.


R1
R2
R3
S1
S2
S3
Frequency
< 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1 1.1
Relative top yield

CHAPTER 7 ADAPTATION TOWARD PARASITISM.

In plant material selected for resistance to nematode pests, the resistance is often only partial; a small number of parasites are able to complete their lifecycle on resistant plants. To date, this has been the case in Grassland's white clover accessions showing resistance to *H. trifolii*; these selections would probably reduce the incidence of the pest in a pasture but are unlikely to eradicate it. To help assess the usefulness of selections, the need has arisen to study the durability and breadth of applicability of such resistance.

Mercer & Grant (1993b) challenged white clover seedlines with nematodes descended from cysts collected from sites throughout the North Island and noted that a few populations were able to develop equally well on resistant and susceptible selections. From this was postulated the existance of more than one biological form in the New Zealand *H. trifolii* population. Further, these data suggested that the resistance was of the "vertical" type (van der Plank 1968); that is, that resistance is the result of a small number of genes.

In the system of van der Plank (1968), individual nematodes able to overcome vertical resistance are said to be "virulent"; the term also describes populations in which the frequency of virulent individuals is high. Such terminology, however, did not meet with the approval of Triantaphyllou (1987) who argued that the issue of concern was not strictly virulence (in the sense that we would use the term of a fungal pathogen) but the nematode's ability to parasitise. Consequently the latter used terminology such as "adaptation toward parasitism" and "parasitism gene"; these are the conventions adopted in this chapter.

Research described here sought to address the issue: might prolonged exposure to resistant white clover alter the

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parasitic ability of a population of H. trifolii? In pondering hypothetical answers to this, consideration of the story of the Mi gene in tomato has been helpful, as there exist obvious analogies between it and the interaction considered in this thesis. The Mi gene can be transferred into the tomato genome from another plant in the same genus and has been incorporated into tomato cultivars since the 1950's. It provides resistance to several Meloidogyne spp. The similarities between Mi gene resistance and that expressed by white clover to H. trifolii are these: both probably rely upon a small number of genes; both offer resistance which is high but nevertheless partial; in both cases the pest nematodes reproduce by mitotic parthenogenesis.

Unfortunately, repeated exposure of some Meloidogyne populations to plants bearing the Mi gene has sometimes resulted in increases in the populations' parasitic ability (Roberts and Thomason 1989). This has occured both in the field situation (Sauer & Giles 1959) and in controlled experiment involving sequential reinoculation with succeeding nematode generations. In an extreme example of the latter, Bost & Triantaphyllou (1982) reinoculated for 12 generations and raised the number of egg masses of M. incognita on a resistant cultivar from 2% to 72% of that on a susceptible cultivar.

Roberts & Thomason (1989) recognised four categories of parasitic variation in Meloidogyne spp. populations on tomato cultivars with the Mi gene. Their first category comprised populations that were unable to adapt toward parasitism, while second category populations, upon prolonged exposure to the host, demonstated a gradual, stepwise adaptation. Other populations - their third group- might by chance display a degree of parasitic ability without prior exposure to Mi-bearing plants. It was postulated that parasitic individuals might carry the advantage by chance association with some

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other trait. A final group were fully parasitic even upon initial exposure to the gene.

The work of Mercer & Grant (1993b) seems to have identified H. trifolii populations akin to Robertson & Thomason's third or fourth categories; virulence in the H. trifolii population is already known to vary on a geographic basis. The aim of this experiment was to determine whether a Palmerston North population has adaptive ability.

Methods and materials

Sequential reinoculation was used to test for the development of a parasitic population. Succeeding generations of inoculum were used to challenge a resistant and a susceptible plant genotype. Plant hosts used in this experiment were propagated cuttings of genotypes S1 and R1 (see Chapter 5). R1 was selected in preference to R2 as it is a little less resistant and therefore more likely to produce sufficient nematode progeny to inoculate the next generation.

From the work described in Chapter 5, values could be assigned to the relative susceptibility of the two plant genotypes. These are the "generation 1" values of Table 7.1. At the same time as the first experiment of chapter 5 was run, an additional seven clones of each genotype were generated from cuttings and inoculated with 6500 eggs. This was done to provide sufficient inoculum from each genotype to inoculate a further round of cuttings; the high inoculum rate was selected to maximise egg production on each cutting (Mercer 1990).

After one nematode generation, elutriation was used to recover cysts from the seven R1 plants. All cysts were picked out under the dissecting microscope and then crushed in the tissue grinder. A total of 6300 eggs was obtained in this fashion. The procedure was repeated for two of the S1 plants; cysts

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were picked at random and crushed until 12600 eggs had been obtained. The eggs were used to inoculate plants of both genotypes in order to set up the following treatments:

R-->R (eggs produced on R1 and inoculated onto R1)
S-->S (eggs produced on S1 and inoculated onto S1)
S-->R (eggs produced on S1 and inoculated onto R1)

This design allowed the R-->R treatment to be compared with two sorts of control. If the R-->R treatment were to result in more cysts than S-->R, the adaption toward parasitism would be indicated. Expressing this as a percentage of S-->S would express the degree of such adaption. Inherent in the latter, of course, is the assumption that S-->S nematodes remain constant in parasitic ability over the generations; put another way, that S1 is 100% susceptible.

Although more plants were available, it was decided to use four plants in each treatment. Distributing the limited inoculum resource over four replicates seemed the best compromise between the twin goals of sufficiently large differences in cyst counts between treatments and sufficient rep number to allow statistical separation of these means. The use of more reps at a lower inoculation rate might have resulted in numerous single-figures cyst counts; any error in the recovery of cysts would then have had a disproportionately large effect on the result. Conversely, fewer reps might have limited my ability to study variability since the difference between three and two degrees of freedom makes for substantial change in the value of the t statistic.

The plants to host "generation 2" nematodes were each inoculated with 1575 eggs and maintained as described in Chapter 3. After one nematode generation, cysts were recovered by elutiation and counted. Eggs were extracted, using the technique described above, to initiate a third generation.

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Unfortunately root growth in the cuttings taken for this next generation was uneven; the R1 plants had put out no more than a single root each and these were generally short. Rather than this unsuitable material, those plants from which cysts had just been elutriated were reused as hosts. The roots of each plant were trimmed to a 1 cm long bundle; this was inspected for cysts and female nematodes before potting in sand/soil medium. The tops were trimmed to leave stolons and petioles about 3 cm long. After potting, inoculation was delayed for three days to allow callusing of the root wounds and thus avoid supplying extra, artificial, penetration sites. During this time the cysts were stored in moist sand at 10° C. They were then removed from the sand by elutriation and their eggs extracted as described above. Experimental design was as for the previous generation, except that each plant was inoculated with only 551 eggs. Again, the cysts resulting from these eggs were recovered and counted after a generation.

Following log $(x+1)$ transformation, the mean number of cysts from each treatment in each generation was compared by ANOVA. Cyst counts of each treatment were expressed as a percentage of the S-->S count in the same generation.

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Results.

Over three nematode generations, the resistant genotype always hosted significantly fewer cysts than the susceptible (Table 7.1).

Table 7.1. Cyst numbers as counts and as a percentage of the S-->S treatment in the same generation.

Generation	Treatment	Mean Cyst Number (n=4)	% of S-->S
1	R	14	19a
	S	73	100b
2	R-->R	6	2a
	S-->R	30	12b
	S-->S	242	100c
3	R-->R	18	18a
	S-->R	31	30a
	S-->S	104	100b

A common letter indicates that values within a generation do not differ ($P<0.05$).

The first inoculation resulted in the resistant genotype supporting 19% the cyst number of the susceptible. In the second generation, numbers of cysts recovered from the resistant genotype depended on the nematodes' ancestry. Fewer cysts were produced by the population whose parents had been raised on R1 than those descended from parents on S2. By the third generation, cyst numbers of the population raised for

two generations on R1 plants were 18% the number of those always raised on S1. However, this did not represent a difference from those on resistant material for the first time (S-->R).

Discussion

Adaptation to a biological form of enhanced parasitic ability is indicated by the difference in cyst numbers between R-->R and S-->R in the second generation. Under the system of Roberts & Thomason (1989), this interaction would be regarded as belonging to the second, rather than the first category; some adaption towards parasitism is possible.

While this result will not particularly please those seeking to improve white clover's resistance, several points will help maintain perspective. Firstly, it must be remembered that a lot was done to help the nematode in this experiment. Temperature, moisture and plant growth conditions were near optimum for nematode growth. Combine this with the fact that large numbers of eggs were placed as close to the roots as possible and it will be appreciated that selection pressure in favour of nematodes with parasitism genes was probably considerable. In spite of this, the number of cysts recovered from resistant plants never exceeded 6% of the number of eggs injected. In the field it may well be the case that there are just too few nematodes with parasitism genes to allow resistance to be overcome; this would be especially true if mutation to new resistance alleles in the host were rapid.

A second point worth noting is that an initial rise in parasitic ability does not necessarily herald a major breakdown in resistance. Such a rise may be followed by no further adaptation, as Forrest & Phillips (1984) noted when they counted Globodera pallida eggs under resistant potato clones for five seasons. Finally, we should remember that the

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resistant white clover host chosen was not the most resistant available. Indeed, since this experiment was completed it seems genotype R1 has been outperformed by many lines from a further cycle of crossing (Mercer pers. comm.).

The well-known concept of Flor (1956) remains a useful starting-point from which to consider the genetics of the long-term host-parasite interaction. Flor wrote that "for each gene that conditions resistance in the host, there is a corresponding gene that conditions pathogenicity in the parasite". Hence, cases of stepwise increase in parasitic ability may reflect a gradual meeting of a series of resistance genes by a series of parasitism genes (Triantaphyllou 1987). In such a case, small-effect mutations in the parasite genome would make a given juvenile slightly more able to parasitise a resistant host than was its parent. Even in the absence of resistant hosts, such mutations may lay latent in the parthenogenetic nematode's gene pool, waiting, as it were, for a resistant host to come along. Provided that such mutations do not result in a selective disadvantage they will not be diluted out of the gene pool, as might happen in species reproducing by cross-fertilisation (Muller 1992). In-depth study of the genetics of parasitism in this interaction will always be hampered by the parthenogenetic nature of the H. trifolii's reproduction. It will more suit Grassland's strategy to study the genetics of resistance in the plant rather than the genetics of parasitism in the nematode; Triantaphyllou (1987) pointed out that such is the case in most research. A small diallele cross has suggested that resistance is a qualitative character (W M Hussein pers. comm.) The apparent contradiction between this and the vertical resistance inference of Mercer & Grant (1993b) begs further research to clarify the issue.

Some features of this experiment's methodology warrant examination. The inclusion of the S-->R treatment was

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instrumental in allowing quick recognition of a population adapting toward parasitism. Without this treatment, the only statistic would have been some ratio of the S-->S and R-->R treatments. Few conclusions could have been drawn from such a design as the large S-->S cyst count values tended to swamp changes in R-->R counts over the generations. A further difficulty is the risk of the S-->S value changing over generations. There may occur changes in parasitic ability of the population on susceptible as well as resistant genotypes; this would result in a "floating control" and perhaps mask adaptation on the resistant genotypes. This point is made because several researchers present results including only the latter two treatments. While such experiments give an intuitive feel that "something is going on" - that adaption is happening - it has to be wondered what influence density-dependant effects (see Chapter 5) and environmental variables are having. This is particularly the case when a parameter is compared across generations; Jarquin-Barberena et al (1991), for example, used ANOVA to compare M. incognita egg-mass numbers across up to ten generations.

A further design involves only a single inoculation. A sample of nematodes (eggs or juveniles) is taken with each new nematode generation, but most of the inoculum is left to reinfest the original hosts. Pf/Pi ratios are compared between plant lines or genotypes. A number of advantages are apparent: such a design lends itself to large-scale field -"real life"- experiments and minimises labour requirements. Eggs remain in a protective cyst or egg-mass so a higher survival rate would be expected. On the other hand, this design must be particularly prone to sampling errors caused by uneven nematode distribution (Ferris 1984) and is particularly open to the "floating control" criticism outlined above. One might imagine that the build up of, for instance, nematode-parasitising fungi would differ between plots of resistant and susceptible hosts in response to the different nematode

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densities after the first generation.

Two difficulties are apparent in the current experiment. Firstly, the decline in available inoculum with each successive generation would have limited the number of possible reinoculations. While the experiment's main aim was achieved, it would have been useful to have more of the selected inoculum available for further study. A secondary goal - to test whether selected inoculum had maintained its parasitic ability against a susceptible host - was not achievable in the absence of suitable eggs. The second concern was the need to reuse plant material for the third inoculation. This may or may not have contributed to the loss of difference in cyst number between R-->R and S-->R after the second generation; certainly it has about it an unsatisfying feeling of lack of experimental precision. Physiological stage of a plant can have an effect on resistance - Powers et. al (1992) found that resistance in alyce clover dissapated after flowering - but it is a moot point whether a trimmed mature plant is physiologically different from its rooted cutting.

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