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# *Ditylenchus dipsaci* Infestation of *Trifolium repens*. II. Dynamics of Infestation Development[1]

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*Abstract:* *Trifolium repens* (white clover) stolons were inoculated with *Ditylenchus dipsaci* (stem nematode), and the development of resulting infestations was monitored. Nematodes initially remained confined to superficial locations, concentrating in petiole axils near inoculation points. They were able to migrate slowly from the initial inoculation points and infest adjacent axils, especially in regions near the stolon tip. As time progressed, in some axils, nematodes migrated through the stolon epidermis and colonized slowly expanding subepidermal pockets of host tissue (ca. 0.2-mm length of stolon/day). In these loci nematodes established exponentially increasing populations, but the rates of locus expansion remained constant, indicating that locus expansion was limited by unidentified host-dependent factors. As a result of increasing population pressure within subepidermal loci, J4 entered a "diapause" state and the rate of egg production by adults declined, thereby reducing rate of population growth to more sustainable levels. Typically, these populations peaked at ca. 10,000 individuals in ca. 160 days occupying 3-cm lengths of stolon. Thereafter, heavily infested regions of stolons started to die, leading to the formation of longitudinal splits in their epidermis. In other axils, nematodes did not migrate into the stolons but remained confined to axils. Some of these populations increased a hundred-fold in 95 days, with population growth ending when petioles started to die. Host plant stolon morphology was affected only when subepidermal stolon populations developed high population levels (>100 nematodes) within close proximity (<2 cm) to active terminal meristems. This occurred either when axillary buds became active on previously infested nodes or when nematodes established endoparasitic populations at locations near the stolon tip during winter and spring, when the rate of stolon extension was limited by low light intensity. Affected stolon tips could "escape" from the influence of such infestations when light intensity and temperature increased. Nematode activity was limited by low temperature rather than light intensity. Global warming is likely to lead to greater damage to infested plants during the winter and early spring because the predicted milder winter temperatures will enhance nematode activity but not necessarily promote stolon growth.

*Key words:* behavior, clover, *Ditylenchus dipsaci*, host-parasite relationship, nematode infestation, population dynamics, stem nematode, *Trifolium repens*.

*Ditylenchus dipsaci* (Kühn) Filipjev (stem nematode) is a widespread and damaging pest of *Trifolium repens* L. (white clover) in the United Kingdom and other temperate regions of the world (Cook et al., 1992b; Cook and Yeates, 1993). White clover, a perennial legume, is a frequent component of the grassland swards that provide the basis for ruminant livestock industries in these regions. The mature plant is composed of horizontal stems (stolons), which bear a petiole, axillary bud, and root primordium at each node (Thomas, 1987). The node and axillary bud are enveloped in a tubular leaf sheath that terminates distally as a stipular tube. A node is considered mature if its petiole bears a fully opened trifoliate lamina. Mature nodes are referred to sequentially in a proximal direction from the first mature node at the terminal (distal) end of the stolon (referred to as node 1). Axillary buds can give rise to flowers or lateral branches (secondary stolons), or remain inactive. Axillary buds on secondary stolons can give rise to tertiary stolons.

Symptoms of attack by *D. dipsaci* include hypertrophy and stunting of infested lamina and petioles, shortening and hypertrophy of infested internodes, and abnormal development of stolon and petiole epidermis (Cook and Yeates, 1993). The life cycle of the nematode has been studied in detail (Griffith et al., 1997; Yuksel, 1960). Developing juveniles go through a series of four molts before becoming adults. The first-stage juveniles molt in the egg and emerge as second-stage juveniles. Rates of development are linearly related to temperature, the basal developmental temperature being ca. 3 °C. The thermal constant for development of gravid females from freshly laid eggs was

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ca. 270 accumulated day-degrees above the basal developmental temperature (Griffith et al., 1997). Rates of egg production by the nematode are also temperature-dependent, ranging from 0.8 to 3.1 eggs per female per day at 10 and 20 °C, respectively (Dijkstra, 1957).

Damage to infested white clover plants is particularly severe in spring after mild winters (Cook et al., 1992a). It has been hypothesized that this is a consequence of stem nematode activity continuing when white clover is inactive due to low temperatures and light intensity (Sackville-Hamilton, 1990). Because of this, it has been suggested that the changes in mean annual temperatures due to global warming may lead to an increased impact and incidence of the disease. The primary aim of this study was to elucidate and quantify the underlying mechanisms and sequence of development of nematode infestations in white clover plants. This information was required for the development of a predictive simulation model of the host-nematode system.

## Materials and Methods

*Development of stem nematode infestation:* Cuttings were taken from susceptible, healthy white clover plants cv. S184. Each cutting was the distal portion of an actively growing stolon severed from the parent plant at internode 3. Cuttings were rooted in tap water, planted in compost, and transferred to a growth room with a day-night temperature cycle of 18 to 15 °C. After 12 days, 100 established cuttings were repotted singly into 9.5-cm-diam. plastic pots filled with 50:50 loam-peat mix and grown for 16 days in a glasshouse. The pots were then placed in boxes (30 × 46 × 20 cm) filled with 50:50 loam-peat mix, and all but one of the stolons on each plant were removed. Remaining primary stolons were grown in straight lines over the soil surface for the next 30 days, when the position and length of all internodes and the condition of associated axillary buds and petioles were recorded.

Stem nematode-infested white clover plants were collected in mid-September from a field at IGER, Aberystwyth, United Kingdom (O.S. reference SN 624838). This plant material was washed and cut up finely, and the nematodes extracted on a mistifier (Hooper, 1986). The suspension of extracted nematodes was concentrated, transferred to a tilted petri dish, and allowed to settle. Adult and J4 *D. dipsaci* were pipetted and washed in several changes of sterile tap water to remove traces of plant phenolics. Finally, they were resuspended in 100 ml sterile tap water and stored in the dark at 2 °C.

The volume of the inoculum suspension was reduced to 10 ml and air was bubbled through it to ensure even mixing and good aeration. Ninety 10-µl aliquots were then removed, and the number and identity of the nematodes in 10 of these droplets assessed. Each droplet contained on average (standard deviation) 42 (3.31) nematodes: 14 males, 15 females, 8 J4, and 5 J2/J3. Each of the 100 plants was assigned at random to one of three treatments: i) primary stolons inoculated with a 10-µl droplet placed among the stipular folds and undeveloped petioles surrounding the stolon tip, ii) as 1 except that the droplet was placed within the stipule surrounding node 2, iii) uninoculated control.

After inoculation, all the boxes were covered with polythene for 24 hours to increase humidity and prevent inoculum droplets from drying too quickly. Boxes were watered twice weekly with 200 ml of tap water per pot, taking care not to allow water to overflow onto the surface of the soil outside of the pots. Temperature was recorded at 60-minute intervals throughout the experiment with thermocouple probes distributed among the boxes.

Plants were sampled 5, 20, 46, 95, and 186 days after inoculation, when morphological characteristics of seven randomly selected plants per treatment were measured. Primary stolons of five of these plants were cut off and fixed in 50% ethanol. These were further analyzed by removing roots, cutting all stolons longitudinally, and staining them (Byrd et al., 1983). Each of the stained sto-

lon systems was dissected under a stereomicroscope, infested regions located, and the nematodes identified to developmental stage and counted. Dead nematodes, recognizable by cuticle breakdown and disruption of their body contents, were counted. Observations were made of any plant symptoms associated with infestations.

Statistical analyses were made with SAS software (SAS Institute, Cary, NC). Due to the possibility of correlations between variates, MANOVA analyses were employed; where these indicated significant overall differences between treatments, univariate ANOVAs and t-tests were performed to confirm the source of these differences. Because of the unbalanced nature of the data sets, general linear models procedures were adopted.

Overall effects of nematode infestation on clover morphology were assessed as follows. For primary stolons, morphological variates (the number of nodes, dead petioles per stolon, active axillary buds per stolon, and stolon length) were compared for each treatment at each sampling time. Secondary stolons were divided into six batches according to their origin: 1-prior to inoculation (0 days), 2-between 0 and 5 days after inoculation, 3-5 to 20 days, 4-20 to 46 days, 5-46 to 96 days, 6-96 to 186 days. Within each batch, morphological variates at each sampling time were compared to test for differences between the secondary stolons in five groups: i) stolons arising from treatment 3, ii) uninfested and iii) infested stolons arising from treatment 1, iv) uninfested and v) infested stolons arising from treatment 2.

Effects of treatments 1 and 2 on infestation development were assessed at each sampling time by comparing number of infested axils per plant, number of infested axils per plant associated with subepidermal infestation loci within stolons, number of infested axils per plant associated with dead petioles, and number of infested axils per plant associated with petioles expressing symptoms of stem nematode attack.

Infested regions of plants were classed into two distinctive types, superficial loci and deep-seated loci, according to whether nematodes had penetrated the stolon epidermis at infested axils and colonized subepidermal pockets of host tissue. For each locus type, six variates describing the nematode population were compared for experimental treatments 1 and 2 at each sampling time. The six variates were number of eggs, number of living nematodes, number of dead nematodes, proportion of adults, proportion of J2 and J3, and proportion of J4. Data for treatments 1 and 2 were combined and the population characteristics of the different locus types compared at each of the sampling times.

*Spread of stem nematode infestation between stolons:* White clover cuttings were established in pots and grown for 30 days as described in the previous experiment. Six pots were then sunk into the center of a box of soil mix and the boxes placed adjacent to each other in a 2 × 3 rectangular array. All but four of the stolons growing from each plant were removed and the remaining four primary stolons were grown in straight lines at right angles to each other toward the corners of each box for 30 days. One of the four primary stolons per plant was then inoculated in the same way as in treatment 1 of the previous experiment. Plants were watered twice weekly by applying 800 ml of water from overhead with a watering can, and were examined 225 days after inoculation. Each box was divided into six equal areas (190 $cm^2$) delimited by a regular rectangular 2 × 3 grid. Stem nematode symptoms on clover in each area were assessed and the number of affected axes recorded.

## Results

*Development of stem nematode infestation:* For primary and associated secondary stolons of uninoculated controls, all the internodes originally proximal to node 1 remained the same length, including 17 internodes originally between nodes 1 and 2 for less than 3 days and which were observed for 20 days or more. Thus, internodes ceased elongation after the trifoliate leaf arising from their proximal end had fully opened (giving rise

to node 1). On primary control stolons the mean (±standard deviation) nodal position of the first active axillary bud at 0, 5, 20, 46, 95, and 186 days after inoculation was 2.4 ± 1.13, 1.9 ± 0.69, 2.3 ± 0.49, 2.7 ± 0.49, 4.1 ± 1.07, and 4.7 ± 3.04, respectively.

Changes in the morphological characteristics of control (uninoculated) primary stolons over time are shown in Fig. 1. Differences between the morphological characteristics of these and inoculated stolons (data not shown) were evident only at 95 days (Wilks' Lambda F-value = 4.15, df = 12, $P < 0.01$), when there was a difference between treatments in the number of dead petioles per primary stolon (F-value = 6.91, df = 2, $P < 0.01$). Primary stolons inoculated at node 2 had more ($P < 0.05$) dead petioles (8.4 ± 1.27) than those inoculated at their tips (5.3 ± 3.04) or uninoculated (4.4 ± 1.62). Differences in morphological characteristics between groups of secondary stolons were neither consistent over time nor clearly associated with stem nematode infestation. Changes over time in the morphological characteristics of secondary stolons are illustrated by those of batch 1, which arose from uninoculated primary stolons (group a) (Fig. 1).

Despite the lack of effect of *D. dipsaci* infestation on the overall morphology of primary or secondary stolons, a small number of the infested primary and secondary stolons did have regions of abnormal morphology associated with subepidermal pockets of colonized stolon tissue. In these stolons, internode lengths were shortened, stolon diameter was increased, and the outer epidermis was puckered and/or distended. Such symptoms were observed only on stolon systems derived from tip-inoculated primary stolons (treatment 1). Eleven primary, 20 secondary, and 15 tertiary stolons had subepidermal infestations, but only 2, 8, and 15, respectively, had symptoms. Forty-six days after inoculation, the mean internode length in symptomless regions (10 ± 4.3 mm, N = 17) was significantly greater (*t*-test, $P < 0.05$, df = 17) than in regions with symptoms (3.7 ± 1.7 mm, N = 8). Internodes tended to be longer at 95 and 186 days after inoculation in symptomless regions (10 ± 5.9 mm, N = 3 and 6.3 ± 5.5 mm, N = 21) than in regions with symptoms (3.7 ± 0.6 mm, N = 3 and 2.1 ± 0.7 mm, N = 15). Internode shortening associated with nematode infestation occurred only when subepidermal loci contained more than 100 nematodes in close proximity (center of the infestation locus within 20 mm) to terminal meristems (Table 1) in regions of internode elongation (stolon region distal to node 1).

The point at which stolons were inoculated had no effect on infestation development except at 20 days (Wilks' Lambda F-value = 4.81, df = 2, $P < 0.05$), when several infested axils on tip-inoculated, but not node 2-inoculated plants, bore petioles with symptoms. The compared variates were extremely variable even within treatments, and there were usually only a few infested axils per plant. On node 2-inoculated plants, none of the axillary infestations were associated with petiole symptoms; however, on tip-inoculated plants there were petioles with symptoms on 20 of the 124 infested axils (Table 2). The mean number of infested axils on both tip and node 2-inoculated plants increased from 1 (the original inoculation point) to ca. 3 over the first 46 days. On node 2-inoculated plants, there was no further increase, but on tip-inoculated plants there was a substantial increase after 95 days. By 46 days after inoculation, nematodes in ca. 50% of axillary loci had penetrated the stolon epidermis and colonized subepidermal pockets of stolon tissue. There was some evidence that penetration occurred earlier and more frequently in tip-inoculated plants (Table 2). From 46 days onward in node 2-inoculated plants, 53% of axillary infestations were found in dead petioles, whereas in tip-inoculated plants, 36% were in dead petioles.

There were no significant differences between population characteristics in superficial and deep-seated loci that could be ascribed to inoculation method. Population data for infestations on node 2- and tip-inoculated stolons were therefore combined. Population characteristics differed significantly (respective Wilks' Lambda F-

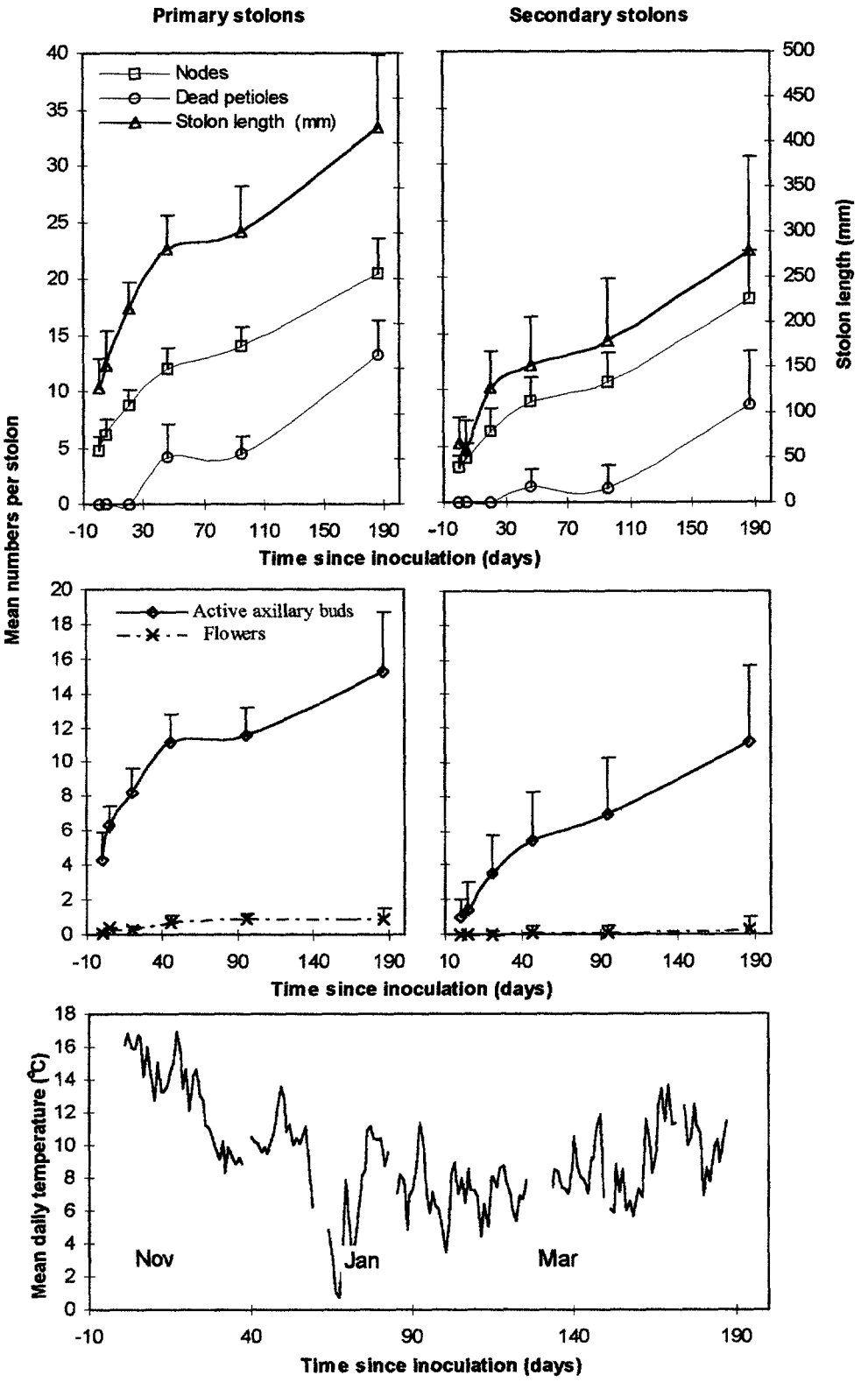


FIG. 1. Morphological characteristics of uninfested stolons of white clover *Trifolium repens* plants and mean daily temperatures during the experiment. All secondary stolons arose from control primary stolons prior to inoculation. Error bars are standard errors of the means; for clarity, only the upper range of each error bar is shown. For primary stolons N = 7 at each time. For secondary stolons N = 10, 27, 27, 29, 32, and 31 at successive time points.

TABLE 1. Locations and characteristics of *Ditylenchus dipsaci* infestation loci in subepidermal stolon tissue of *Trifolium repens*.[a]

| Distance from stolon tip to center of locus (mm) | Distance from stolon tip to distal end of locus (mm) | Nematodes/ infestation locus | Symptoms[b] | Stolon order[c] |
|---|---|---|---|---|
| 1–20 | 2 | 2 | – | 3 |
| 1–20 | 3 | 2 | – | 2 |
| 1–20 | 13 | 78 | – | 2 |
| 1–20 | 7 | 88 | – | 2 |
| 1–20 | 5 | 107 | + | 1 |
| 1–20 | 8 | 164 | + | 2 |
| 1–20 | 5 | 2,849 | + | 3 |
| 1–20 | 1 | 5,027 | + | 3 |
| 1–20 | 6 | 5,183 | + | 2 |
| 1–20 | 1 | 6,701 | + | 2 |
| 1–20 | 10 | 8,040 | + | 2 |
| 1–20 | 10 | 11,994 | + | 2 |
| 1–20 | 1 | 12,346 | + | 3 |
| 1–20 | 18 | >5,892 | + | 2 |
| 1–20 | 9 | >8,500 | + | 2 |
| 21–40 | 28 | 151 | – | 1 |
| 21–40 | 27 | 534 | + | 2 |
| 21–40 | 24 | 761 | – | 1 |
| 21–40 | 30 | 830 | – | 2 |
| 21–40 | 19 | 5,027 | – | 2 |
| >40 | 31 | 8 | – | 1 |
| >40 | 101 | 52 | – | 2 |
| >40 | 41 | 55 | – | 1 |
| >40 | 65 | 92 | – | 1 |
| >40 | 60 | 169 | – | 1 |
| >40 | 117 | 717 | – | 1 |
| >40 | 30 | 803 | – | 2 |
| >40 | 106 | 903 | – | 2 |
| >40 | 39 | 940 | – | 2 |
| >40 | 162 | 1,258 | – | 1 |
| >40 | 128 | 9,488 | – | 1 |
| >40 | 45 | 39,790 | + | 1 |
| >40 | 84 | >1,901 | – | 2 |
| >40 | 73 | >2,945 | – | 2 |

[a] Stolons were inoculated with a droplet of water containing 42 *D. dipsaci* placed either among the stipular folds and undeveloped petioles surrounding the stolon tip or within the stipule surrounding node 2.

[b] Symptoms, if present (+), included internode shortening, increased stolon diameter, and puckering and/or distention of the stolon epidermis.

[c] Primary stolons (1) give rise to second-order stolons (2), which give rise to third-order stolons (3).

values = 2.94 and 8.99, df = 6, $P < 0.05$) with many more living nematodes, eggs, and dead nematodes in deep-seated loci than in superficial loci at 95 and 186 days (Fig. 2). At 186 days the proportion of adults was lower ($P < 0.05$) in deep-seated loci (Fig. 3).

In superficial infestation loci, nematodes were usually restricted to the epidermal tissues of lower portions of petioles, associated stipules, and regions of stolon enveloped by the stipules. Occasionally, nematodes penetrated into the lumen of petioles and then moved up to the base of the trifoliate lamina, but they were rarely found in the actual lamina. In many loci, the number of nematodes per locus increased only slightly or decreased (Fig. 2); however, in about 25% of the loci there was a substantial increase for up to 96 days after inoculation, followed by a population decline.

Deep-seated infestation loci were discrete pockets within cortical and pith tissues of stolons. They often extended along a stolon for several internodes and were always associated with at least one infested axil from which nematodes had invaded. In these loci

TABLE 2. Development and characteristics of axillary infestations by *Ditylenchus dipsaci* on inoculated *Trifolium repens* stolons.

| Point at which primary stolons inoculated[a] | Time after inoculation (days) | Number of infested axils per plant[b] | | | Percentages of axils associated with: | | |
|---|---|---|---|---|---|---|---|
| | | Mean | σ | n | Deep-seated infestations[c] | Petiolar symptoms[d] | Dead petioles |
| Tips | 0 | 1.0 | 0.00 | 5 | 0 | 0 | 0 |
| | 5 | 1.8 | 0.58 | 5 | 33 | 0 | 0 |
| | 20 | 2.2 | 1.09 | 5 | 0 | 27 | 0 |
| | 46 | 3.4 | 3.13 | 5 | 76 | 29 | 0 |
| | 95 | 4.0 | 1.41 | 5 | 50 | 40 | 10 |
| | 186 | 24.0 | 22.54 | 3 | 39 | 1 | 51 |
| Node 2 | 0 | 1.0 | 0.00 | 5 | 0 | 0 | 0 |
| | 5 | 1.2 | 0.45 | 5 | 0 | 0 | 0 |
| | 20 | 1.6 | 0.89 | 5 | 0 | 0 | 0 |
| | 46 | 2.8 | 1.09 | 5 | 14 | 0 | 21 |
| | 95 | 2.0 | 1.41 | 5 | 0 | 0 | 80 |
| | 186 | 2.3 | 2.31 | 3 | 43 | 0 | 86 |

[a] Stolons were inoculated with a droplet of water containing 42 *D. dipsaci* placed either among the stipular folds and undeveloped petioles surrounding the stolon tip or within the stipule surrounding node 2.
[b] Mean number of axils containing *D. dipsaci* per plant with estimated standard deviation (σ) and number of plants sampled (n).
[c] Deep-seated infestations were longitudinal pockets of subepidermal stolon tissue colonized by *D. dipsaci*.
[d] Petiole symptoms characteristic of *D. dipsaci* infestation include hypertrophy and stunting.

nematode populations the number of eggs increased exponentially over time (Fig. 2). The egg-to-adult ratio was highest at 95 days and declined thereafter (Fig. 3). Locus length increased linearly except between 46 and 95 days (Fig. 4). This period corresponded to winter, when mean daily temperatures were low and photoperiods short (Fig. 1). Dead nematodes were observed only after 95 days, and their number increased slowly thereafter (Fig. 2). The fraction of adults in the population peaked after 20 days and declined thereafter (Fig. 3).

In 25 of the 26 deep-seated loci sampled at 46 days or earlier, nematodes remained restricted to the subepidermal cortex (Table 3). In 4 of the 11 loci sampled after 95 days, nematodes had penetrated the vascular sheath and spread into the pith. By 186 days, this had occurred in 15 of the 17 loci sampled. After 186 days, stolon epidermis in 12 of the 69 infested internodes (15 cm of the total 25-cm length of infested stolon) was split longitudinally and underlying and surrounding tissues were senescing.

Many deep-seated infestation loci (21 of 40) did not expand from the stolons in which they were first established into connected stolons. In six other regions, deep-seated infestation loci had extended into connected stolons but the population structures were markedly different, suggesting that at these locations two separate loci had developed.

There was a clear relationship between the length of deep-seated loci and population size, with longer loci containing more nematodes (Fig. 5). There was also a marked tendency for loci in more proximal regions of stolons to be longer; at 186 days, loci in primary axes were longer than those in secondary axes (Fig. 6).

At 95 days after inoculation, the mean population density in loci with symptoms was significantly greater than that in other loci (350 ± 242 nematodes per mm (N = 3) vs. 97 ± 97 (N = 10)). At 96 days, population density was 433 ± 392 nematodes per mm (N = 16) in loci with symptoms compared to 204 ± 204 (N = 7) in loci without symptoms, but this difference was not statistically significant.

*Spread of stem nematode infestation between stolons:* In the second experiment, after 225 days, nematodes had spread from the original inoculation points in all six boxes. Symptoms were present on stolons up to 52 cm from original inoculation points. On average, each box contained 203 ±140 (N = 6) axes with distinctive symptoms of *D. dipsaci*

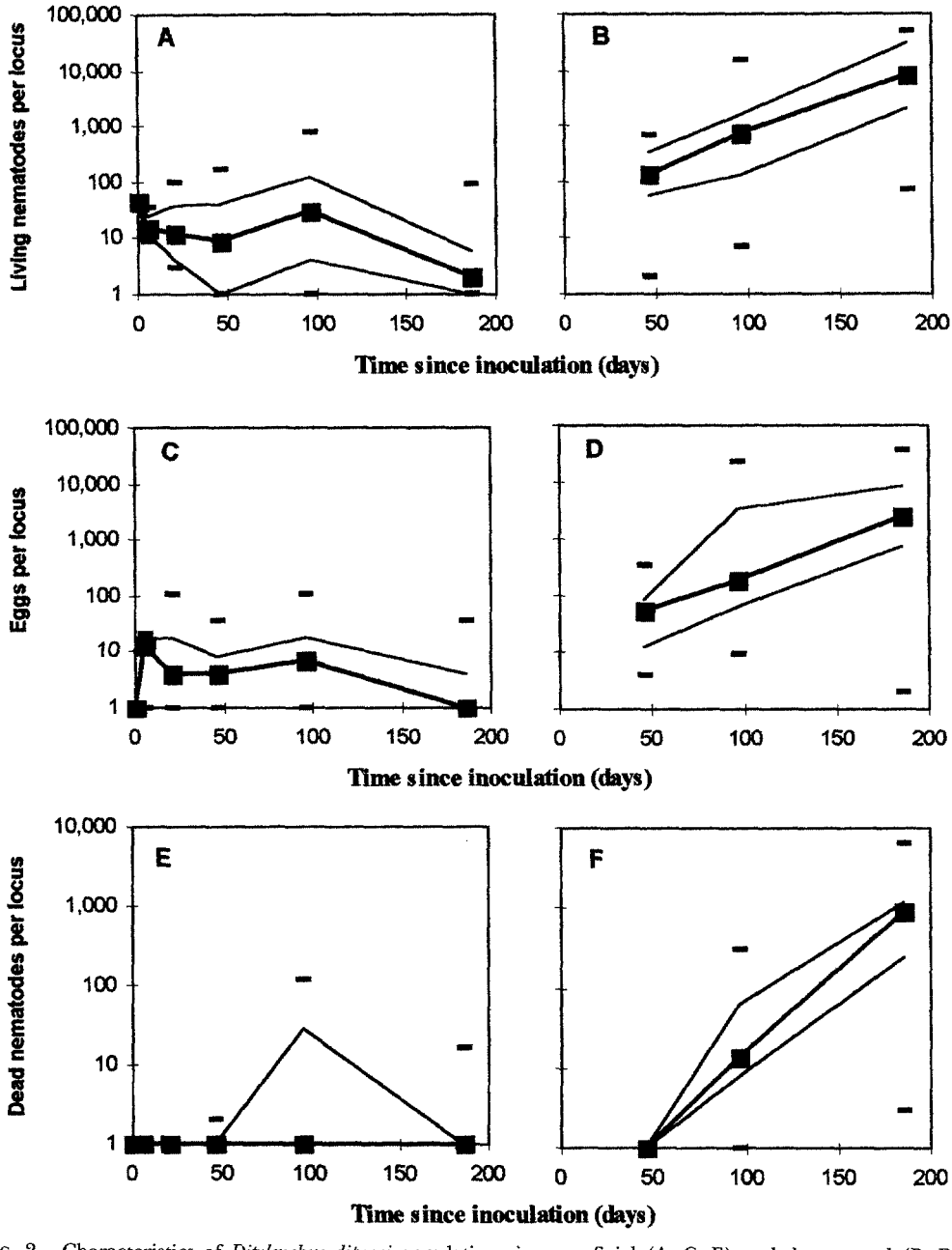


FIG. 2. Characteristics of *Ditylenchus dipsaci* populations in superficial (A, C, E) and deep-seated (B, D, F) infestation loci on inoculated *Trifolium repens*. Superficial infestations were restricted to axillary locations. Deep-seated infestations were pockets in subepidermal stolon tissue and associated axils colonized by *D. dipsaci*. Y axes are logarithmic scales. Short horizontal bars indicate ranges, thin lines indicate interquartile ranges, and plotted points are medians. For deep-seated loci N = 8, 17, 16, 21, and 48, respectively. For other loci N = 17, 9, and 16, respectively.

infestation. Most of these axes were concentrated within 18 cm of the original inoculation points, where there were between 2 and 6 infested axes per 10 $cm^2$ of clover sward.

## DISCUSSION

With no overhead watering, the number of axillary infestations increased over 46

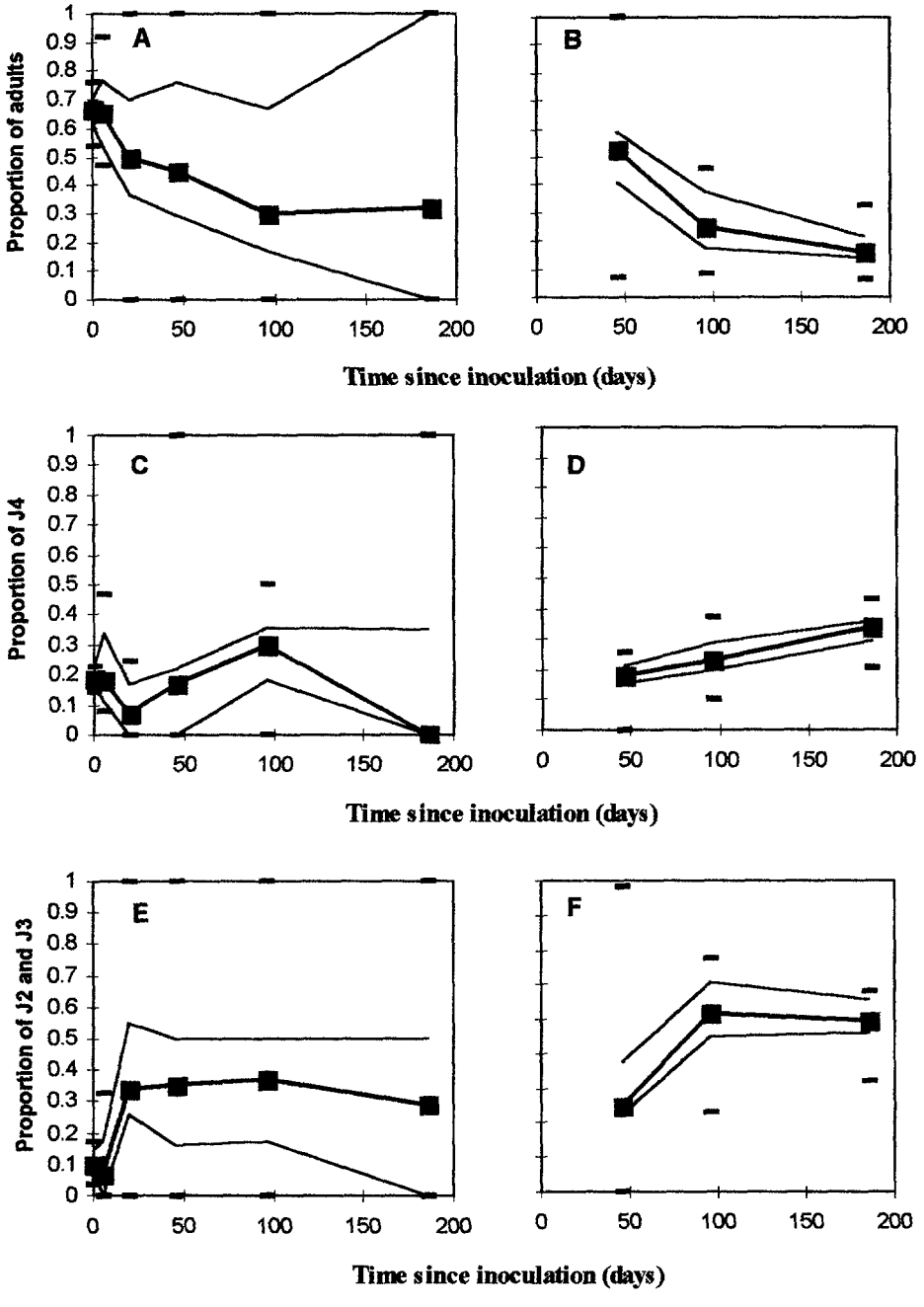


FIG. 3. Characteristics of *Ditylenchus dipsaci* populations in superficial (A, C, E) and deep-seated (B, D, F) infestation loci on inoculated *Trifolium repens*. Superficial infestations were restricted to axillary locations. Deep-seated infestations were pockets in subepidermal stolon tissue and associated axils colonized by *D. dipsaci*. Short horizontal bars indicate ranges, thin lines indicate interquartile ranges, and plotted points are medians. For deep-seated loci N = 8, 17, 16, 21, and 48, respectively. For other loci N = 17, 9, and 16, respectively.

days from 1 to ca. 3, with nodes immediately adjacent to inoculation points becoming infested. This increase must have required active but limited nematode migration from inoculation points across the surfaces of the plants. On primary stolons inoculated at node 2, nematodes could not have been passively transferred, and movement of nema-

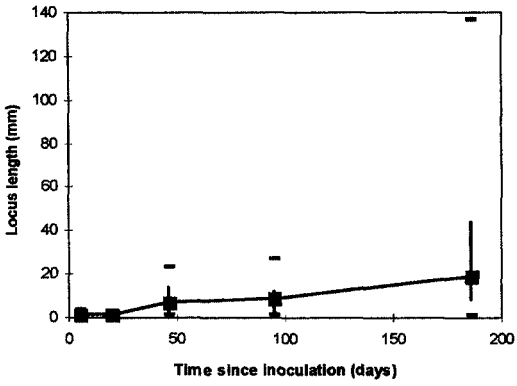


FIG. 4. Expansion of subepidermal pockets of stolon tissue colonized by *Ditylenchus dipsaci* in inoculated *Trifolium repens* plants. Medians are plotted, the vertical bars indicating interquartile ranges and the horizontal bars representing maximum and minimum values. For successive times N = 3, 3, 20, 11, and 17.

todes inside stolons was extremely limited. Approximately one-fourth of the nematodes inoculated into stipules were deposited in the immediate vicinity of suitable feeding sites (axils). The majority of these nematodes presumably remained at axils but some adults may have laid eggs and then migrated, since the proportion of adults at axils decreased while the total population remained constant. Adult nematodes inoculated onto seedlings have been observed to behave similarly (Griffith et al., 1997). Most of the migrating nematodes were probably those not deposited in the immediate vicinity of axils.

A thin layer of condensation must have formed on the plants since nematode migration across a surface requires a suitable water film (Wallace, 1963). When clover swards were watered from overhead, nematode migration was much more pronounced, presumably because of widespread persistent water films on plant and soil surfaces. The rate of infestation spread was approximately 2 mm per day, which is similar to that observed for other races of stem nematode (Wallace, 1962; Webster, 1964).

Multiplication of the white clover race of stem nematode in some of the superficial infestation loci shows that it is not an obligate endoparasite. Similar observations have been made for other races of the nematode (Hodson, 1926). The observed population reduction in the majority of superficial loci after 95 days is almost certainly associated with the onset of petiole death. An important factor in determining the maximum population that could be produced at any specific superficial locus was the time between locus establishment and petiole death.

The increase in the number of superficial infestations in tip-inoculated plants at 186 days after inoculation was probably due to nematode migration from deep-seated infestation loci into adjacent axils. This increase was particularly marked in stolon regions where localized stolon symptoms had developed (such regions were absent on node 2-inoculated plants). At these locations, nematodes migrating across the relatively short internodes also could have invaded axils not directly overlying deep-seated infestations.

The following hypothetical sequence of deep-seated locus development is suggested by and fully consistent with the observations made during this study. Some nematodes start to penetrate the stolon epidermis in the vicinity of petiole/stolon junctions (axils) soon after inoculation, but these initial incursions are of limited duration and depth

TABLE 3. Development and characteristics of *Ditylenchus dipsaci* infestation loci in subepidermal stolon tissue of *Trifolium repens*.

| Point at which primary stolons inoculated[a] | Time after inoculation (days) | Number of loci per plant[b] | | | Percentages of loci with nematodes in pith and cortex |
|---|---|---|---|---|---|
| | | Mean | σ | n | |
| Tips | 5 | 0.6 | 0.55 | 5 | 0 |
| | 20 | 0.6 | 0.55 | 5 | 33 |
| | 46 | 3.4 | 1.67 | 5 | 0 |
| | 95 | 2.0 | 1.22 | 5 | 40 |
| | 186 | 3.7 | 2.89 | 3 | 82 |
| Node 2 | 5 | 0.0 | 0.00 | 5 | |
| | 20 | 0.0 | 0.00 | 5 | |
| | 46 | 0.6 | 0.89 | 5 | 0 |
| | 95 | 0.2 | 0.45 | 5 | 0 |
| | 186 | 2.0 | 1.00 | 3 | 100 |

[a] Stolons were inoculated with a droplet of water containing 42 *D. dipsaci* placed either among the stipular folds and undeveloped petioles surrounding the stolon tip or within the stipule surrounding node 2.

[b] Mean number of discrete deep-seated loci containing *D. dipsaci* per plant with estimated standard deviation (σ) and number of plants sampled (n).

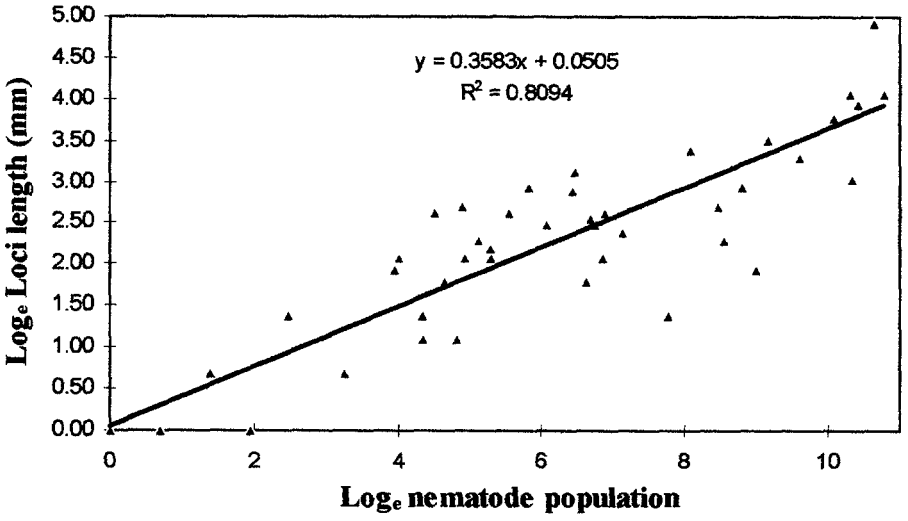


FIG. 5. Relationship between the length of individual subepidermal pockets of stolon tissue colonized by *Ditylenchus dipsaci* in inoculated *Trifolium repens* plants and their total *D. dipsaci* population. Scatter plot is for combined data for 46, 95, and 186 days.

because the structure of the host tissue imposes severe constraints on nematode movement. The initial penetrations into the stem trigger a series of metabolic changes in host tissue that spread from the penetration sites into and along the stolon. These metabolic changes cause structural modifications of host tissue including cell separation, swelling, and/or collapse, which allow nematodes to move around within it (Blake, 1962; Newhall, 1943; Riedel and May, 1971; Seinhorst, 1956). Thus, an expanding pocket of host tissue capable of sustaining nematodes is centered around initial penetration points. This locus is then colonized by nematodes present at the axil. The rate of formation and expansion of such loci varies from plant to plant and at different locations on the same plant, and may be influenced by temperature, internode age, and light (Seinhorst, 1956). The length of deep-seated loci increased slowly at a linear rate except over the winter, when the rate of locus expansion was reduced. Locus expansion rate is independent of the number of nematodes since the rate remained constant over periods of time when nematode population increased exponentially. When rates of locus formation and expansion were very low, the axillary infestation remained superficial.

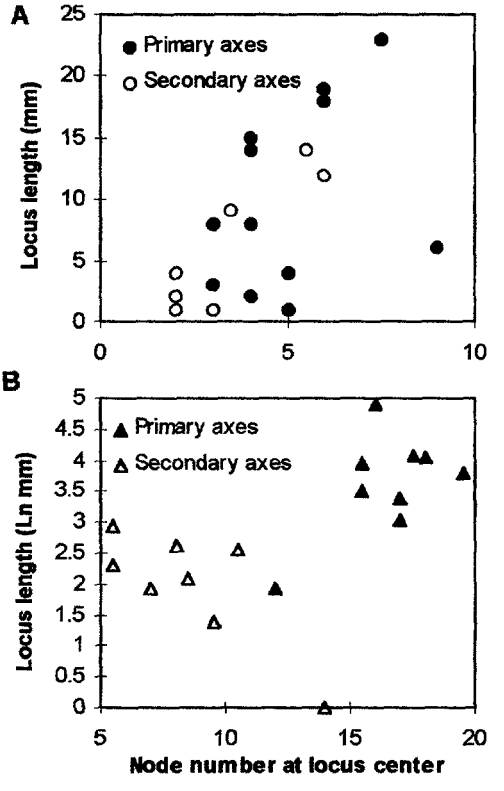


FIG. 6. Variation in length of individual subepidermal pockets of stolon tissue colonized by *Ditylenchus dipsaci* in inoculated *Trifolium repens* plants located at various positions along stolons A) 46 days after inoculation. B) 186 days after inoculation. Primary axes were the main stolons. Secondary axes (stolons) arose from the primary axes.

If a petiole supporting a superficial infestation locus dies, presumably there is no further invasion of subepidermal tissues from that locus. The sample of node 2-inoculated plants taken at 95 days was unusual in that the primary stolons bore significantly more dead petioles than control and tip-inoculated plants. This sample was also anomalous in that the plants had fewer deep-seated infestation loci than in the preceding sample, with only one locus being found in the five plants examined. It is probable that these two effects are interrelated.

Nematode populations developing within deep-seated infestation loci increased exponentially while remaining confined within discrete, linearly expanding pockets of host tissue, leading to increasing population pressure within these loci. At high population densities, there were decreases in the proportion of adults in the populations and in the rates of egg production, but an increase in nematode death. It has been reported that in unfavorable conditions J4 do not molt into adults but instead enter a diapause-like state (Clayden, 1985; Feil, 1991). In *Caenorhabditis elegans* (Maupas) Dougherty, J3 enter a diapause state (dauer stage) if the J1 from which they developed were exposed to an appropriate balance of chemosensory cues involving a "dauer-inducing" pheromone and a "food signal" produced by bacteria (Riddle and Georgi, 1990). Populations of *C. elegans* therefore adjust their rate of increase to match resource availability, channeling declining resources to survival/dispersal stages. Several other species of parasitic nematodes are known to behave similarly (Evans and Perry, 1976; Michel, 1974). Our results suggest that the white clover race of the stem nematode also behaves in this way.

Other important changes also occur as population pressure increases within deep-seated loci. Nematodes penetrate the vascular sheath surrounding the pith, which may accelerate the rate of locus expansion since nematodes can move freely through voids within pith parenchyma. At the same time, stolons in the older regions of loci start to senesce, opening longitudinal splits in the stolon epidermis. In the presence of moisture it is probable that this splitting triggers a mass migration of nematodes from the stolon (Griffith et al., 1997; Robertson, 1928).

Symptoms on petioles occurred only on those infested before they were fully differentiated, indicating that symptoms develop because petiole morphogenesis is disrupted. The presence of just one nematode in a developing petiole was enough to trigger abnormal morphogenesis.

For white clover stolons not watered from overhead, even large *D. dipsaci* infestations typically had little effect on growth and morphology. Stolon morphology was affected only when subepidermal loci containing more than 100 nematodes were present within 20 mm of actively growing meristems. Such situations arose when nematodes invaded developing tissues close to terminal meristems (distal to node 1) and subsequent stolon growth was slow, as in tip-inoculated stolons over winter. It also occurred when meristems in axillary buds on infested regions of stolons became active after nematode populations in adjacent loci had increased to critical levels. This explains why symptoms were progressively more common on secondary and tertiary branches arising from infested primary stolons. Affected meristems can "escape" from the influence of infestation loci when the rate of stolon growth exceeds the rate of locus expansion. Such "escapes" of stolon tips through internode elongation were observed in this study and have been observed in field infestations (Cook et al., 1992a).

Symptom development in white clover is therefore dependent on the balance among nematode population growth, locus expansion, and internode elongation. In white clover, internode growth is limited both by temperature and duration of bright sunshine (Sackville-Hamilton, 1990). At temperatures less than 4 °C, internode growth is negligible, and at less than 4 hours of sunshine per day internode growth is minimal even at temperatures above 8 °C. This explains the observed reduced rates of stolon development over winter. During mild winters nematode population levels in loci will in-

crease more rapidly due to higher mean daily temperatures (Griffith et al., 1996, 1997). However, the rate of growth of terminal meristems away from infestation loci will not change and so the probability of symptoms occurring will increase. This explains why damage to infested plants is more marked in spring, especially after mild winters (Cook et al., 1992a) and indicates that predicted climatic changes due to global warming (Murphy and Mitchell, 1995) would lead to more pronounced damage by *D. dipsaci* to white clover in the United Kingdom.

Development of symptoms on stolons, as on petioles, is almost certainly a consequence of nematode activity interfering in some way with morphogenesis at the meristem. The resulting changes in stolon structure, including cell separation, swelling, and rounding-off, enhance rates of locus expansion and nematode reproduction. In red clover (*Trifolium pratense* L.) the degree of multiplication of stem nematode in host tissue is correlated to symptom expression, especially swelling (Dijkstra, 1957).

The details of the dynamics of deep-seated infestation locus development deduced from this study have been combined with information derived from an earlier investigation of the population dynamics of stem nematode on white clover (Griffith et al., 1997) to produce a simulation model of the host-nematode system (Griffith et al., 1996). This simulation model will be used to make quantitative predictions of the effect of predicted global warming on the system.

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