Journal of Nematology 28(4):492–500. 1996.

Effects of Resistance in White Clover (*Trifolium repens*) on *Heterodera trifolii*

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Abstract: Clones of two partially resistant and two susceptible white clover, *Trifolium repens,* genotypes were exposed to eggs of *Heterodera trifolii* and nematode development in stained roots measured at 2, 4, 7, 11, 18, 23, and 37 days after inoculation. The differences in development between nematode populations in resistant and susceptible genotypes showed that resistance operated after infection during feeding and development. At 7 days after inoculation, counts of second-stage juveniles did not differ between genotypes, whereas at 37 days more adults had developed in the susceptible than in the resistant genotypes. In a separate experiment, cysts hosted by susceptible genotypes were larger and contained more eggs than those on resistant genotypes so that the product of the values for cysts per plant and for eggs per cyst resulted in a more sensitive measure of resistance than from using cysts per plant alone.

Key words: clover cyst nematode, *Heterodera trifolii, nematode, pasture, Pf/Pi, resistance, Trifolium repens,* white clover.

Herbage yield and nitrogen fixation by white clover (*Trifolium repens* L.) in New Zealand pasture may be suppressed by plant-parasitic nematodes (20). An important parasitic species is the clover cyst nematode (*Heterodera trifolii* Goffart), which is particularly damaging to seedlings (7). An attractive method of improving white clover productivity is the selection and breeding of nematode-resistant material, which has resulted in plant genotypes that are resistant to the clover cyst nematode (19). It has not been shown, however, which mechanisms are associated with the low numbers of cysts recovered from resistant plants. Low cyst counts may be the result of a single mechanism or a combination of factors, and resistant genotypes may differ in the type of resistance mechanism.

Plant species are known to express resistance to nematodes at several stages in the host-parasite relationship (3,9,16). Preinfectional 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, other plants may be more chemically attractive. Physically impenetrable roots have been postulated as a resistance mechanism (5). Post-infectional mechanisms may deter nematodes from feeding, slow or stop development, or inhibit reproduction.

In describing a plant's efficiency as a host of parasitic nematodes, the definitive feature is its effect on nematode reproduction (4), which is measured by the number of healthy eggs produced. In practice, nematode reproduction is frequently estimated by measurement of other parameters, such as some symptom of parasitism, or counts of nematode life stages other than eggs. Resistance to root-knot nematodes, for example, is often assessed by the number of root galls; in the case of cyst nematodes, the number of cysts produced is a common screening parameter. These approaches provide a compromise between the ideal measurement of nematode reproduction and the need to process large numbers of samples. Data on nematode fecundity are relatively few and difficult to summarize (22), even in the case of the Heteroderidae where the enclosure of eggs in a cyst and(or) egg mass greatly facilitates counting. Among *Meloidogyne* spp., the few studies of the effect of plant resistance upon nematode fecundity reveal a range of possibilities from more eggs per female on resistant compared with suscep-

Received for publication 12 April 1996.

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The authors thank G. W. Yeates for comments on the manuscript.

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tible plant genotypes (16) to no effect or fewer eggs per female on resistant plants (1,15). In the case of *H. trifolii,* it has been conjectured that cysts from resistant genotypes may be smaller than those from susceptible ones (7), but no statistical evidence exists for this view nor have any data on egg-per-cyst counts been presented. The objectives of this study were to identify on which lifestages resistance was having its effect and to measure the effect of resistance on cyst size and fecundity.

Materials and Methods

Development experiment: The initiation of the *H. trifolii* culture was as described by Mercer (12). Plant genotypes that had supported either high or low numbers of cysts in a resistance screening trial were chosen for these experiments. Five genotypes 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-sterilized sand and kept moist with a 100-ppm solution of captan fungicide. Five rooted cuttings from each genotype were repotted into a pasteurized 50:50 sand-top soil medium (12) in 180-ml plastic plant pots, one cutting per pot. A 3-ml aqueous suspension containing 2,000 *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. Pots were maintained in a glasshouse at a soil temperature of 18 °C to 25 °C. They received complete nutrients fortnightly and were watered as necessary between nutrient applications. Forty-two days atter inoculation, cysts were extracted by means of an elutriation tower (21) and counted. Based on these counts, two resistant (R1 and R2) and two susceptible (S1 and S2) genotypes were selected and propagated by the techniques described above for use in the development experiment. Two weeks after potting into a pasteurized 50:50 sand-top soil medium, all plants were washed free of soil and their roots trimmed to approximately equal volume. Plants were repotted in pasteurized medium, one per pot, and grown for 2 more weeks before inoculation as described above. The pots were randomized, maintained in a glasshouse, and received nutrients, all as above. Soil temperature was recorded 30 mm deep every 6 minutes by a computer-controlled transducer. The computer stored this information as accumulated degree-days over 10 °C (DDA).

Eight days after inoculation, the plants were carefully washed and repotted into fresh pasteurized sand-soil mix to limit the time available for invasion by J2. Four plants of each genotype were selected at random at 2, 4, 7, 11, 18, 23, and 37 days after inoculation. Soil was removed under a gentle flow of water, the roots were separated from the tops and weighed, and the nematodes within roots stained with aniline blue (2). Beginning at day 30, four plants of each genotype were washed over a sieve with 106-μ openings to capture dislodged nematodes following rupture of the cortex. On day 37, an elutriation tower and sieves were used to recover nematodes dislodged from individual plants.

On the day after staining, nematodes were counted under a dissecting microscope. To facilitate counting, roots were spread in glycerol in the lid of a glass petri dish with the base of the dish inverted on top. Sandwiching the roots in this way was found to minimize the need for refocusing as the roots were examined. Nematodes were assigned to life stage (14). 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). The presence of a gelatinous matrix was used to distinguish adult nematodes from advanced fourth-stage juveniles (14). For each harvest, counts of stages from each genotype were compared using analysis of variance (ANOVA). Because the counts covered a wide range and included some low counts, data were transformed with $\log_{10}(x + 1)$ before analysis (18). Means were separated by Tukey's test. As dislodged adults and 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 parasitizing 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 data were transformed by arcsin ($\sqrt{x}$) before analysis (18).

Fecundity experiment: The effects of resistance on cyst size and egg content were investigated. Clones of each of the genotypes R1, R2, S1, and S2 were raised from nodal cuttings. After taking root, the cuttings were transferred to the pasteurized sand-soil mix in 180-ml 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 randomized and after 2 more weeks' growth a suspension of 2,200 *H. trifolii* eggs was injected under each plant. The plants were maintained as above except that plants were not repotted at 8 days. After 42 days, cysts were recovered by elutriation and those from each plant counted under a dissecting microscope. In order to select five cysts at random, each plant's cyst count was divided by 5 to give a value 'n'. As a path was traced through a 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 cysts were transferred to a drop of water on a microscope slide and their length and breadth measured under the dissecting microscope at ×50 magnification. 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 magnification, under a compound microscope. Empty eggshells, but not juvenile nematodes, were included in the egg count.

Pf/Pi values were calculated by dividing the final number of eggs on a plant by 2,200, the number of eggs in the inoculum. 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 (17). The products were compared by ANOVA and Tukey's HSD test. Egg numbers per cyst were compared using the Mann-Whitney U test.

Results

Development experiment: Reproduction of *H. trifolii* was less on clones of *T. repens* genotypes R1 and R2 in both experiments ($P < 0.05$) (Table 1). Second-stage juveniles (J2) penetrated genotype S1 in greater numbers, and genotype R1 in fewer numbers ($P < 0.05$), than they did the other genotypes whose numbers were not different (Fig. 1). Four days after inoculation J2 numbers ranged from a mean of 17 in R1 to 109 in S1, with the other two genotypes intermediate. Three days later, however, differences in the counts were not significant. Although J2 had started to swell by day 11 (96 DDA), the percentage of swollen J2 did not differ between genotypes (Fig. 2). By day 18 (150 DDA) J3,4 were present in greater numbers on the susceptible genotypes than on the resistant

Table 1. Mean numbers of *Heterodera trifolii* females and cysts recovered from four *Trifolium repens* genotypes.

Genotype	Nominal resistance status	Development experiment[a]	Fecundity experiment[b]
R1	resistant	18a	2a
R2	resistant	4a	2a
S1	susceptible	364b	23b
S2	susceptible	517c	65b

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

[a] Four clones of each genotype were grown for 42 days after inoculation with 2,000 eggs.

[b] Five clones of each genotype were grown for 42 days after inoculation with 2,200 eggs.

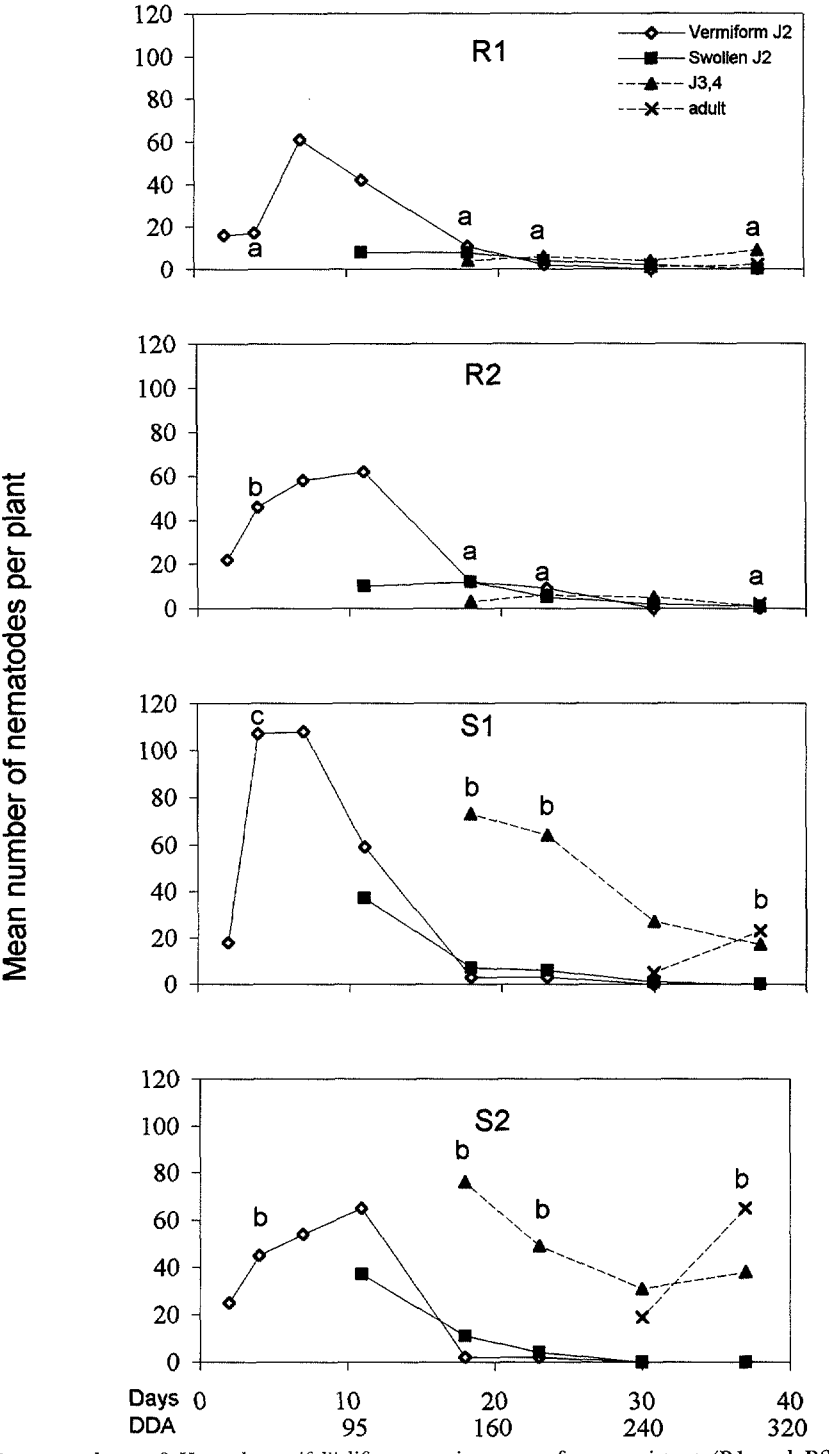

FIG. 1. Mean numbers of *Heterodera trifolii* life stages in roots of two resistant (R1 and R2) and two susceptible (S1 and S2) *Trifolium repens* genotypes on eight occasions following inoculation. Letters indicate points of comparison between genotypes on a particular day as follows: vermiform J2 (day 4), J3,4 (days 18 and 23), adults (day 37). Comparisons between genotypes on a particular date with a letter in common do not differ ($P = 0.05$).

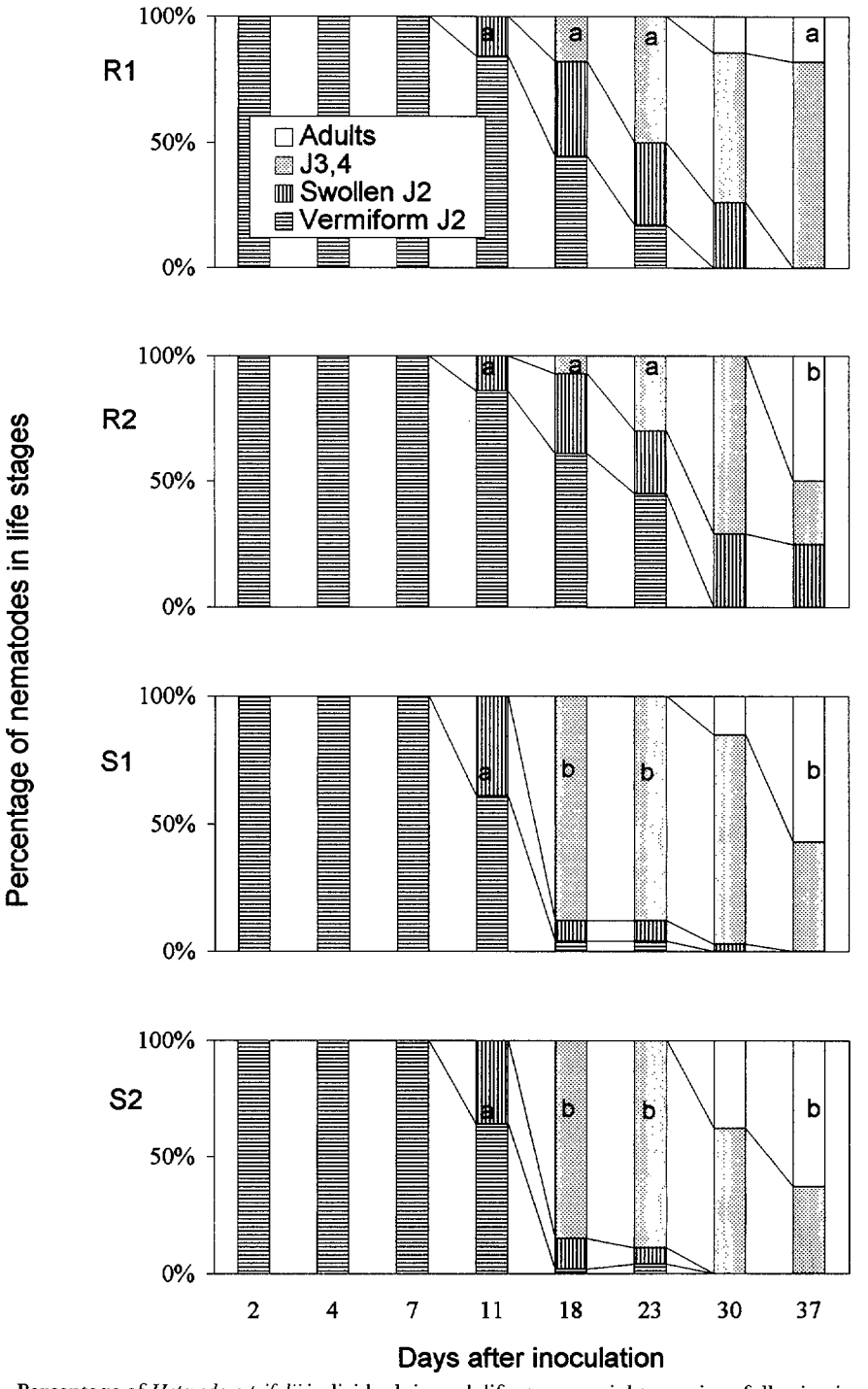

FIG. 2. Percentage of *Heterodera trifolii* individuals in each life stage on eight occasions following inoculation of two resistant (R1 and R2) and two susceptible (S1 and S2) *Trifolium repens* genotypes. Letters indicate points of comparison between the four genotypes on a particular day as follows: swollen J2 (day 11), J3,4 (days 18 and 23), adults (day 37). Comparisons between genotypes on a particular date with a letter in common do not differ ($P = 0.05$).

ones ($P < 0.05$). Numbers of J3,4 greatly outnumbered the J2 nematodes on susceptible genotypes whereas, on the resistant genotypes, numbers in these categories were similar. This pattern was repeated at the day 23 harvest. When expressed as a percentage of total nematodes, the J3,4 population on the susceptible genotypes on days 18 and 23 was higher than on R1 and R2 ($P < 0.05$) (Fig. 2). By day 30, the first adults were found 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 J3,4 stages were still present (Fig. 1). On genotypes S1 and S2, the percentages of adults were 58 and 63, respectively. Few nematodes remained in the resistant genotypes. On R1 the small population of remaining parasites consisted mostly of juveniles; only some 15% had reached adulthood (Fig. 2, day 37). The four clones of genotype R2 hosted only 10 nematodes among them.

Fecundity experiment: Cysts hosted by susceptible genotypes were larger than those on resistant genotypes ($P < 0.05$) (Table 2). Those from genotype S2 were larger than those from all other genotypes ($P < 0.05$). A similar pattern was observed in the eggs-per-cyst counts where S2 cysts contained more eggs than those from other genotypes and S1 cysts contained more cysts than R1 cysts. A large proportion of the cysts hosted by S1 and the resistant genotypes had produced fewer than 20 eggs (Fig. 3). 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 with a highest count of 250 eggs per cyst. Cyst size and egg numbers were correlated positively ($R = 0.63$, $P < 0.001$).

Discussion

Final counts of nematodes were determined by post-infectional resistance as similar numbers of juveniles were seen in resistant and susceptible genotypes in the early samples, yet there were marked differences in J3,4 and adult stages in the last sample. Resistance at this stage has been observed in other forage legumes resistant to the Heteroderidae, such as red clover (*Trifolium pratense*) with *Meloidogyne javanica* (3) and Kenya white clover (*Trifolium semipilosum*) with *Meloidogyne* sp. (13). Our result confirms the observation of Cook and Mizen (6), who described similar numbers of *H. trifolii* J2 invading resistant and susceptible white clover and that resistant seedlines affected subsequent development.

Inhibition of development might result from J2 inability to initiate syncytium development; alternatively, such a feeding site may develop but later disintegrate. The latter is the case in potato resistant to *Globodera rostochiensis*, where initial syncytial cell walls break down close to the feeding site; even when a syncytium continues to enlarge, such breakdown close to the

Table 2. Parameters of *Heterodera trifolii* cysts raised on resistant (R1, R2) or susceptible (S1, S2) genotypes of *Trifolium repens* 42 days after inoculation with 2,200 eggs.

Genotype	Cysts per plant[a]	Cyst length × width (mm^2)[b]	Eggs per cyst[b]	Eggs per plant	Pf/Pi[c]
R1	14 b	0.14 a	17 a	238 a	0.11
R2	5 a	0.16 a	24 ab	120 a	0.05
S1	34 bc	0.25 b	48 b	1,632 b	0.74
S2	73 c	0.31 c	85 c	6,205 c	2.8

Within a column, means followed by the same letter do not differ at $P = 0.05$.
[a] Cyst per plant data are means of five replicates.
[b] Cyst size and eggs per cyst data are means of 25 cysts from each genotype.
[c] Pf/Pi values were calculated by dividing the final number of eggs on a plant by 2,200.

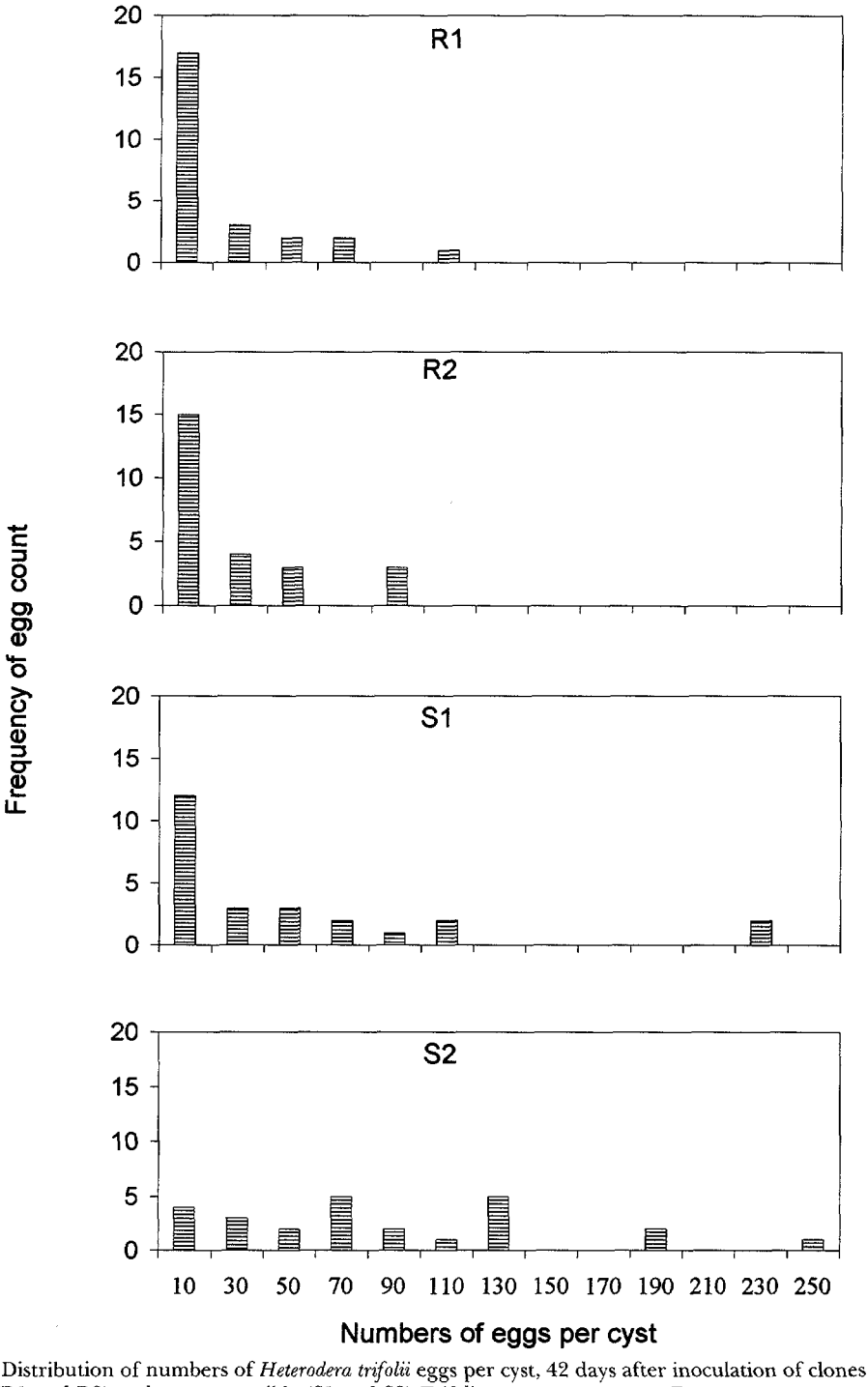

FIG. 3. Distribution of numbers of *Heterodera trifolii* eggs per cyst, 42 days after inoculation of clones of two resistant (R1 and R2) and two susceptible (S1 and S2) *Trifolium repens* genotypes. Eggs per cyst categories are of the progression 0–19, 20–39, 40–59, etc.

nematode is thought to lead to complete syncytial destruction (8). 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 syncytia were at least initiated.

However, values of P close to 0.05 indicate that this is of marginal significance.

The results of the fecundity experiment identify a further manifestation of resistance. Use of a simple length × breadth calculation to express cyst size effectively treats the cyst as two-dimensional. Volume would be more valid for cyst size. The fact that significant differences were identified using this conservative approach strongly indicates the extent to which cyst size was reduced on resistant genotypes.

To the encouragement of the breeding program, it is clear that the progress made by screening based on cyst count in fact underestimates the true effect on nematode reproduction. Table 2 indicates that the 14-fold difference between R2 and S2 in terms of cysts per 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 (Pf/Pi < 1) would categorize them as nonefficient hosts (10). Genotype S2 is an efficient host, while we might regard S1 as slightly inefficient or neutral.

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 the cysts from R2. The distribution of eggs per cyst in S1 cysts more closely resembled those of R1 and R2 than that of S2. The plant-parasitic nematode genera have been categorized according to their population strategies (11). The "r-strategists" or "exploiters" are adapted 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, meaning they will increase resource utilization efficiency when resources are limited (22). The practical implication is 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 the second experiment, but on day 37 of the first experiment the mean S1 root weight was below half that of the other three genotypes. Although hosting fewer nematodes than S2 in absolute terms, S1 hosted nearly 50% more cysts per gram of root weight than S2 on day 37. It seems likely that competition between nematodes occurred on genotype S1 and that the cysts partitioned the limited available resources toward body development rather than egg production.

Root size can determine J2 numbers as larger roots provide more penetration sites. However, root weight was not a factor in determining nematode numbers in these white clover roots. The only difference in root weight on day 4 was between genotypes R1 and S1; R1 had larger roots (mean fresh weight 1.78 versus 0.53 g, $P < 0.01$) but only about one-fifth as many nematodes as S1.

While the Pf/Pi ratio was lower and the cyst size was smaller in plants selected for resistance, further work is needed to determine to what extent resistance benefits the host plant and to compare the sensitivity of seedlings and mature plants.

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