Culturing and Population Studies of *Ditylenchus dipsaci* under Monoxenic Conditions

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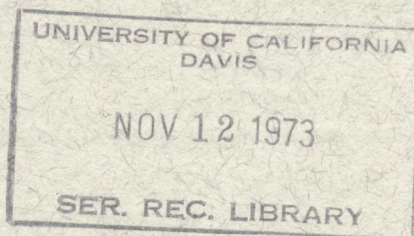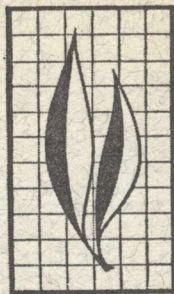
Ditylenchus dipsaci is well known for its "race" characteristics in terms of host range preferences. This study established isolates of *D. dipsaci* occurring in California in monoxenic laboratory cultures to account for different host properties as reflected in nematode growth and development. Microbial contaminants in hosts as well as seed lots varied, thus no single axenization method was found acceptable. Multiple treatments, varying in kinds and sequences of axenizing agents and duration of exposures, were required. Growth rate of all tissues were increased two-fold by the addition of casein hydrolysate and yeast hydrolysate to a literature-recommended medium.

Three biotypes of the "onion race"—Mexican, French, and California—and one biotype of the "alfalfa race," Antelope Valley biotype, were studied. In some regimes, the "type-host" response was observed for "non-type host" tissues, while in other regimes a bias towards maleness or femaleness occurred. This bias, however, was not always directly correlated with density changes. Differentiated rather than undifferentiated or callus tissue of a suitable "non-type host" was requisite for satisfactory population buildup. Blockage in callus cultures probably occurred in embryogenesis. With reduced population buildups on "non-type hosts," as compared with "type host," blockage in the nematode life cycle occurred at different steps depending upon the kind of host tissue— whether from the same or from different plants. Our results with two "races" of the stem and bulb nematode indicating "inter-racial" and "intra-racial" characteristics in the nominal species, *D. dipsaci,* host-parasite complex would suggest that this phenomena occurs more generally than has been thought.

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INTRODUCTION

THE STEM AND BULB NEMATODE, *Ditylenchus dipsaci*, was originally described from specimens obtained from flower heads of Fuller's teasle *Dipsacus fullonum* L. (Kühn, 1857); however, diseases of some cereals and legumes subsequently shown to be caused by this nematode were known nearly 30 years earlier (Goodey, 1933). Some 30 years after the description of this nematode, it was established (Ritzema Bos, 1888) that although morphologically indistinguishable, all members of this species were not identical; populations parasitizing one host group were unable to transfer to another host group parasitized by a second population, while a third population could attack successfully a number of host groups. Currently, this nematode is believed to attack more than 400 plant species (Thorne, 1961) comprising nearly 50 botanical families among the bryophytes and monocotyledonous and dicotyledonous plants (Filipjev and Schuurmans Stekhoven, 1941).

The initial discovery that populations of *D. dipsaci* from different sources, though identical morphologically, could vary greatly in their host preferences eventually led to the notion of host races. This concept developed to the point where 11 distinctive races were recognized in Europe (Seinhorst, 1957); subsequently this grew to 21 races (Hesling, 1966).

Preference for a given host seems to be based mainly on two factors: adaptation and genetic constitution (Goodey, 1931; Mayr, 1970; Steiner, 1925). Mayr (1970) suggested that in a biological race with a preferred host range, some individuals should be capable of parasitizing another host species as well. He noted that the definition of a "biological race" consists of a heterogeneous assemblage of phenomena, but that only one of them, the host race, has the best claim to the designation "biological race." Some of the early hypotheses on the genesis of biological races included such diverse concepts as "physiological accommodation" (Steiner, 1925), "food memory" (Goodey, 1931), and "gene pool" (de Bruijn Ouboter, 1930).

The "gene pool" conclusion by de Bruijn Ouboter (1930), that populations of *D. dipsaci* consisted of a mixture of genotypes such that under environmental pressures, biological races gradually evolved through natural selection, offers the most promise in explaining the experimental observations on the question of races. Attempts over the years to separate the races of *D.*

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dipsaci on morphological criteria have been unsuccessful. Gibbins and Grandison (1968) were unable to distinguish the races by serological techniques; this lack of success may have been in part a consequence of the use of eggs, multiple stages, and adults as antigenic material. Polyacrylamide gel electrophoresis of soluble protein from homogenates of different races of *D. dipsaci* (Eriksson and Granberg, 1969) would appear to have more promise as a means of characterization. Smith (1951) with alfalfa, and subsequently Bingefors (1957) with red clover, increased the complexity of the race phenomena when they found that different populations of each race of *D. dipsaci* varied in ability to parasitize different host varieties. In contrast to observations in Italy, *D. dipsaci* from oats in England were reported not to damage wheat (Goodey and Hooper, 1958). Other population differences among the races of *D. dipsaci* are known, *e.g.*, those reported for the alfalfa race on various host plants (Barker and Sasser, 1959; Grundbacher, 1960).

The question of biological races is further confounded by isolating or swamping pressures resulting in segregation or interbreeding and recombination of characters controlling host preference. Sturhan (1966) observed that the host preference of the hybrid populations resulting from interbreeding among 11 biological races differed from those of the parental types. Subsequently, Webster (1967) reported that continuous backcrossing and a slow rate of reproduction in the resulting progeny could account for variation in host preference of the biological races of *D. dipsaci*. As a consequence of the substantial variation in host preferences of the various biological races and biotypes and the ability of these lines to interbreed, Sturhan (1969) concluded that the physiological differences among the races of *D. dipsaci* as currently recognized are in-

sufficient to warrant the designation of these races by a "type" host. Consistent with these arguments is the report (Viglierchio, 1971) of an extremely polyphagous race from garlic able to reproduce on a number of plant tissues including "type" hosts of the races suggested by Seinhorst (1957).

Although the complex phenomenon of biological races has been investigated frequently over the years and recently reviewed (Sturhan, 1971), the nature of the physiological polymorphism involved in host preference is poorly understood. *Ditylenchus dipsaci* has been known for years in California on a number of crops (Godfrey and Scott, 1935). In addition, biotypes were subsequently found among the populations isolated from alfalfa throughout the state; the host resistance reflected in these biotypes was conferred by a single dominant gene factor (Grundbacher, 1960). It was of interest, therefore, to explore the properties of the various population types of *D. dipsaci* found in California on a range of hosts of varying susceptibility representing a situation as might occur in nature. One expeditious means of pursuing such a study would be to utilize *in vitro* monoxenic tissue culture systems to rear nematodes in the laboratory. In such a fashion it would be possible to improve control of environmental conditions, reduce variability in results, and increase efficiency in inoculations and recovery. Furthermore, it would facilitate the study of cytological and biochemical reactions of host-tissue response to the parasite as a possible reflection of the nature of resistance.

The culture of tissue of *Medicago sativa* (alfalfa) under axenic conditions has been reported for the rearing of *D. dipsaci* (Krusberg, 1960, 1961; Bingefors and Eriksson, 1963). These established techniques, however, were soon found to be only partially satisfactory. For our large-scale studies, it was essential to improve procedures for

the axenization of both plant and nematode material and to develop a common culture medium able to support the improved growth of a number of plant tissues to be tested.

This report describes techniques for the culture of *D. dipsaci* monoxenically on a number of plant tissues, population development of two biological races and their biotypes on several plant tissues, effects of community and sequence cultures on population development, together with the modifying properties of tissue types, and population development as a function of culture, age, and nematode biotype.

AXENIZATION OF HOST MATERIAL

A prime requisite of *in vitro* culture studies is maintenance of sterility or freedom from undesirable microbial interference. Atmospheric or other accidental contaminations are usually avoidable by rigorous adherence to the common aseptic procedures already developed for such investigations (White, 1963). The difficult problems of contamination usually arise as a result of an intimate association of the microorganisms with the nematode or the plant tissue which is being processed for axenic culture. The techniques and procedures reported in this section were developed through modifications of existing techniques and found acceptable for these investigations.

Storage organs

Tissues of storage organs (*e.g.*, carrots, *Daucus carota;* potatoes, *Solanum tuberosum;* sugarbeets, *Beta vulgaris;* bulbs, and the like) were prepared for culture .by modifications of the method of White (1963). These storage organs, selected for health and uniformity, were first scrubbed with warm detergent solution and rinsed in tap water. Using a potato peeler, a surface layer was removed to eliminate dirt-containing crevices and other necrotic or blemished areas. The peeled tissue was washed with water before being placed into axenizing solutions (usually 15 minutes in 0.5 to 1 per cent aqueous sodium hypochlorite prepared from commercial household bleach or for four hours in 2 to 5 per cent aqueous Chloramine T). Then it was removed and rinsed at least three times in sterile, distilled water. These axenizing solutions penetrated the surface of the storage organ to various depths depending upon the tissue, the concentration of chemical, and the length of exposure. This affected tissue was surgically removed by aseptic techniques before suitably sized explants were prepared for culture. The explants from potato and sugarbeet were washed with sterile, distilled water and dipped into a sterile 10^{-3} molar solution of ascorbic acid to retard subsequent suberization and browning of exposed surfaces.

Exploratory tests and investigations were conducted periodically with various storage organs and populations of *D. dipsaci*. The essentials of these studies have already been reported in a separate publication (Viglierchio, 1971).

Seeds other than alfalfa and clover

Tissue cultures from onion (*Allium cepa*), broad bean (*Vicia faba*), oats (*Avena sativa*), rye (*Secale cereale*), phlox (*Phlox drumundi*), and Fuller's teasle (*Dipsacus fullonum*) were established as follows:

Seeds with hard seed coats were placed in stainless steel baskets and scarified in concentrated sulfuric acid for 5 to 10 minutes and then washed in tap water for 30 minutes. The scarified seeds and seeds which did not require scarification were usually then placed into axenizing solutions, either 0.5 to 1 per cent aqueous sodium hypochlorite for 15 minutes or 2 per cent aqueous

Chloramine T solution for two hours. Both were found to be effective; however, the latter was preferred because it permitted higher seed germination. The seeds were rinsed several times in sterile, distilled water and transferred individually from the baskets to sterile, 1 per cent water agar plates for germination in the dark at 25° C. Broad bean seeds were treated overnight in a 2 per cent Chloramine T solution, after which their seed coats were surgically and aseptically removed then placed in a 0.1 per cent aqueous streptomycin sulfate solution for two hours before finally rinsing several times in sterile, distilled water. The removal of seed coat and the subsequent streptomycin sulfate treatment were necessary because of the microbial contamination harbored under the coat. Upon germination, the sterile seedlings were selected and transferred to the sterile culture media.

Seeds of alfalfa and clover

For practical considerations, a 30 to 50 seed inoculum of alfalfa (*Medicago sativa*), red clover (*Trifolium pratense*), and white clover (*T. repens*) per culture was required to assure nonlimiting quantities of proliferating tissue within the time parameters prescribed by the large-scale routine testing procedures. The axenization methods outlined in the previous section and that of Krusberg (1961) were found unsuitable for alfalfa, red clover, and white clover because of a persistent bacterial contamination. The contamination gradually appeared in more and more cultures. Most of the subcultures from the apparently axenic parent cultures also showed the presence of this contaminant within 48 hours. The contamination, apparently present in all seed lots, assumed a milky white appearance and formed finger-like colonies on the culture medium. Microscopical examinations

invariably showed these contaminants to be gram negative short rods.

Growth and control of bacterial contaminants

Because of the superficial similarity of the contaminants, a series of tests were run to establish an optimal growth medium for these microorganisms and to search for control antibiotics. To test the growth of these bacterial contaminants on different substrates, nine growth media were prepared and poured in 9-cm petri plates and inoculated with a bacterial loop. The plates were incubated at 30° C, and the colony diameters were measured 15 and 39 hours after inoculation. The bacterial growth from whatever source was maximum on APT agar both after 15 and 39 hours and on NIH thioglycolate media only after 39 hours. Little or no growth was observed on Lithman oxgall and A.C. broth media, whereas only moderate growth was observed in the other five media (table 1). Because of these results, stock cultures of the bacteria from contaminated seeds were maintained on brain-heart infusion slants.

To evaluate the effect of several common antibiotics on the bacterial growth, a series of 9-cm petri dishes with 25 ml of standard brain-heart

TABLE 1
RATE OF GROWTH OF THE
PERSISTENT SEED-BORNE
BACTERIUM ON SELECTED
GROWTH MEDIA

Growth medium	Mean diameter of inoculum in 9-cm petri dish after:	
	15 hours	39 hours
	cm	*cm*
APT agar	covered	covered
NIH agar	0.7	0.9
Neurospora	0.6	0.8
NIH thioglycolate	0.05	covered
Sabourand	0.8	0.8
Mycological	0.6	0.9
Lithman oxgall	0.05	0.05
A.C. broth	0.05	0.05
Brain-heart infusion	0.7	0.8

infusion (BHI) agar medium were inoculated with the bacterial contaminants. A commercial bacto-sensitive disc, impregnated with antibiotic was placed in each plate. Three concentrations of antibiotic were used except for aureomycin and terramycin, in which cases 0.5 ml of antibiotic solution was added to a filter paper disc to simulate the commercial preparations. Three replicates were used for each antibiotic concentration. The presence or absence of a clear area around the discs, representing the area where the antibiotic had diffused into the medium, was used to assess the qualitative effectiveness of an antibiotic. Tetracycline showed antibiotic action at all concentrations; penicillin and aureomycin were active at the intermediate and high concentrations; neomycin and novobiocin showed antibiotic action only at the high concentration (table 2). In preliminary experiments, penicillin was found to inhibit bacterial growth; but its properties appeared to be bacteriostatic rather than bacteriocidal, as its activity did not persist over prolonged periods of tissue culturing.

TABLE 2

EFFECTS OF VARIOUS ANTIBIOTICS IN VARYING DOSAGES ON PERSISTENT SEED-BORNE BACTERIUM

Antibiotic	Concentration: effects (+ = positive; − = negative) of antibiotic per disk		
	Low	Medium	High
	μg	μg	μg
Chloromycetin	5.00 −	10.0 −	30 −
Erythromycetin	5.00 −	10.0 −	30 −
Kanamycin	5.00 −	10.0 −	30 −
Neomycin	5.00 −	10.0 −	30 +
Novobiocin	5.00 −	10.0 −	30 +
Penicillin	2.00 −	5.0 +	10 +
Streptomycin	2.00 −	5.0 −	10 −
Tetracycline	5.00 +	10.0 +	30 +
Aureomycin	0.10 −	1.0 +	10 +
Tetramycin	0.25 −	2.5 −	25 −

Axenization methods

In all varieties the seeds occurred in a range of color forms from deeply pigmented to cream-colored. Each vari-

ety was arbitrarily separated into light and pigmented types and tested on the medium of Krusberg (1961) to assess the percentage germination. There was no significant difference between the germination of color types. With Pennscott, however, the light seeds of all other varieties gave improved per cent germination and therefore were used for all subsequent experiments.

On the basis of the above results with the persistent bacterial contamination, the following tests were made to axenize alfalfa (variety DuPuit, Moapa), red clover (variety Kenland, Pennscott), and white clover (variety White Dutch, Ladino). Seeds were placed in the stainless steel wire baskets and scarified in concentrated sulfuric acid for 30 minutes and then rinsed in tap water for 30 minutes. The baskets were then transferred to a 2 per cent aqueous solution of Chloramine T for varying lengths of time (from 1.5 hours to 24 hours). To insure gaseous exchange and uniform exposure of seeds to Chloramine T solution, the beakers were placed on a shaker. These seeds were used to evaluate the effects of sulfuric acid and Chloramine T on microbial contaminants and seed germination. Bacterial growth tests were conducted following axenization by placing treated seeds in culture tubes of APT and NIH agar media at 27.5° C for seven days. These media were selected because the seed-borne bacterial contaminant grew best on these two (table 1). For germination tests, a portion of the treated seeds were placed on sterile Krusberg's culture media (Krusberg, 1961; Krusberg and Blickenstaff, 1964), while untreated control seeds were germinated on sterile 1 per cent water agar.

A four-hour treatment of 2 per cent Chloramine T successfully axenized alfalfa (variety Moapa), red clover (variety Pennscott), and white clover (variety Ladino). Red clover (variety Kenland) and white clover (variety

TABLE 3

PERCENTAGE OF TUBES CONTAINING SEEDS SHOWING BACTERIAL
CONTAMINATION AFTER H_2SO_4 AND TAP WATER SOAKING, THEN
VARYING LENGTHS OF TIME IN 2 PER CENT AQUEOUS CHLORAMINE T

Seed variety*	Duration of Chloramine T soak (hr):					No treatment (Control)
	1.5	4.0	8.0	16.0	24.0	
DuPuit	100	100	20	0	20	100
Kenland	100	60	20	60	0	100
Ladino	20	0	40	0	0	100
Moapa	60	0	0	0	0	100
Pennscott	0	0	0	0	0	100
White Dutch	60	20	60	0	0	100

* Five seeds per tube, five tubes—after 30 minutes in concentrated H_2SO_4, 30 minutes in tap water.

TABLE 4

PERCENTAGE GERMINATION OF SEEDS AFTER 30 MINUTES IN
CONCENTRATED H_2SO_4, 30 MINUTES IN TAP WATER AND VARYING
LENGTHS OF TIME IN 2 PER CENT AQUEOUS CHLORAMINE T

Seed variety	Seed germination after following period spent in Chloramine T (hr):					No treatment (Control)
	1.5	4.0	8.0	16.0	24.0	
	Per cent					*Per cent*
DuPuit	94	91	48	6	6	92
Kenland	96	82	46	24	6	93
Ladino	94	86	78	17	31	78
Moapa	98	72	53	18	2	88
Pennscott	92	49	22	6	0	86
White Dutch	90	84	48	24	4	90

White Dutch) were only poorly axenized after an 8-hour treatment (table 3) and showed a considerably reduced germination (table 4). DuPuit alfalfa seeds which were still partially axenized after 24 hours in 2 per cent Chloramine T showed only a 6 per cent germination. As a result of a series of pilot experiments, including modifications of the above measures as well as supplementary antibiotic treatments indicated effective in table 2, DuPuit was axenized. The results for DuPuit alfalfa seeds that were treated for five minutes in concentrated sulfuric acid, rinsed for 30 minutes in tap water, shaken for 1.5 hours in 2 per cent Chloramine T and shaken again for five to 45 hours in tetracycline, neomycin, or aureomycin are given in table 5. Since prolonged exposure (45 hours) to neomycin produced no adverse effect, it was selected for treatment of DuPuit alfalfa seeds. Fungal contamination was seldom a problem

TABLE 5

PERCENTAGE OF BACTERIAL
CONTAMINATION AND GERMINATION
OF DUPUIT SEEDS AFTER 5 MINUTES
IN CONCENTRATED H_2SO_4, 30
MINUTES TAP RINSE, 1.5 HOURS IN
CHLORAMINE T, THEN TREATMENTS
IN OTHER ANTIBIOTICS

Seed treatment	Bacterial contamination	Germination
Five hours in:	*Per cent*	*Per cent*
Aureomycin	0	92
Neomycin	0	88
Tetracycline	0	92
(Control)	..	...
15 hours in:		
Aureomycin	0	92
Neomycin	20	88
Tetracycline	0	88
(Control)	..	...
45 hours in:		
Aureomycin	0	0
Neomycin	0	100
Tetracycline	0	0
(Control)	40	96

with any seed, so long as concentrated sulfuric acid treatment was not eliminated. Apparently, the combination treatments of acid followed by Chloramine T in the axenization procedures

TABLE 6
AXENIZING PROCEDURES FINALLY SELECTED FOR SEED VARIETIES WITH
RESULTING BACTERIAL CONTAMINATION AND GERMINATION

Seed variety	Time spent in:					Bact. cont.	Germ.
	Conc. H_2SO_4	Tap water rinse	Chloramine T	Sterile distilled water	Neomycin		
	Minutes	*Minutes*	*Hours*	*Minutes*	*Hours*	*Per cent*	*Per cent*
DuPuit	5	30	1.5	–	5	3.1	80.0
Kenland	5	30	1.5	–	5	0.0	70.0
Ladino	5	30	4.0	30	–	3.3	74.0
Moapa	5	30	16.0	30	–	0.0	59.0
Pennscott	5	30	4.0	30	–	6.6	73.0
White Dutch	5	30	16.0	30	–	0.0	60.0

was fungicidal, whereas either alone was not reliably so.

Selected treatments for the seed varieties

Since no single concentration of sulfuric acid and Chloramine T was suitable for uniformly axenizing all seeds, a series of treatment schedules indicated in table 6 were accepted as suitable for the seed lot samples of the California varieties used. These treatments were selected, because they provided complete freedom from the bacterial contamination and yielded 93 per cent or more seed germination. The acid wash was reduced to five minutes because of the adverse effect of longer exposure on germination with some seed lots. Prolonged incubation in Chloramine T was also phytotoxic in varying degrees to different types of seeds, hence different incubation periods were selected (table 6).

NEMATODE PREPARATION AND UTILIZATION

Sources of nematodes

Preliminary studies indicated that properties of *D. dipsaci* field populations could vary with the source; therefore, stock supplies of each population were kept to provide a standard source of inoculum. In the case of populations from garlic (*Allium sativum*), the garlic scales were placed in polyethylene bags and stored in a cool, dry place for long periods with only gradual loss in the recovery of fourth-stage larvae, which were collected by placing scales in a mist-extraction chamber. On the other hand, *D. dipsaci* populations from alfalfa were normally comprised of mixed developmental stages, hence it was impractical to store them in dry conditions. Therefore, stock supplies of three biotypes of the onion race were established and maintained in axenic tissue cultures:

1. Mexican Biotype (extracted from *D. dipsaci*-infested garlic [variety Chileano]) imported from Celeya, Mexico.

2. French Biotype (extracted from California-grown *D. dipsaci*-infested garlic) originating from an unknown location in France.

3. California Biotype (extracted from *D. dipsaci*-infested garlic) from Gilroy, California.

The alfalfa race, known as the Antelope Valley Biotype, was extracted from *D. dipsaci*-infested alfalfa from the Antelope Valley in southern California.

Axenization of nematodes

Surface axenization of phytoparasitic nematodes has usually been adequate for subsequent transfer and rearing of monoxenic cultures (Crosse and Pitcher, 1953; Mountain, 1955;

Lownsbery and Lownsbery, 1956; Feder and Feldmesser, 1957; Krusberg, 1961; McClure and Viglierchio, 1966; Johnson and Viglierchio, 1969). However, our nematode sources, as do almost universally all field sources, carried rhabditid contaminants. Rhabditids carry bacteria and fungal spores within their gut, and surface axenization of these nematodes is not sufficient to establish clean cultures. Normally, such troublesome nematodes can be removed by picking before surface axenization of the phytophagous ones. Inasmuch as our investigations required large populations of axenic *D. dipsaci* at frequent intervals, picking was clearly impractical.
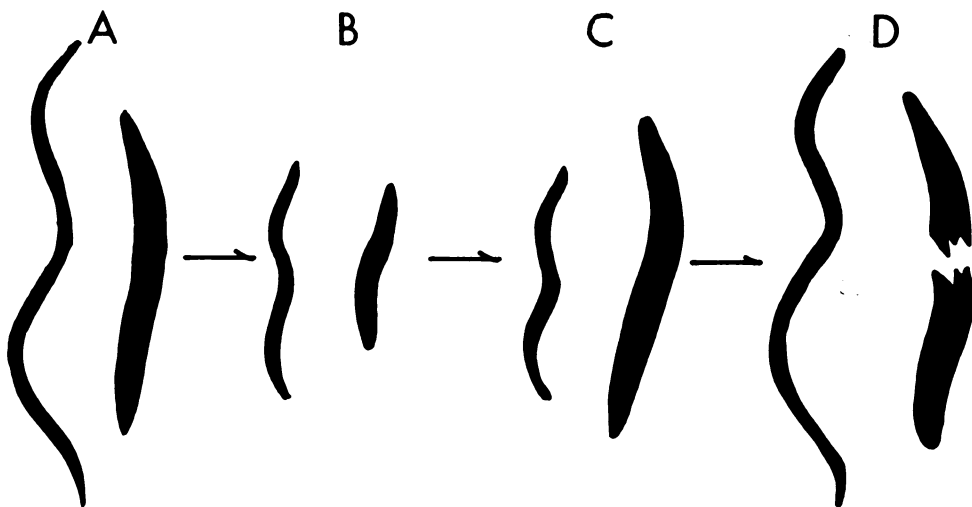
Fig. 1. Size changes in the osmotic treatment for separating *D. dipsaci* (left) and *Rhabditis* spp. (right). A, in distilled water; B, 5 minutes after transfer to hypertonic media; C. 24 hours in hypertonic media, *Rhabditis* spp. has recovered its original size. *D. dipsaci* remains dehydrated; D, on return to distilled water, *D. dipsaci* recovers and *Rhabditis* spp. bursts through excessive uptake of water (from Viglierchio, Croll and Görtz, 1969).

After an attempt to kill rhabditids selectively by chemical treatment failed (Viglierchio and Croll, 1969), an osmotic technique was devised, which exploited the differences in the physiological responses of *D. dipsaci* and rhabditid contaminants to osmotic stress (fig. 1). The nematodes extracted from garlic scales were treated with 0.8 molar NaCl for 24 hours, and then rinsed in distilled water. The rhabditids in the hypertonic medium slowly accommodate to the osmotic pressure by taking up ions from the bathing solution which causes them to burst when they are returned to distilled water; *D. dipsaci*, on the other hand, recovered its normal dimension and mobility (Viglierchio, Croll, and Görtz, 1969). After several washings in distilled water *D. dipsaci* were ready for surface axenization.

Preliminary tests with combinations of penicillin, streptomycin sulfate, mycifradin sulfate, and aretan were successful in yielding surface-axenized nematodes in batches of up to 5,000. *Ditylenchus dipsaci* were concentrated and pipetted into the top chamber of the pre-sterilized Lownsbery apparatus (Lownsbery and Lownsbery, 1956) which contained about 20 ml of aretan solution (133 ppm). The nematodes were treated in

aretan, by gentle bubbling with sterile air for 16 hours. The mixing was stopped, the nematodes were allowed to settle and then were transferred to the second chamber containing a sterile solution of penicillin (5,000 units/ml) and streptomycin sulfate (1.0 mg/ml). The nematodes were finally transferred to the collecting tube after eight hours in the antibiotic solution. A magnetic stirrer was placed into the collecting vial to aid in achieving a uniform concentration of nematodes for inoculation.

Nematode inoculation, extraction, and estimation

The desired number of axenized nematodes was introduced into each culture tube or jar containing the tissue and medium using a sterile, graduated 1-ml syringe. The suspension of axenized nematodes was diluted with sterile, distilled water depending on the density desired. The nematodes were placed either on or near the tissue, and the cultures were incubated at 25° C, unless otherwise stated.

At the end of each experiment, the tissue was macerated with a specially designed Hamilton Beach blender equipped with rubber blades. The contents of each tube, including the tissue and medium, were placed individually on Baermann funnels for two days. At the end of this period, most of the nematodes had come out of the tissue and settled in the stem of the funnel. The nematodes were collected and their numbers were estimated either directly or, in the case of high nematode populations, by taking a representative aliquant from a known volume of nematode suspension. The relative number of each developmental stage in a 1-ml sample was counted, using the criteria of developmental stage determination described by Yuksel (1960) for *D. dipsaci*.

PLANT TISSUE CULTURE MEDIA

The rearing of plant-parasitic nematodes on plant tissues *in vitro* called for a restricted set of conditions that were satisfactory both to host and parasite. Besides environmental factors, it was essential that the support matrix, either particulate or in the nature of a gel, be able to sustain plant tissue growth and yet provide suitable conditions for the nematode to gain sufficient purchase to penetrate the host. Agar gels have been most commonly used as a matrix for *in vitro* culturing.

Liquid medium

Tulecke and Rutner (1965) indicated that some plant tissue can be cultured in relatively large quantities in moderate amounts of liquid medium. Because of large requirements for callus tissues in our experiments, it was of interest to test the suitability of the liquid system. Explants of alfalfa (variety DuPuit and A14-X), red clover (variety Kenland), white clover (variety Ladino), garlic, and onion were transferred to a series of 250-ml flasks each containing 100 ml of Tulecke medium. The flasks were subsequently placed on a shaker and supplied with continuous diffused light at 25° C. After 46 days of growth, DuPuit alfalfa and onion tissue increased by one and one-half times, and the friable portions were white. However, tissues of Kenland and Ladino clovers, garlic and A14-X alfalfa did not grow well. Since the growth of different tissues was slow and disproportionate, the Tulecke medium was unsuitable for our purpose. The use of media of differing composition was undesirable as it would unnecessarily confound all nematode-rearing responses. Hence, it was preferable to improve upon the

already developed media systems that had previously been found successful with other tissues rather than to attempt to develop new ones.

Agar media for stem callus tissue of broad beans

In addition to susceptibility to *D. dipsaci*, *Vicia faba* (broad bean) tissue possesses other sufficiently interesting characteristics, *e.g.*, habit, development, and ease of culture to warrant its establishment in culture. To obtain axenic etiolated tissue, broad bean seeds were soaked for varying periods in Chloramine T, rinsed in sterile, distilled water, their seed coats removed aseptically, treated with 0.1 per cent streptomycin sulfate for two hours and rinsed several times with sterile, distilled water. The seeds were then placed in bottles containing sterile vermiculite and allowed to germinate in the dark. Germination was observed after four days at 24° C for seeds soaked for two hours in 2 per cent Chloramine T and 22 hours in sterile, distilled water. Seed germination was greatly reduced with an increase in Chloramine T concentration. The etiolated stem tissue was excised and placed on each of the following media: 1) 10 × normal White's medium (White, 1963), 2) Krusberg's medium (Krusberg, 1961), and 3) Tulecke's medium (Tulecke and Rutner, 1965), supplemented with 2 per cent agar and 200 mg/l 2,4-D (2,4-dichlorophenoxyacetic acid) instead of the usual NAA (naphthalene acetic acid). Of the three media tested, Krusberg's medium gave the best callus growth after one and one-half months. However, the etiolated sections showed a considerable variation in the amount of growth.

For the proliferation of green callused tissue of *Vicia faba*, sterile stem sections were placed on media containing different concentrations of 2,4-D (Klein and Mannos, 1960; Bach and Feliz, 1961). Sterile green stems were prepared after the method of Ball and Soma (1965). Five 7 to 10 mm long sections, taken between the second and third internodes above the primary leaf, were planted vertically with either apical or basal end up in a test tube each containing 6 ml of medium and incubated at 25°C. Callus proliferation took place within 10 days at all levels of 2,4-D; therefore the lowest concentration of 2,4-D was selected for subsequent routine use in the medium composition that follows.

MEDIUM TO INVESTIGATE PROLIFERATION OF GREEN TISSUE OF *Vicia Faba* AT DIFFERENT CONCENTRATIONS OF 2,4-D

0.200 g Ca $(NO_3)_2$ · H_2O
0.200 g $Na_2 SO_4$
0.065 g KCl
0.170 g NaH_2PO_4
0.360 g $Mg SO_4$ · $7H_2O$
1.00 ml Hoagland microelements
(1000 × normal strength):

  Mn SO_4 ....3.000 g
  Zn SO_4 ....0.500 g
  H_3BO_3 ....0.500 g
  Cu SO_4 · 5 H_2O ....0.025 g
  Na_2MoO_4 ....0.025 g
  H_2SO_4 ....0.500 ml
  H_2O .... 1000 ml

(Adjust above to 6.5–7.0 pH before autoclaving.)
  3.0 ml Fe-EDTA stock
  1.0 ml 2,4-D (10^{-4}, 10^{-5}, 10^{-6}M)
30.0 g sucrose
15.0 g agar
Glass distilled water to make 1000 ml of nutrient medium.
2,4-D concentrations (in 50% ethanol):
  10^{-4}M .... 0.2210 g/10 ml (1000×)
  10^{-5}M .... 0.0221 g/10 ml (1000×)
  10^{-6}M .... 0.0221 g/100 ml (1000×)

Callus tissue growth in relation to temperature and water loss

When tissue from alfalfa varieties DuPuit and A14-X were grown in

modified Krusberg's medium at different temperatures between 15° and 32° C, the rate of growth increased significantly until 25° C, at which point growth remained essentially constant. Water loss from tissue and medium increased with increase in temperature. However, callus tissue could be grown at 25° C for about four months before dehydration of the medium, and salt imbalance necessitated callus subculturing on fresh medium.

Henk's medium

Preliminary experiments had shown that different tissues grew — some poorly—at unequal rates on Krusberg's medium. To promote more rapid and uniform growth and to reduce the possibility of slow host tissue growth as a limiting factor in nematode development and reproduction, Krusberg's medium was supplemented with various addenda and the growth rates of six tissues compared. The following stock solutions of vitamins and hormones were maintained for the preparation of nutrient media:

STOCK SOLUTIONS FOR NUTRIENT MEDIUM

Stock salts I:

Na_2SO_4 ....8.00 g
$MgSO_4 \cdot 7H_2O$ ....1.80 g
KNO_3 ....0.80 g
KCl ....0.65 g
$NaH_2PO_4 \cdot H_2O$ ....0.33 g

Dissolve in enough H_2O to make 1 liter = 10 × normal strength

Stock salts II:

$MnSO_4 \cdot 4H_2O$ ....0.4500 g
or $MnSO_4 \cdot H_2O$ ....0.3410 g
$ZnSO_4 \cdot 7H_2O$ ....0.6000 g
H_3BO_3 ....0.0375 g
KI ....0.3000 g

Dissolve in enough H_2O to make 1 liter = 100 × normal strength

Vitamin stock:

Glycine ....0.300 g
Thiamine · HCL ....0.010 g
D-Ca-Pantothenate ......0.250 g

Dissolve in enough H_2O to make 1 liter = 100 × normal strength
Filter-sterilize before refrigeration

Ferric and calcium stock:

$FeNH_4$ citrate ....0.82 g
$Ca(NO_3)_2 \cdot 4H_2O$ ....4.00 g

Dissolve in enough H_2O to make 1 liter = 10 × normal strength

Hormone stock:

Kinetin ....0.050 g
2,4-Dichlorophenoxy-
      acetic acid ....0.200 g

Dissolve in 100 ml of 50 per cent aqueous ethanol = 100 × normal strength. May be heated slightly when crystalized after storage in the refrigerator.

Casein and yeast hydrolysates were found to be beneficial to tissue growth. The constituent analyses of each hydrolysate indicated that certain components present in one were absent in the other. Hence, a solution of the pure components at the proper concentration was substituted for the corresponding hydrolysate:

SIX MEDIA FOR GROWTH OF TISSUE OF
SIX SEED VARIETIES

(A) Krusberg
(B) Krusberg + 0.1% yeast hydrolysate
(C) Krusberg + 0.1% casein hydrolysate
      + 0.0301 g arginine/l
      + 0.0230 g phenyla-
            lanine/l
      + 0.0151 g histidine/l
      + 0.006 g tryptophane/l
      + 0.250 mg thiamine/l
      + 0.0080 mg riboflavin/l
(D) Krusberg + 0.1% yeast hydrolysate + 0.1% casein hydrolysate
(E) Krusberg + 0.1% casein hydrolysate

(text table cont.)

(F) Krusberg + 0.1% yeast
hydrolysate
+ 0.090 g serine/l
+ 0.1412 g glutamine/l
+ 0.0240 g alanine/l
+ 0.0412 g proline/l
+ 0.0372 g aspartic
acid/l
+ 0.020 g tyrosine/l

A comparison of growth on different media indicated that all supplemented media provided better tissue growth than that obtained on Krusberg's medium (table 7). Media containing casein and yeast hydrolysates as supplements promoted a twofold increase in growth for all six tissues tested. It is evident that the two varieties of each plant species tested have different requirements for proliferation of callus tissue. Supplementation of pure components for the principal missing constituents of the corresponding hydrolysate seldom completely substituted for the hydrolysate, thus indicating that other factors, *i.e.*, deficiency of minor components, imbalance of nutritional components, presence of inhibitors and imbalance of electrolytes, were involved.

It appeared that supplements of casein and yeast hydrolysates supported tissue growth better over a long period (table 8).

In view of these findings, Henk's medium (so named in appreciation of the efforts of Jan Henk Görtz, who worked so diligently to develop it) was found to be the most suitable substrate for a wide variety of callus tissues. Unless otherwise indicated, Henk's medium described as follows was used for culturing different tissues:

COMPOSITION OF HENK'S MEDIUM

To make 1 liter of Henk's nutrient medium, use:

Water ....760 ml
Stock I ....100 ml
Stock II ....10 ml
Fe-stock ....100 ml
Hormone stock ....1 ml
Adjust pH to 5.7
Sucrose ....20.0 g
Agar (purified) ....7.5 g
Yeast hydrolysate ....1.0 g
Casein hydrolysate ....1.0 g
Vitamin stock ....10.0 ml

TABLE 7

RELATIVE WEIGHTS OF TISSUE AFTER THREE WEEKS FOR SIX PLANT
VARIETIES ON KRUSBERG'S MEDIUM, AND KRUSBERG'S MEDIUM
PLUS SUPPLEMENTS

Media	Relative weight of tissue*					
	Medicago sativa		*Trifolium pratense*		*Trifolium repens*	
	DuPuit	Calverde	Pennscott	Kenland	Ladino	White Dutch
Krusberg	100	100	100	100	100	100
Krusberg + casein hydrolysate	118.2	113.3	244.0	172.3	131.8	183.6
Krusberg + casein hydrolysate + supplements	148.7	147.8	245.7	177.4	108.5	188.0
Krusberg + yeast hydrolysate	166.2	130.1	242.3	174.8	137.0	208.6
Krusberg + yeast hydrolysate + supplements	184.8	232.1	223.5	193.2	242.6	242.8
Krusberg + casein hydrolysate + yeast hydrolysate	221.6	208.5	254.0	205.1	260.3	244.6

* Growth on Krusberg's medium = 100; initial inoculum weights for each kind of tissue were identical.

TABLE 8
'RELATIVE TISSUE PROLIFERATION AFTER TWO GROWTH PERIODS
FOR SIX TISSUES ON TWO MEDIA

Media	Relative tissue proliferation of:					
	Medicago sativa		*Trifolium pratense*		*Trifolium repens*	
	DuPuit	Calverde	Pennscott	Kenland	Ladino	White Dutch
Krusberg + casein and yeast hydrolysates..........	100	100	100	100	100	100
Krusberg + casein hydrolysate (3 weeks)...	53.5	54.3	96.1	83.8	50.7	75.2
Krusberg + casein hydrolysate (17 weeks)......................	46.0	40.1	86.8	110.0	32.1	55.6

POPULATION STRUCTURE AND DEVELOPMENT OF
D. DIPSACI ON TISSUES OF VARIOUS HOSTS

This investigation of the properties of the nominal species of *D. dipsaci* was based upon the premise that the race designation was a strong phenomenon and that conflicting reports in the literature about the race designation was either due to geographical variations in the inoculum or incomplete observations. An experiment with different tissues with nematodes from different geographical locations and host was designed to elucidate the characteristics of races and a possible insight into the nature of resistance.

Clover mixed tissue

Ditylenchus dipsaci has been cultured, with varying degrees of success, and from a small inoculum considerable numbers of nematodes have been recovered after several months of growth (Krusberg, 1961; Bingefors and Eriksson, 1963). We were able also to recover appreciable numbers of nematodes; however, frequently, under identical conditions of tissue type and age, environment, and inoculum, there was often a wide variation in the number of nematodes recovered. Since each replicate usually involved 30 to 50 seeds placed on 15 ml of Henk's medium in a 25×100 mm tube with an inoculum of 100 or more nematodes, this erratic response in nematode reproduction was not explicable in terms of sex ratio or lack of uniformity in host tissue sources. This phenomenon is illustrated by *D. dipsaci* from garlic (French Biotype) when cultured on red clover (variety Kenland). The cultures were sampled at five-day intervals from 10 to 30 days. Nematode population levels and structures were determined as described earlier. Inactive nematodes would not have been collected with the Baermann funnel and therefore were not counted.

Second-stage larvae began to appear by the tenth day as was first determined by Yuksel (1960), and the population continued to increase to six- to eight-fold by the twentieth day and then leveled off for the next five days (fig. 2A). After 25 days, however, there occurred a wide variation in population increases; some cultures reaching 3,000 nematodes by the thirtieth day, while others dropped to 300 nematodes.

Population structure also reflected the changes in population levels just discussed (fig. 2B). As the inoculum consisted mainly of the fourth-stage larvae, approximately half of the popu-
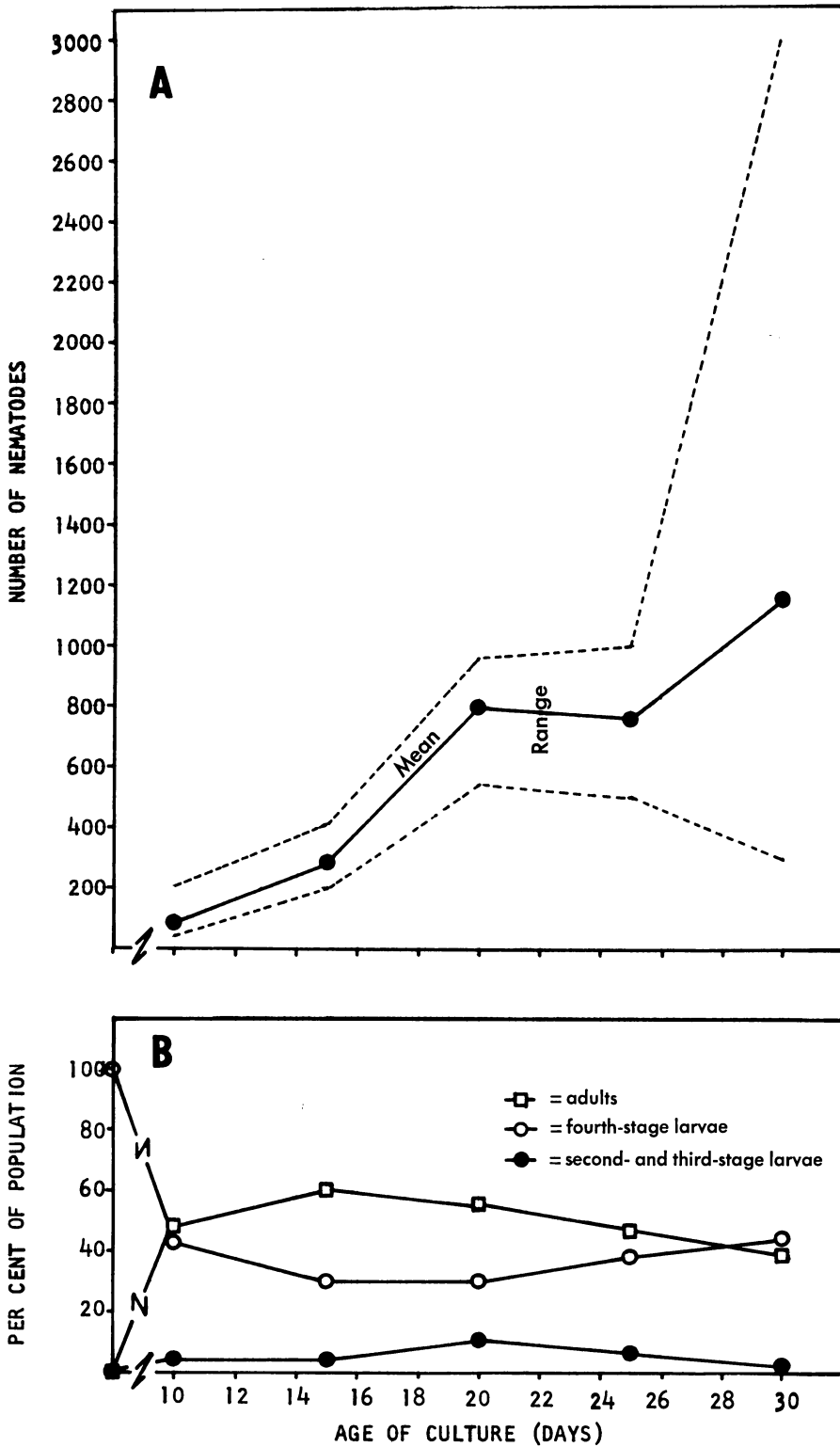
Fig. 2. (A) population levels and structure of *D. dipsaci* (Onion Race—French Biotype) on Kenland red clover mixed tissue at different culture ages. (Mixed tissue-seed, stem, or root con‑ sisting of differentiated tissue grown on a hormone-containing medium to promote undifferenti‑ ated callus growth. (B) population structure of a composite sample from cultures of different ages.

lation developed into adults within the first 10 days. The egg-laying and subsequent hatching resulted in the appearance of second and third-stage larvae. In cultures where population increase was moderate, the second and third developmental stages never represented more than 10 per cent of the total. In contrast, the cultures which showed low population levels, the population consisted largely of fourth-stage larvae and adults.

Much older cultures, *i.e.*, 16 to 20 weeks, showed an even greater decrease in numbers, although occasionally some cultures maintained high population levels. This divergence in the populations confounded replication due to a randomness of populations that entered the second numerical increase. Hence, the first phase, *i.e.*, up to 21 to 25 days, was considered to be the period of emphasis. In the first generation, a certain fraction of inoculum successfully adopted to parasitism, that is, nematodes penetrated the tissue and completed their life cycle with varying degrees of success. This degree of efficiency in parasitism was, in turn, reflected in their population structures.

The wide range of population levels obtained on red clover cultures with identical inoculum levels from the same stock suspension was explored further. The usual mixed tissue (seed, stem, or root consisting of differentiated tissue) culture was prepared from Ladino clover seeds on Henk's medium. After one week of growth, the cultures were inoculated with 100 to 150 fourth-stage larvae of the French Biotype from garlic scales (first culture). After rearing periods of 4 to 6 weeks, population levels were determined and nematodes from the highest reproducing replicates saved as inoculum for the subsequent subculture. For the fifth culture, different levels of inocula obtained from the fourth culture were used. The results (fig. 3) indicate that by selection it is possible to increase the reproductive rate and homogeneity of a population. According to the results of the fifth culture, the reproductive rate is inoculum-level dependent. The variability evident at the low inoculum level suggests that continued selection would have further increased the reproductive potential of this population.

Population dynamics in sequence cultures

Preliminary evidence for sex ratio dependence upon host tissue by populations developing from the same initial source of inoculum (Viglierchio and Croll, 1968) was thought to be due to chemical, perhaps transmissible, factors that could in part explain these observations. The tissue culture techniques were especially useful, since tissues in cultures are known to release a number of chemicals into the nutrient media (Heller, 1965). Sequence culture permits the examination of one aspect of this system. For example, if one tissue were grown on a medium for a certain period, then replaced by a second explant of the same or different tissue, allowed to establish for a short period, inoculated with nematodes, and the population allowed to develop for a specified period, it would be possible to examine the population levels and structures of the nematodes as a function of the exsorbates of the first tissue.

This method was used for a number of tissue combinations. The initial tissue was grown for 12 days on the medium then replaced by the second tissue which was allowed to equilibrate for two days, then inoculated with 150 fourth-stage larvae of *D. dipsaci* from garlic (French Biotype) and harvested after four weeks.

An analysis of the data indicates that in tissue sequences containing the red clover (variety Kenland) as one of the explants, the sex ratios were similar to that normally expected for this nematode while reproducing on a
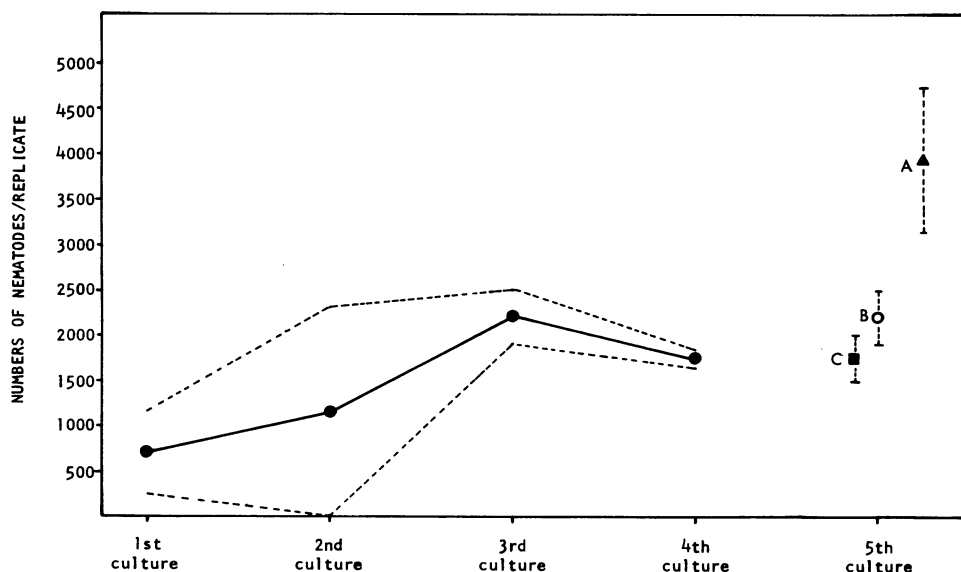
Fig. 3. Average population per replicate and Standard Deviation boundaries of *D. dipsaci* (Onion Race—French Biotype) on Ladino white clover cultures. Rearing period, 4 to 6 weeks. Inoculum for first cultures, 100 to 150 nematodes. Fifth-culture inoculum (obtained from fourth culture) was A = 50; B = 100; C = 200. Nematodes were selected from highest increase replicates for subsequent reinoculation.

"type host," *i.e.*, onion or garlic. Those tissue sequences containing the white clover (variety Ladino) and the alfalfa (variety DuPuit) tissues in some combination appeared to promote a bias towards maleness (fig. 4A). For reasons unknown, the effect of the alfalfa (variety DuPuit) tissue on a nematode population reared on the red clover (variety Kenland) was biased towards maleness 2 to 1. However, when these two tissues were used in the reverse sequence, the sex ratio was biased towards femaleness 3 to 1.

Figure 4B indicates the combined second and third larval stages as per cent of total population at the harvest time for the corresponding sequences. The percentage of second and third developmental stages in the population varied according to tissue sequence but did not appear to be correlated with sex ratios at harvest time (28 days).

When sex ratios of populations reared on pure cultures of red clover (variety Kenland and Pennscott), white clover (variety Ladino), and alfalfa (variety DuPuit) were determined at different intervals, it was evident that the male bias, as noted in sequence cultures, persisted (fig. 5). The predisposition of the red clover (variety Kenland) towards a sex ratio either similar to that on "type host" or more femaleness and that of the alfalfa (variety DuPuit) and the white clover (variety Ladino) towards a typical maleness in populations was evident at 14, 21, and 28 days. The increase in maleness of the population was, in turn, reflected in reduced population levels when grown on these tissues. This sex ratio correlation on single host tissue is, therefore, supported by our observations on sequence cultures.

Population dynamics in community cultures

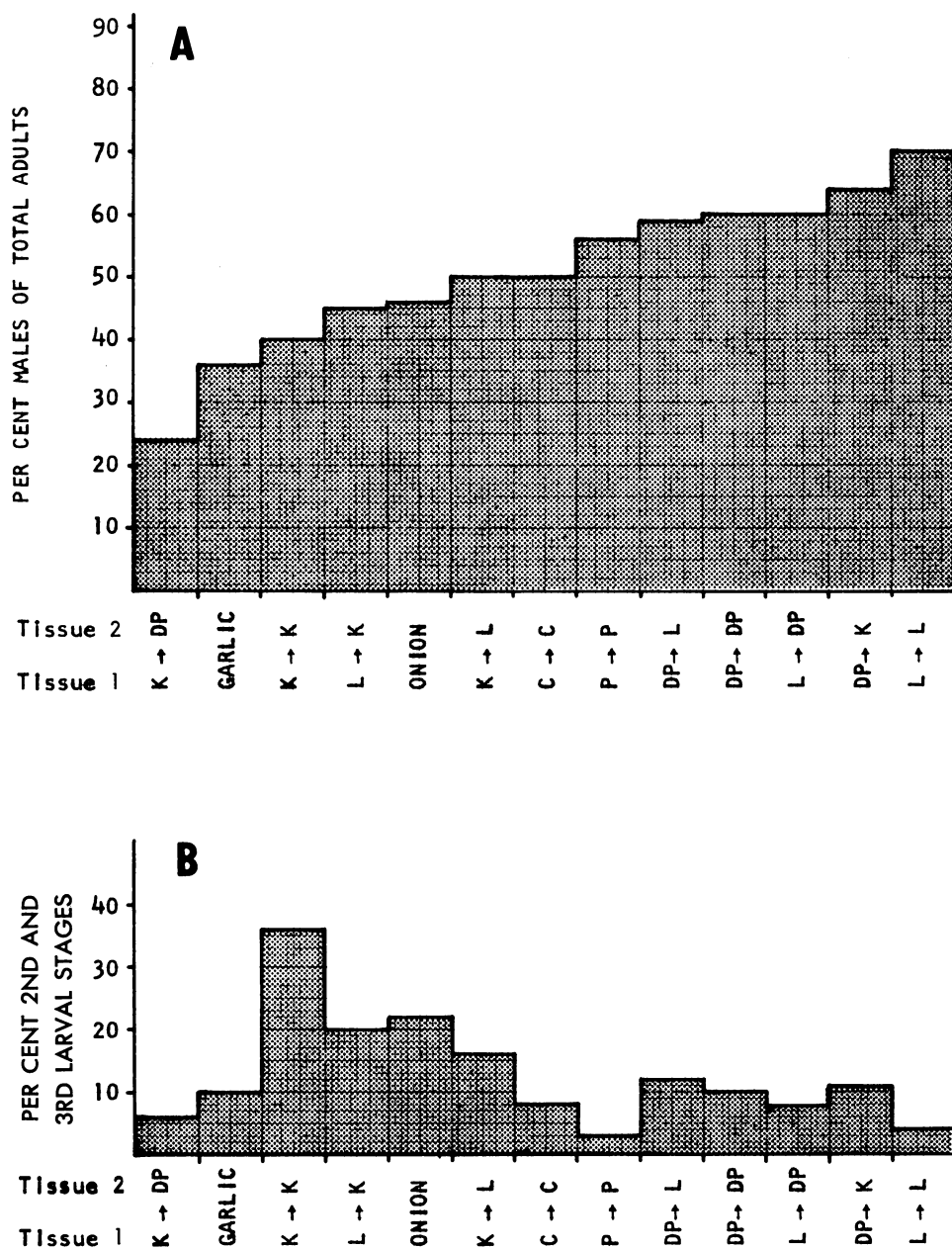In view of our findings from the sequence culture experiments, it was of

Fig. 4. Populations of *D. dipsaci* (Onion Race—French Biotype) on sequence cultures. Tissue 1 cultured two weeks followed by tissue 2 for one week before inoculation with 100 fourth-stage larvae (N = 6). DP = DuPuit alfalfa; C = Caliverde alfalfa; K = Kenland red clover; P = Pennscott red clover; L = Ladino white clover.
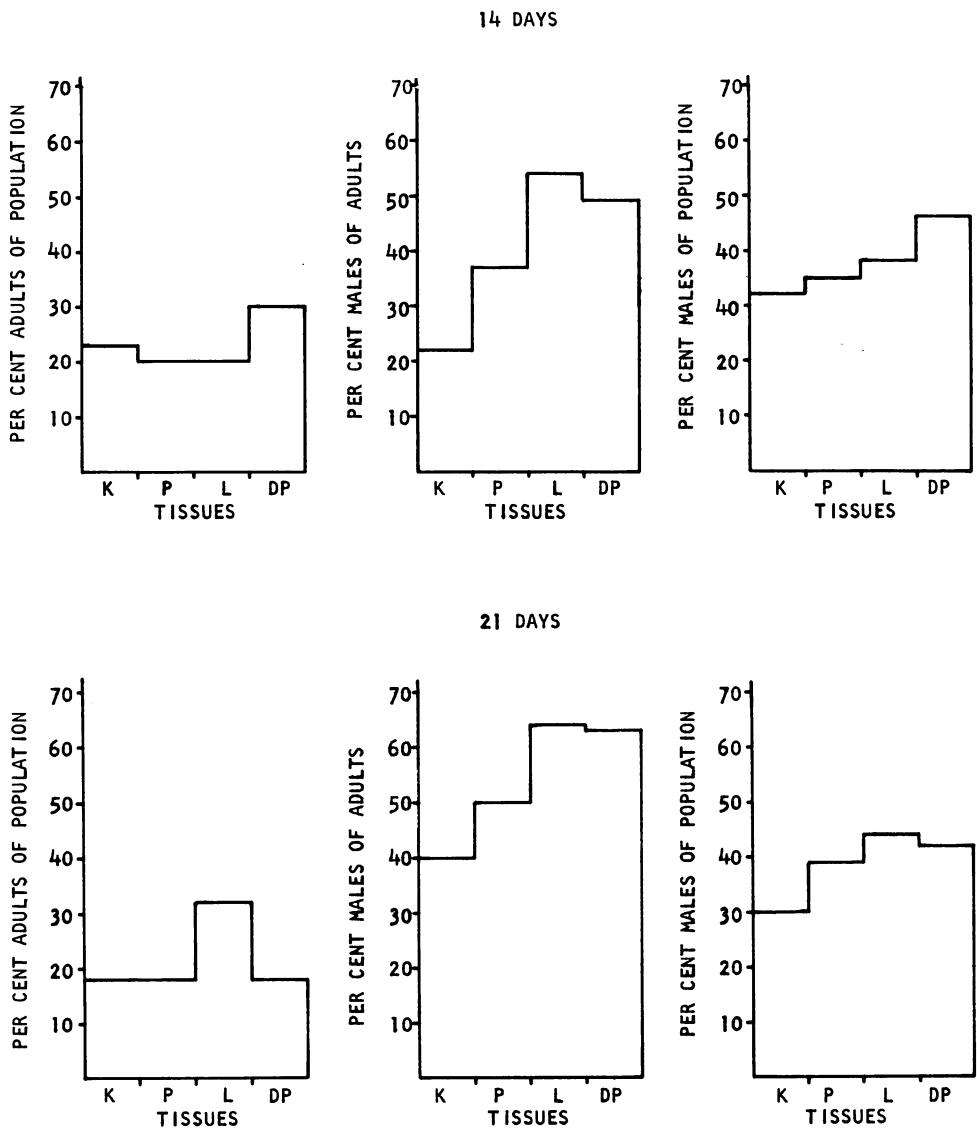
14 DAYS

21 DAYS

Fig. 5. Proportion of males in populations of *D. dipsaci* (Onion Race—French Biotype) on different mixed tissues at 14 and 21 days after inoculation. K = Kenland red clover; P =Pennscott red clover; L = Ladino white clover; DP = DuPuit alfalfa (N = 8).

interest to compare the effects of community cultures on nematode populations, especially on sex ratios. The following technique was used in preparing community cultures of different tissues: one end of a piece of wetted 25-mm dialysis tubing was knotted to hold water. The free end was then ligatured to a glass ring 20 mm in diameter and 30 mm in height. The assembly with dialysis bag downward was suspended in a 125-ml screw-cap bottle, and warm medium was added so that its level in the bag was the same as in the bottle. The final level of medium in the community culture assem-

bly was 5 to 10 mm above the lower edge of the glass ring. After autoclaving, the upper edge of the glass ring was coated with a thin layer of sterile lanolin as a barrier to nematode migration. Callus tissue was placed on the medium inside the dialysis bag (internal explant), and approximately three-fold quantity of modifying plant tissue was placed on the medium outside the dialysis bag (external explant). After some 10 days of callus tissue growth, nematode inoculum of known numbers was placed on the internal explant.

Population levels of *D. dipsaci* (Onion race: French Biotype) were considerably modified in community cultures (fig. 6A, B). Alfalfa (variety DuPuit) and white clover (variety Ladino), which were biased towards maleness and the Ladino-Kenland combination which allowed a sex ratio similar to that on the type host, respectively, in sequence cultures promoted the highest population increase among the tissues tested in the community culture. Furthermore, the effect of Kenland red clover upon DuPuit alfalfa, which strongly biased towards femaleness in the sequence cultures, promoted a lower population increase in the community cultures than did their reverse combination, which was found to induce a maleness bias in the sequence cultures. It is readily apparent (fig. 4,6) that the tissue interaction is complex, involving such factors as differential mineral absorption and mineral imbalance, as well as differential release and uptake of exsorbates.

Population structure as a function of age of culture

Explants of red clover (variety Kenland) and white clover (variety Ladino) were placed into culture tubes containing approximately 15 ml of Henk's medium, and the tissue was allowed to equilibrate for a week. The cultures were then inoculated with ap-

proximately 100 fourth-stage larvae of *D. dipsaci* (Onion race: French Biotype) and the populations were allowed to develop. Beginning the fifteenth day after inoculation, six replicates of each tissue were harvested every five days until the fiftieth day. The levels and structures of these populations were determined as described earlier. In the fifteenth-day harvest there were no second- and third-stage larvae; however, the population structure had stabilized by the twenty-fifth day (fig. 7). After the thirtieth day, population structures began to shift such that the structures by 40, 45, and 50 days were similar to those illustrated for the fortieth day (fig. 7). Between the fifteeenth and thirtieth days, the initial inoculum of fourth-stage larvae had developed to a population which consisted of 45 per cent fourth-stage larvae, 45 per cent adults with approximately equal males and females, and 5 to 10 per cent of second- and third-stage larvae on both types of tissues. Subsequently, the population structures on the two kinds of tissue changed; the proportion of second- and third-stage larvae decreased to 1 per cent or less of the total population on both tissues. After 40 days on white clover (variety Ladino), the proportion of fourth-stage larvae increased to 50 per cent, while the adult population remained the same as in young cultures (fig. 7).

In contrast, the proportion of fourth-stage larvae and adult males and females was of the same order after 40 days on red clover (variety Kenland). Apparently, the initial inoculum of fourth-stage larvae developed into adults with moderate facility on all tissues; in older Kenland tissue, this balance shifted even further in favor of the adult stage. The primary hindrance in development of *D. dipsaci* appears to be between the egg-laying and subsequent fourth larval stages. Development which proceeded with difficulty
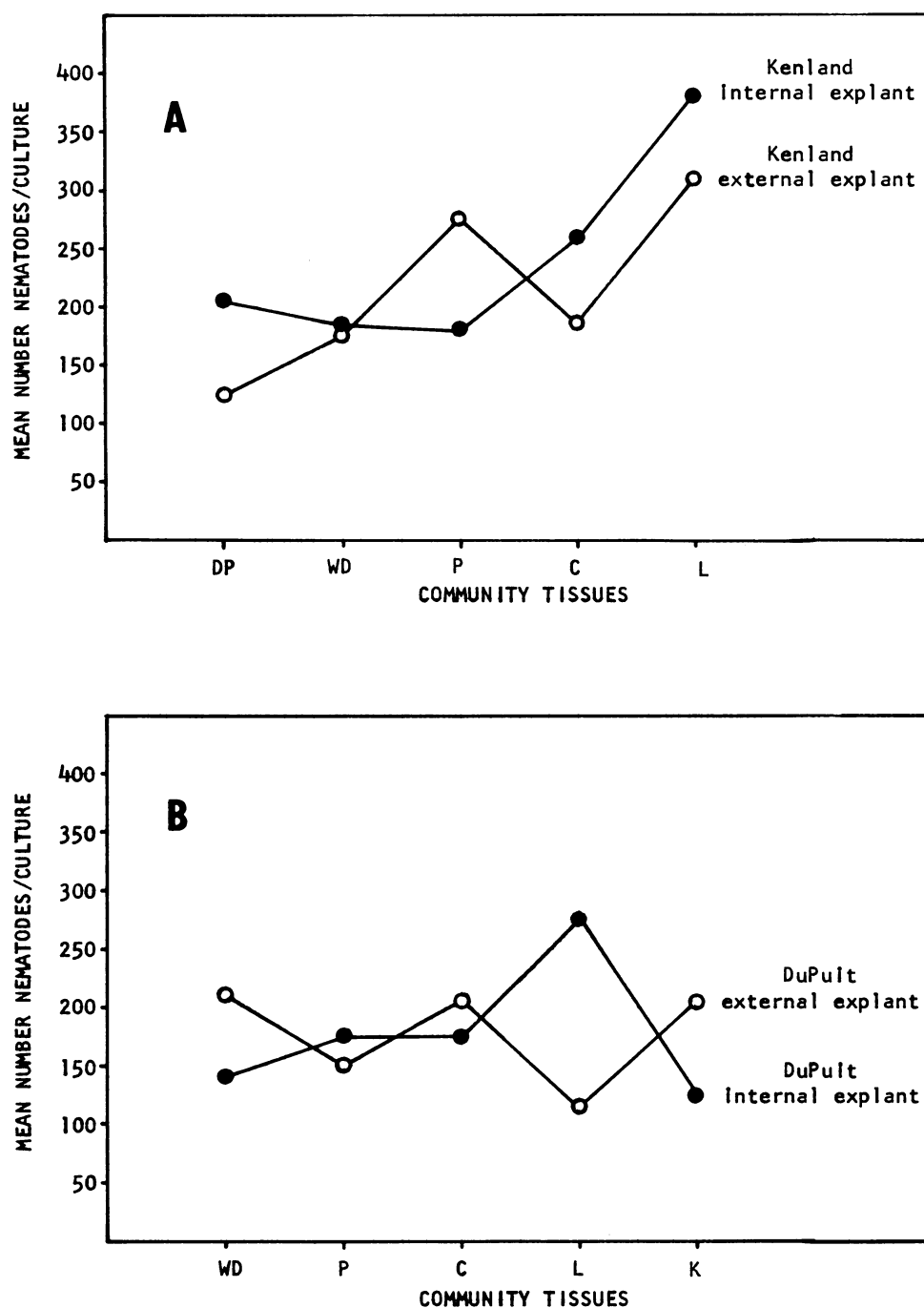
Fig. 6. Population levels after 21 days of community culture. Inoculum of 100 fourth-stage larvae of *D. dipsaci* (Onion Race—French Biotype) were placed upon the internal explant. DP = DuPuit alfalfa; WD = White Dutch clover; P = Pennscott red clover; C = Caliverde alfalfa; L = Ladino clover; K = Kenland red clover (N = 6).
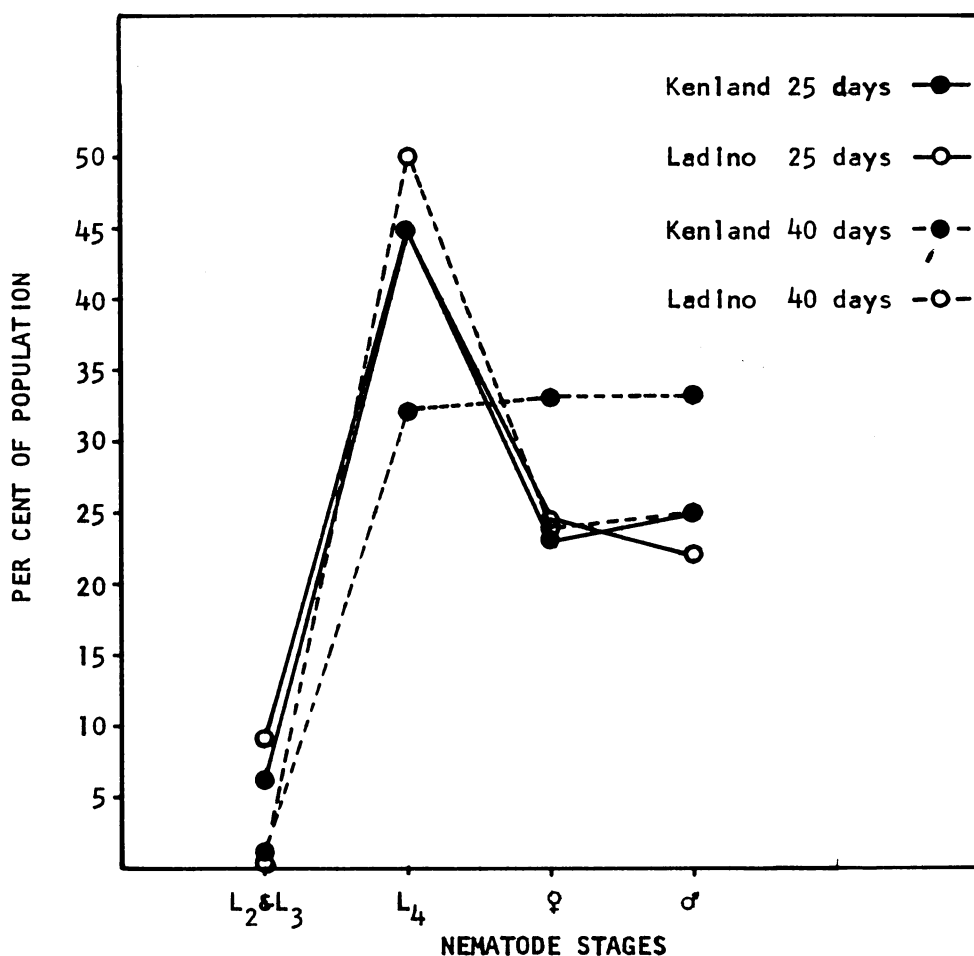
Fig. 7. Population structure of *D. dipsaci* (Onion Race—French Biotype) on Kenland and Ladino clover explants at 25 and 40 days after inoculation. L_2 = second larval stage; L_3 = third larval stage; L_4 = fourth larval stage; ♂ = adult males; ♀ = adult females.

on young tissue nearly stopped on old tissue. Analysis of data indicates that the blockage in nematode reproduction and development probably occurred in embryogenesis.

Effect of tissue age at inoculation time on population increase and development

Conventional plant cultures used to rear nematodes were of mixed tissue, *i.e.*, seed, stem, or root consisting of differentiated tissue grown on a hormone-containing medium to promote undifferentiated callus growth. It was

evident from the previous studies that age of tissue could modify the levels and structures of populations reared on it; therefore, it was of interest to better define the nature of this modification. The usual practice was to allow the explants to become established for a week to ten days before inoculation with nematodes. Tissue age was measured from the time of explant transfer, and culture age was, therefore, determined from the time of inoculation. For this study, the axenized white clover (variety Ladino) seeds or pure callus subcultures were
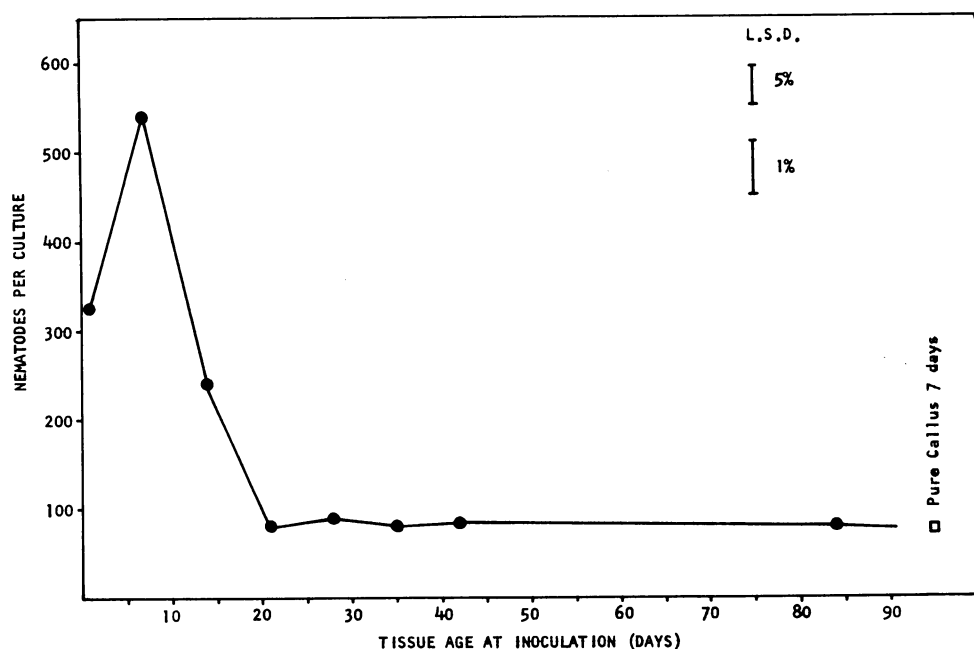
Fig. 8. Population increase of *D. dipsaci* (Onion Race—California Biotype) on mixed tissues of Ladino white clover of different ages at time of inoculation (N = 12).

allowed to establish for varying periods before inoculation with approximately 200 fourth-stage larvae of *D. dipsaci* (Onion race: California Biotype). All the cultures were allowed to incubate four weeks before nematode population level and structure analyses were made.

With mixed tissue, the greatest population increase occurred when the tissue was allowed to establish for one week before inoculation (fig. 8). Apparently, a one-day establishment period permitted less tissue growth and so restricted population growth. Even so, the population increase was greater than that on tissues that were allowed to equilibrate for two weeks or longer before inoculation. After an establishment period of three weeks and longer, the population levels on mixed tissue were similar to that achieved on pure callus. Apparently, after the one-week establishment period, the mixed tissue became less suitable for population increase. The structures of

these populations explain, in part, that the population increase was a function of the length of establishment period (fig. 9). The population structures of mixed tissues allowed to establish two weeks or less showed the presence of 4 to 7 per cent of second-stage and 11 to 13 per cent third-stage larvae, therefore, reflecting the dynamic state common for an increasing population.

In contrast, if cultures of mixed tissue were allowed to incubate three weeks or longer, the proportion of second and third-stage larvae was reduced to 4 per cent or less, with a consequent increase in the adult stage, thus reflecting a more static condition. The structure of populations, whether from three-week-and-older tissues or pure callus, suggests severe hindrance in the completion of the life cycle. The development of fourth-stage larvae to adults, however, continued unabated. As observed in the previous experiment, the blockage in the reproduction
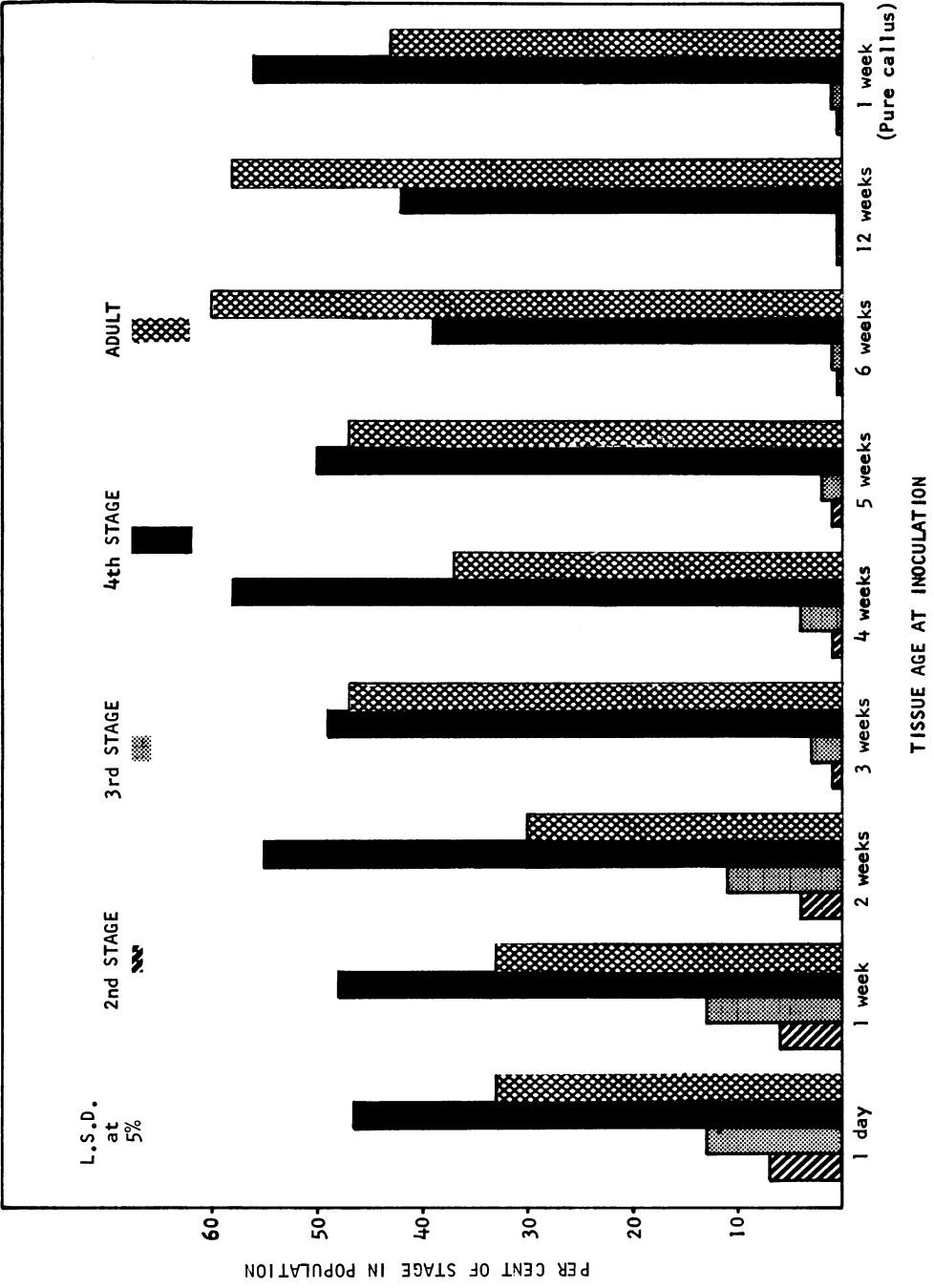
Fig. 9. The structure of *D. dipsaci* populations (Onion Race—California Biotype) established on mixed and pure callus tissues of Ladino cloer of different ages four weeks after inoculation (N = 12).

and development was apparently in embryogenesis.

Tests using other varieties of alfalfa and clovers gave similar results with the California Biotype. In a mixed-tissue culture, the initial tissue was differentiated immediately after germination and able to support the parasitic nematodes. With increasing age, however, the hormone-containing medium favored undifferentiated cell growth which was unsuitable for the nematode and which, in all probability, partially occluded the small amount of residual differentiated tissue able to support the parasite.

Population development on three kinds of mixed tissues

In view of previous findings, an investigation of the population development of *D. dipsaci* (Onion race: California Biotype) under monoxenic conditions using mixed tissues of onion (variety White Globe), broad bean (variety Red Purple), and alfalfa (variety El Dorado) was undertaken. The first two tissues were known to be susceptible to this race of *D. dipsaci*, whereas El Dorado alfalfa was described to be resistant to some field populations of the alfalfa race of this nematode. Mixed tissue cultures of alfalfa and broad beans were obtained by germinating the seeds directly on Henk's medium, while onion seeds were first germinated on 1 per cent water agar, then transferred to Henk's medium. Since both root and shoot apices were allowed to grow under callus proliferating conditions on a hormone-containing medium (Henk's medium), such cultures were termed mixed tissue cultures.

Analysis of population levels six weeks after inoculation indicated that the increase in population was in the order of onion (29): broad bean (6): alfalfa (1): (fig. 10). On onion mixed tissue, after six weeks, population

levels of about 8,000 were obtained from an inoculum of 200 fourth-stage larvae. However, the same nematode population also reproduced on broad beans, although only moderately. Even though alfalfa is not considered to be a host of this race, some reproduction occurred on alfalfa as indicated by the presence of second- and third-stage larvae.

Population structure was also different on the three tissues tested. The structure on El Dorado alfalfa was similar to that observed on other varieties of alfalfa and clover in the previous experiments. In comparison to onion and broad bean, the proportion of second- and third-stage larvae on alfalfa was lower, but the proportion of fourth-stage larvae and adults was higher. Therefore, it further supports our conclusion derived from the previous results that with alfalfa and clover tissue, the hindrance in reproduction lay somewhere in embryogenesis. However, on broad bean, which was intermediate between onion and alfalfa in supporting population increases, the proportion of second- and third-stage larvae was higher, and fourth-stage larvae was lower than on onions, thereby suggesting that hindrance in the life cycle took place between the third and fourth larval stages. It appears that in comparison to onion, the preferred host of the *D. dipsaci* population used in this study, the hindrance in life cycle takes place at a different step in broad bean than in alfalfa.

Population development of three biotypes of *D. dipsaci* on five types of tissue from *Vicia faba*

Under field conditions, *D. dipsaci* prefers different parts of different plants as sites for reproduction. The purpose of this study was to compare the suitability of different plant parts for reproduction under monoxenic
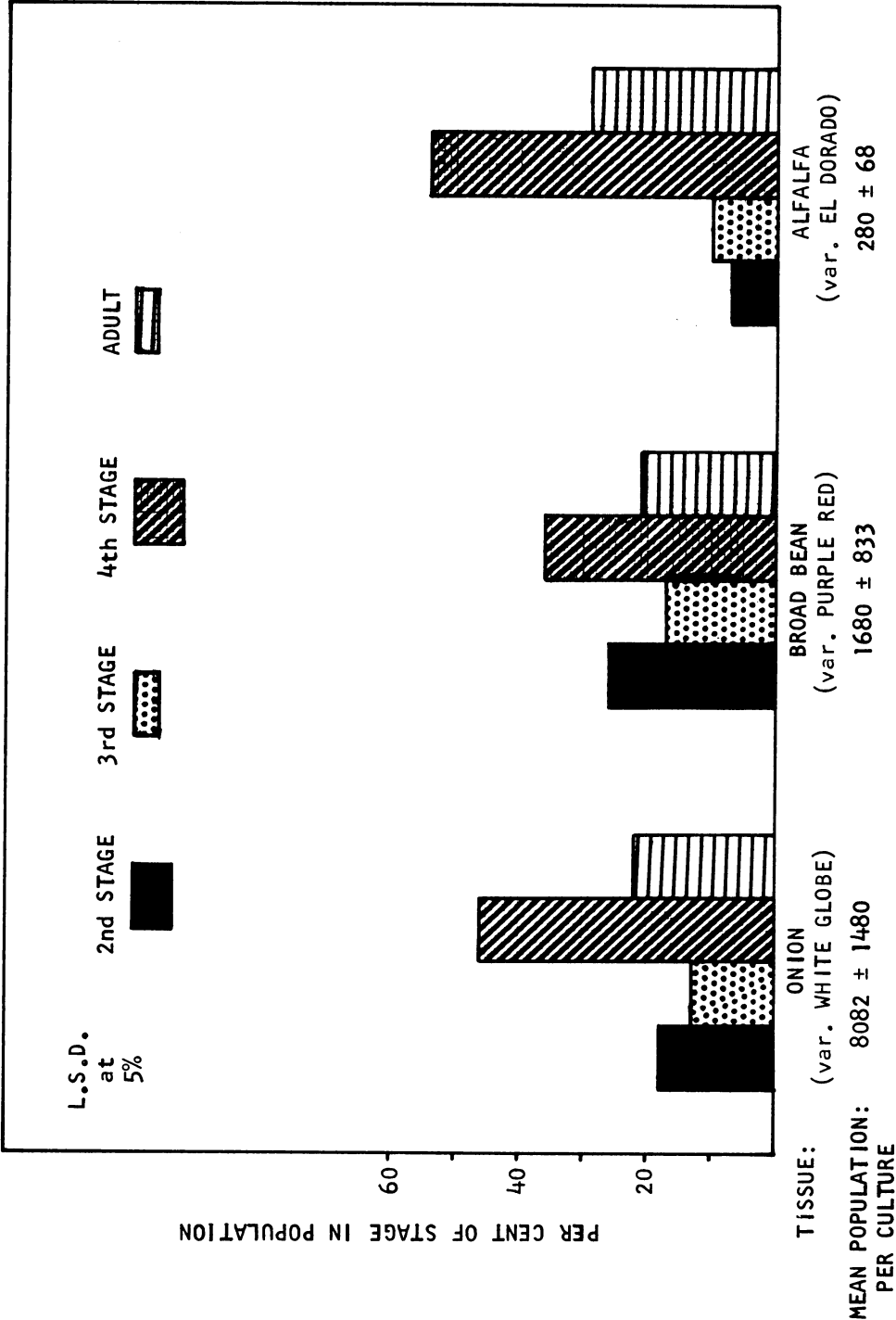
Fig. 10. Population levels and structures of *D. dipsaci* (Onion Race—California Biotype) on three kinds of mixed tissues six weeks after inoculation with 200 fourth-stage larvae (N = 10).

conditions and to evaluate adverse effects of those tissue types on population structure which reduce or do not support nematode reproduction. Broad beans (*Vicia faba*, variety Red Purple) were selected as the seeds were more suitable to excisement and to manipulation. The seeds were axenized as described earlier and placed on 1 per cent agar for germination. After three days, the following tissues were prepared, then placed on Henk's medium in 125-ml screw-cap bottles: (1) cotyledons, separated from the radical and stem; (2) radical and stems of seedlings without cotyledons and excised to remove root and shoot apices; (3) whole seedlings with cotyledons—with 2,4-D in the medium; (4) whole seedlings with cotyledons—without 2,4-D in the medium, and (5) pure callus tissue of broad bean obtained from subcultures of old cultures initiated from stem and radical tissue.

Figure 11 shows population levels six weeks after each of the cultures were inoculated with the three biotypes (200 fourth-stage larvae). California Biotype reproduced in the greatest and Mexican Biotype reproduced in the lowest numbers; French Biotype reproduced in the intermediate range. On mixed tissue, from broad bean stems and radicals, however, the order of population levels was reversed. With all three biotypes the population levels were the lowest on pure callus tissue. Whole seedlings grown on the medium without 2,4-D allowed higher reproduction of both French and California Biotypes than the seedlings grown on 2,4-D-supported medium. In contrast, Webster and Lowe (1966) noted that 2,4-D added to the tissue culture medium increased the susceptibility of otherwise resistant red clovers to the alfalfa race of *D. dipsaci*. Since reproduction of the Mexican Biotype was generally low on all of the five tissue types tested, their suitability is questionable. Nevertheless, reproduction of

both French and California Biotypes was significantly higher on differentiated tissue than on callus or mixed tissue.

According to the structure analysis of the California Biotype on the five types of tissue (fig. 12), the proportion of the various stages was essentially the same on the populations reared on cotyledons, seedlings and seedlings on medium without 2,4-D, although there is over a three-fold increase in the population of each succeeding member of this sequence. The structures of populations reared on cotyledons or on whole seedlings, grown on Henk's medium either with or without 2,4-D, were representatives of a dynamic population as indicated by 19 to 29 per cent of individuals still in their second and third larval stages. The lower populations appeared to result from a rather nonspecific general depression of all stages of the life cycle. With pure callus of *Vicia faba*, the transition of fourth stage to adult, and perhaps embryogenesis, appeared to be blocked. The small amount of differentiated tissue in the stem and radical cultures permits some reproduction of the California Biotype.

Analysis of the population structures of the French Biotype on different types of *Vicia faba* tissues shows that the proportion of second- and third-stage larvae was significantly higher on whole seedlings than on the undifferentiated tissue (mixed tissue) from stems and radicals (fig. 13). The structures of the populations on whole seedlings and to some extent those on cotyledons resembled the proportions of different developmental stages that are typical of a dynamic culture. On the other hand, population structures of the French Biotype on pure callus and mixed tissue proliferated from stems and radicals were composed of 93 to 98 per cent fourth-stage larvae and adults. The stem and radical mixed tissue allowed the development of
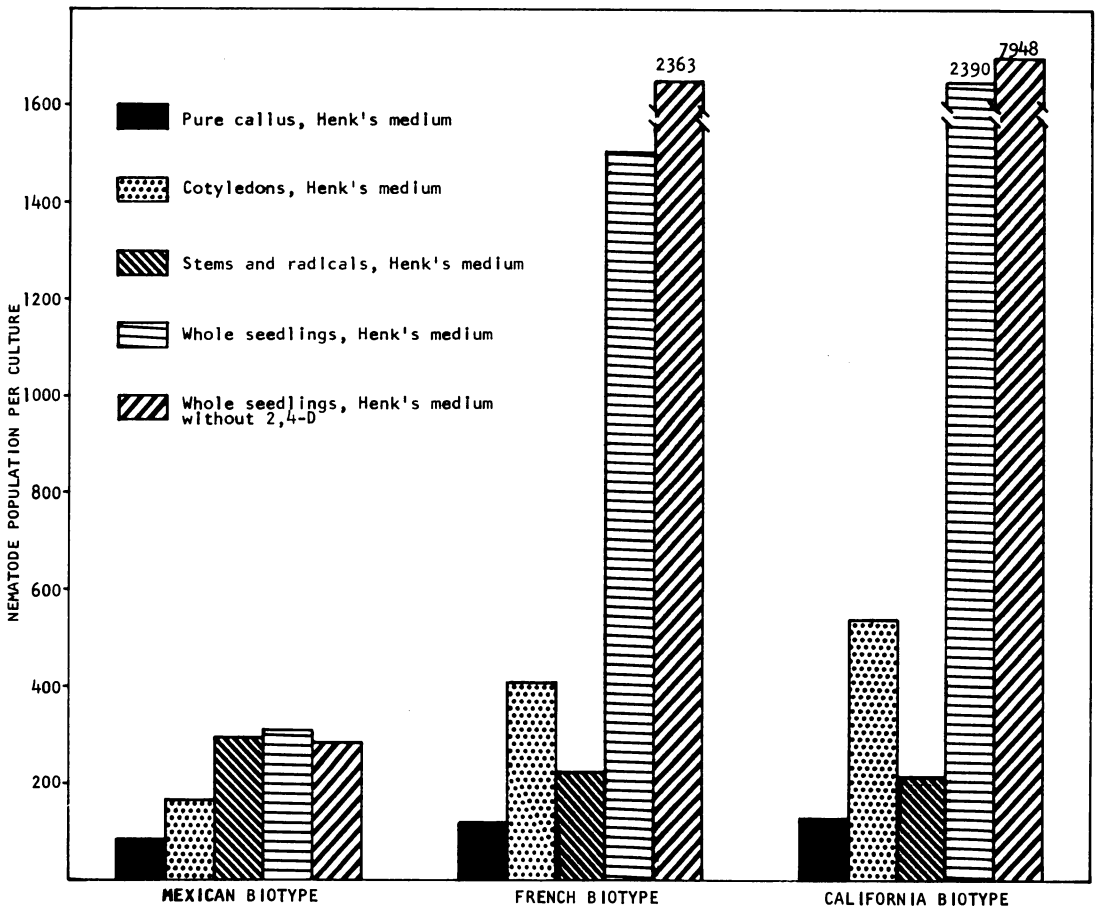
Fig. 11. Population levels of three biotypes of *D. dipsaci* (Onion Race) on different types of *Vicia faba* tissue six weeks after inoculation with 200 fourth-stage larvae (N = 8).

fourth-stage larvae to adults, but the steps from egg laying to fourth-stage were only slightly better than those on pure callus tissue (fig. 13). There appears to be a relatively higher proportion of second-stage larvae to other stages of the French Biotype relative to the California Biotype. This may indicate that the reduced reproductive capacity of the French Biotype was due, in part, to a hindrance of the step leading from the second to the third stage in the life cycle.

Analysis of the population structure of the Mexican Biotype on various types of tissue indicates that differences in the proportion of the various

stages were hardly significant (fig. 14). It is noteworthy that the proportions of the various stages in the population reared on seedlings grown on Henk's medium without 2,4-D (differentiated tissue) and that reared on pure callus (undifferentiated tissue) were essentially the same, but different from the other three tissue types which were also similar to each other. This is further confounded by the observation that the population increase achieved on stem and radical tissue, seedlings on Henk's medium, and seedlings on Henk's medium without 2,4-D is the same, while that on pure callus or cotyledons is appreciably lower.
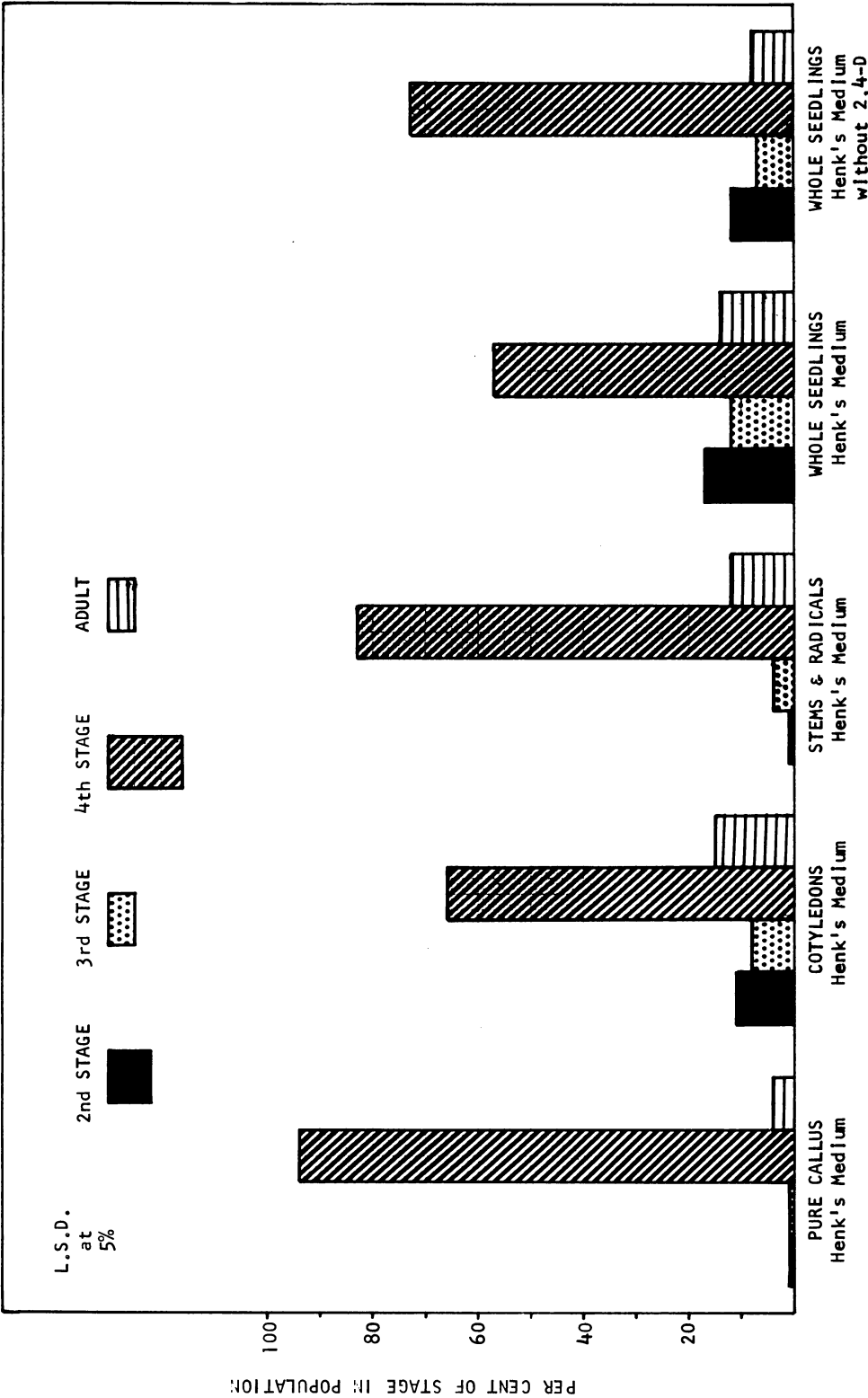
Fig. 12. The structure of *D. dipsaci* (Onion Race—California Biotype) populations on five different types of *Vicia faba* tissue six weeks after inoculation (N = 5).
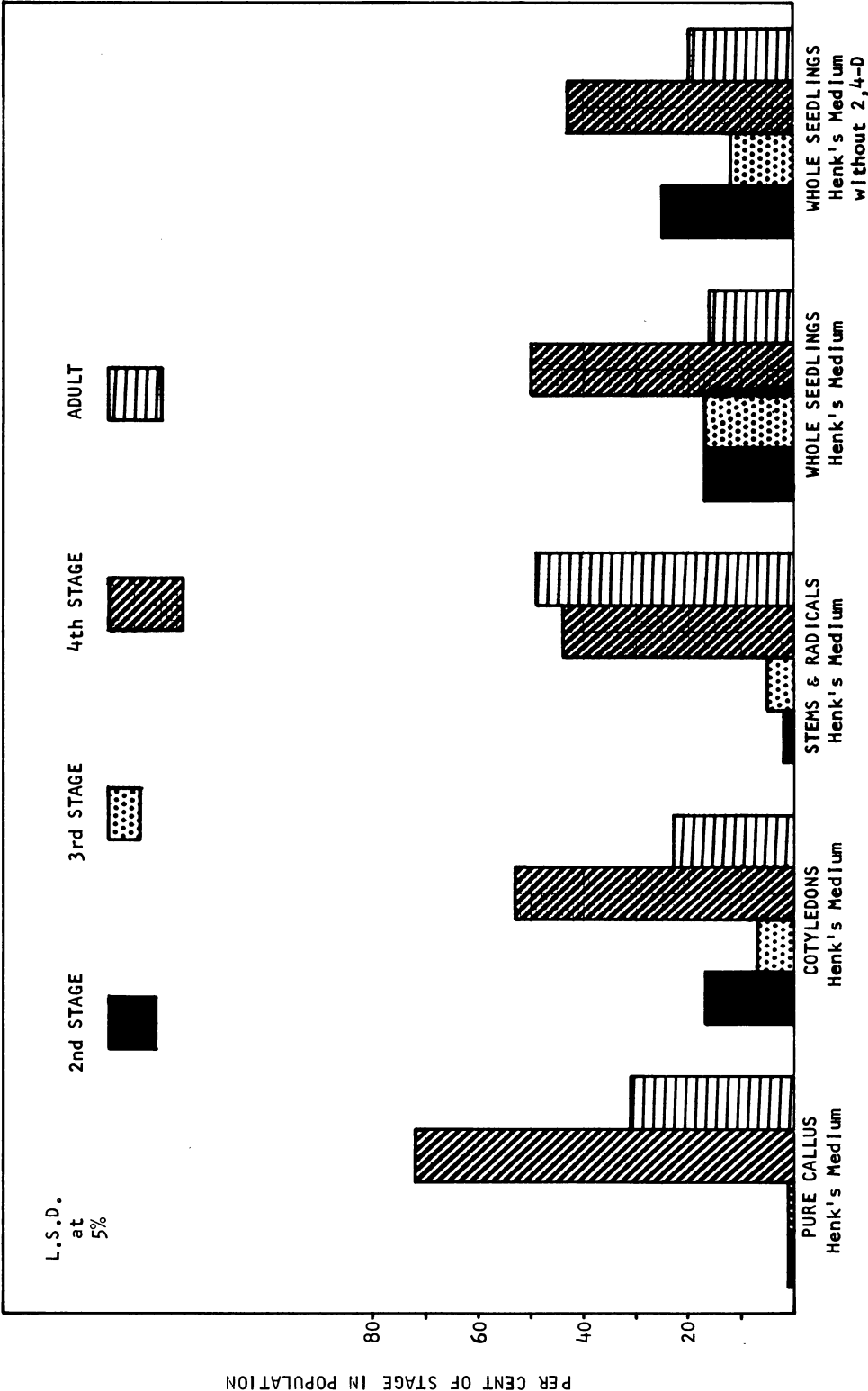
Fig. 13. The structure of *D. dipsaci* (Onion Race—French Biotype) populations on five different types of *Vicia faba* tissue six weeks after inoculation (N = 5).
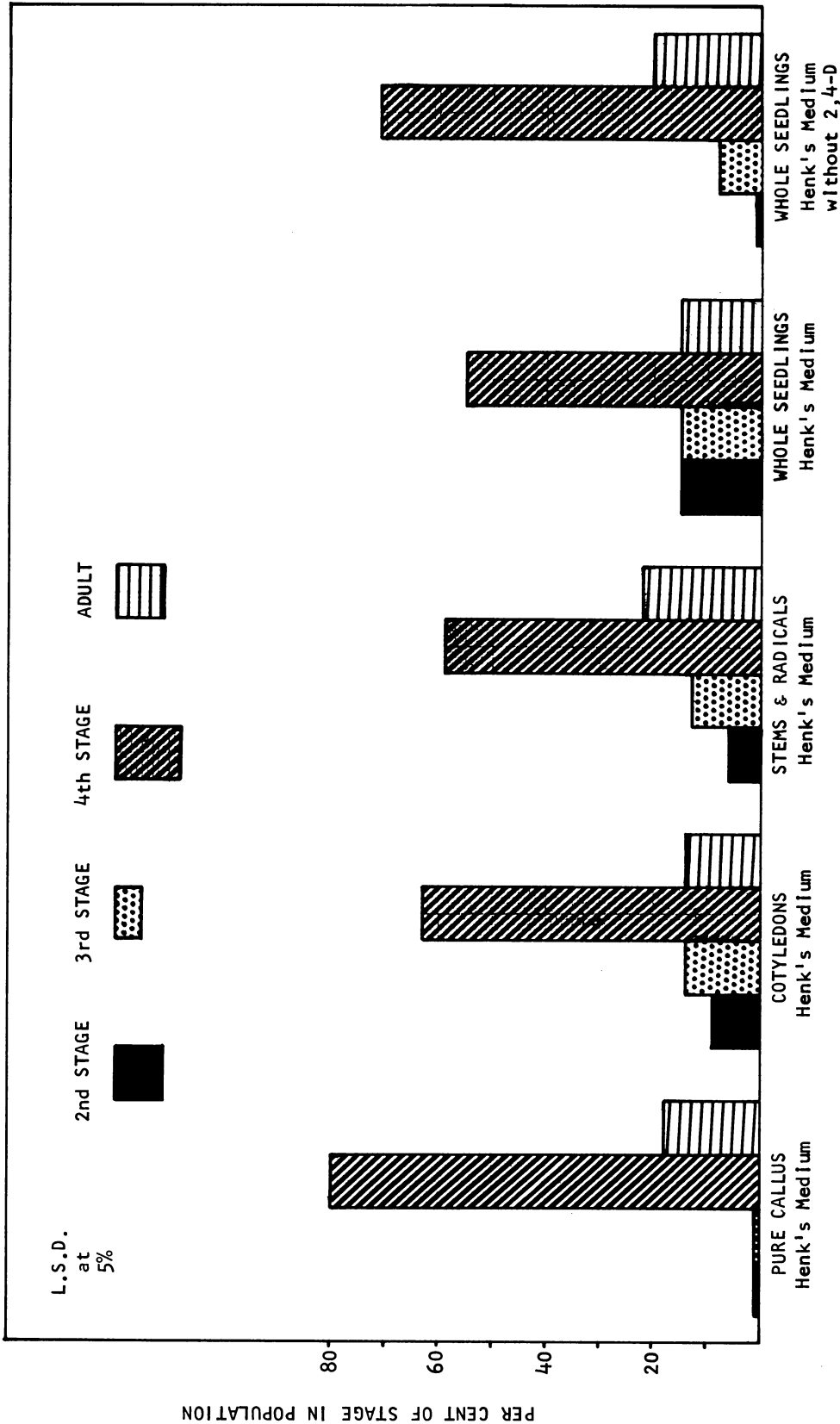
Fig. 14. The structure of *D. dipsaci* (Onion Race—Mexican Biotype) populations on five different types of *Vicia faba* tissue six weeks after inoculation (N = 5).
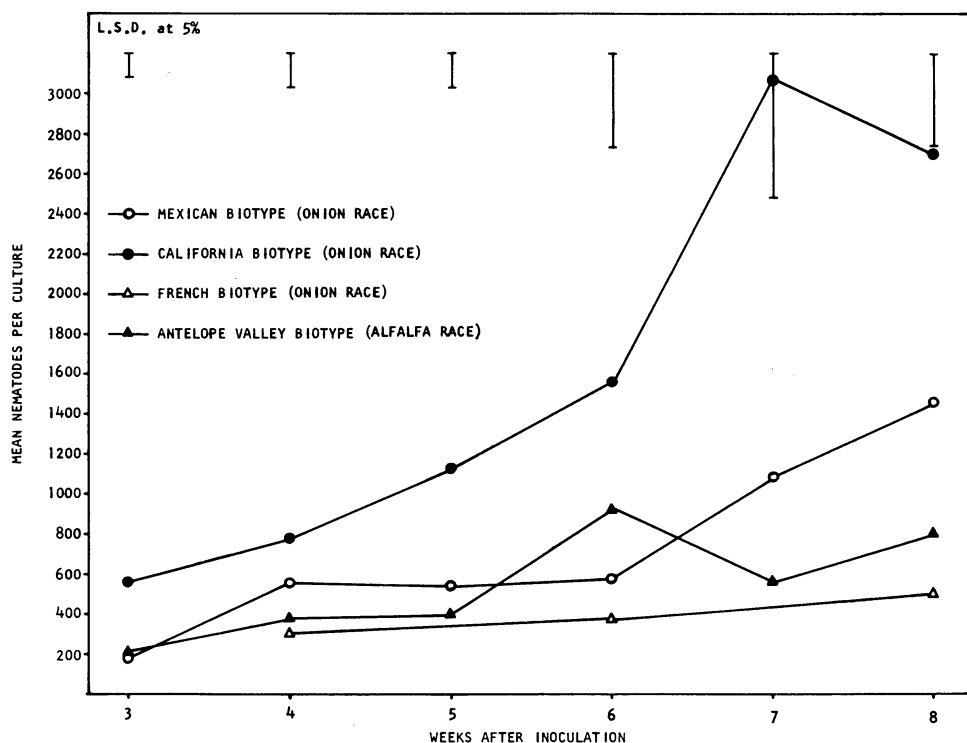
Fig. 15. The population increase of three biotypes of the Onion Race and a biotype of the Alfalfa Race of *D. dipsaci* on onion mixed tissue at different periods after inoculation.

On the basis of population structure analysis of the three biotypes of *D. dipsaci* on the five types of *Vicia faba* tissue, it appears that the French and California Biotypes behave similarly though differing in their physiological properties; both, however, are considerably different from the Mexican Biotype. If the three populations of *D. dipsaci* obtained from garlic of different geographic origins are considered to belong to one race, our data strongly suggest evidence of sub-racial characters in the different populations.

Population development of three biotypes of the onion race and a biotype of the alfalfa race on onion mixed tissue

The sub-racial differences observed in studies with *Vicia faba* tissue were investigated. Onion tissue, considered to be the "type host" of all the three

biotypes, was used, along with a biotype of the alfalfa race for purposes of comparison.

Mixed tissue of onion (variety White Globe) was grown by germinating axenized onion seeds on 1 per cent water agar. The seedlings were transferred to Henk's medium about seven days after germination. Within a week after transfer to the hormone-supplemented medium, the cultures had grown sufficiently to be ready for inoculation. Approximately 200 surface-axenized nematodes of mixed developmental stages were introduced into each culture, using a graduated microsyringe. Beginning with the third week, eight cultures of each of three biotypes were harvested every week until the eighth week, when their population levels and structures were determined. In the case of the French Biotype, the cultures were harvested
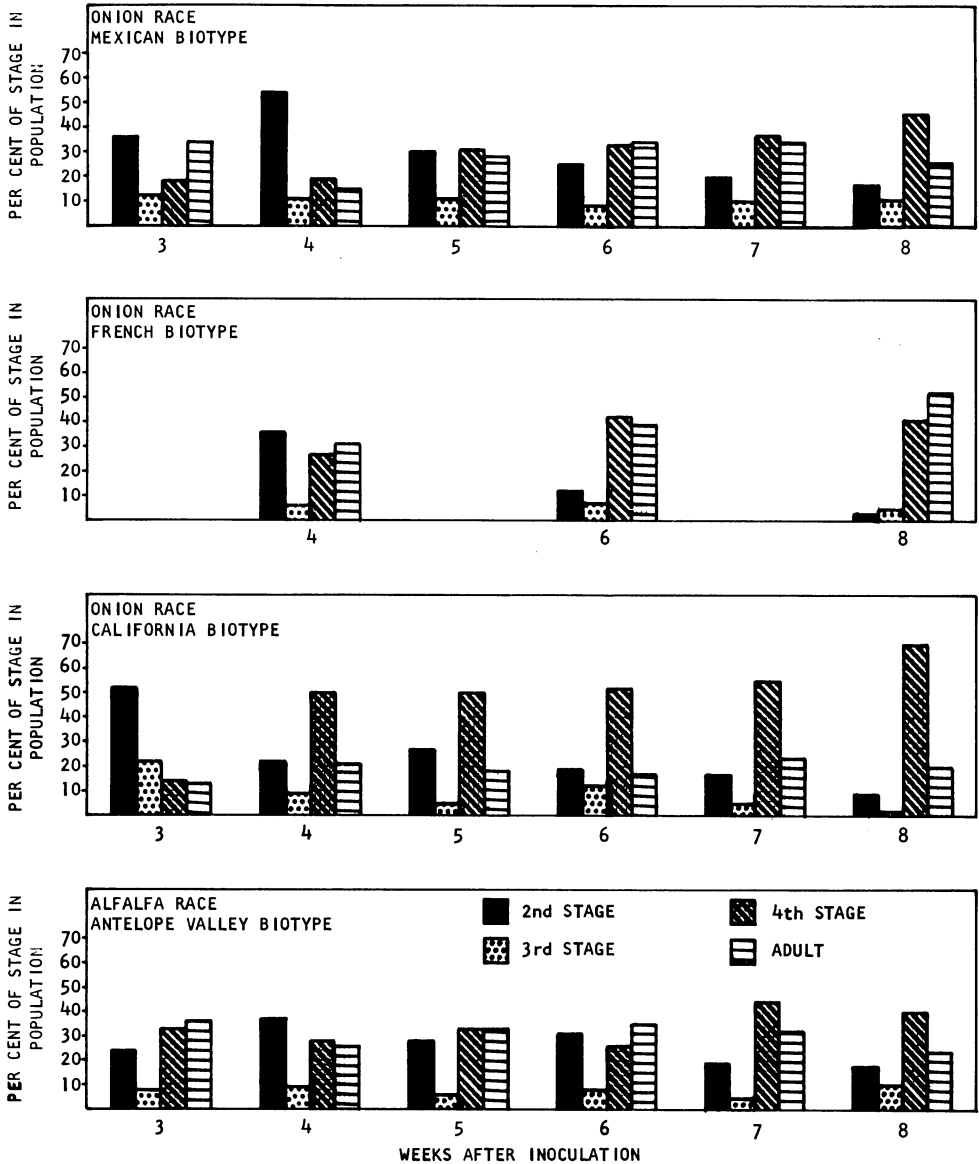
Fig. 16. Population structures of *D. dipsaci,* (three biotypes of the Onion Race and one biotype of the Alfalfa Race) on onion mixed tissue at different periods after inoculation. The proportion of each stage is indicated as per cent of total population (N = 8).

only four, six, and eight weeks after inoculation.

Population levels of the California Biotype were significantly higher than the other three biotypes tested on all harvest days (fig. 15). The population buildup of the California Biotype con-tinued until the seventh week and then declined. The French Biotype, which reproduced better on *Vicia faba* seed-lings than did the Mexican Biotype, reproduced only slightly on onion mixed tissue. Reproduction of the Mexican Biotype was moderate, and its

population levels did not display an upward trend until the seventh week after inoculation (fig. 15). Population levels of the Antelope Valley Biotype, isolated from alfalfa and considered to belong to the alfalfa race, were higher than those of the French Biotype of the onion race. The above data illustrate not only the intra-racial but also the inter-racial variations in *D. dipsaci*. They also raise some questions about the rigid separation of races on the basis of "type hosts."

Differences in population structures of different biotypes were difficult to assess precisely because the initial inoculum consisted of mixed developmental stages, including the eggs. Figure 16 shows the structures of all four biotypes tested on different harvest dates. The presence of second and third developmental stages of the French Biotype on the fourth and sixth weeks indicated some reproduction which, however, appears to be somewhat hindered between the second and third larval stage and in embryogenesis as visible in the old culture (fig. 16). The population structure of the Antelope Valley Biotype indicated a hindrance in the transition between the second and third larval stage but less obstruction in embryogenesis than the French Biotype. The Mexican Biotype population structure and development is similar to that of the Antelope Valley alfalfa race biotype. In contrast the California Biotype manifests the characteristics of a dynamic population.

GENERAL CONCLUSIONS

Axenization procedures reported in the literature should not be accepted without reservation. Though any recommended procedure may have been perfectly satisfactory for a given investigation, duplication can be precluded by differences in seed lots, areas of origin, and environmental factors during the growing season. Our investigations supported this view; for this reason, meticulous attention was paid to seed selection, different axenizing agents, and treatment periods. Hopefully, the procedures may serve as guidelines for the future.

Surface axenization of nematodes is relatively straightforward and presents little difficulty in cultures. Difficulties, however, usually arise when the plant-parasitic population is accompanied by appreciable numbers of free-living forms which usually harbor microorganisms within the gut. There is no reliable method by which field populations containing such forms may be axenized so that internal microorganisms can be eliminated. For occasional needs and small inocula, separation of the undesirable forms can be accomplished by manual picking. For larger inocula and routine large scale uses, other methods must be devised to remove the undesirable components. Separation methods based upon differential osmoregulatory properties, as reported in these investigations, may be used to advantage.

There appeared to be no perfect medium for tissue culture—particularly when a range of tissues were used. Compounding the problem was that as the tissue grew, the balance of components changed, with constituents being taken up at different rates and others released by the tissue. The effect upon nematodes of the changing solute composition of the environment were unknown. To reduce the confounding effects of these unknown factors, cultures were allowed to develop on one basic medium. When this commonly used medium produced unequal results, supplements were added—whereupon tissue growth rates were more than doubled. Even so, the experimental results indicated that the optimal conditions for each tissue

was other than that given by the supplemented medium which was adopted as providing acceptable growth support.

It has been well established that one "race" can attack, develop, and reproduce on the preferred host of other "races." Our results not only confirmed these observations, but further indicated that a selection process could increase uniformity in reproductive potential. This capacity has been demonstrated in *in vitro* cultures only, but there is no reason why it cannot occur under natural field conditions.

It has been established that the population structure of *D. dipsaci* is host-dependent; certain plant hosts can favor maleness, while others favor femaleness, and this bias need not be correlated with either high or low population levels. Furthermore, the factors effecting this bias towards maleness or femaleness are diffusible through a dialysis membrane and can persist in a medium for some time. Since these factors affect development, it would appear that they were manifested through host tissue.

The nature of plant tissue can be very important in nematode rearing. These investigations have shown that a population reared upon juvenile tissue suitable as a host and able to support good reproduction manifested a dynamic balance in the proportion of the individual stages. For example, Ladino clover mixed tissue one week old at time of inoculation promoted, after four weeks of culture, a population consisting of 5 to 7 per cent second-stage, 11 to 15 per cent third-stage, 42 to 54 per cent fourth-stage, and 28 to 37 per cent adults. On tissues older than two weeks at time of inoculation the proportion of second and third larval stages dropped sharply, while the proportion of adults increased. On pure callus the proportion of second and third stages was equally low to those tissues three weeks and older at time of inoculation. However, the proportion of adults to fourth-stage larvae was much lower than that developing on old tissues at time of inoculation. Inasmuch as the population levels upon tissues three weeks or older at time of inoculation, including pure callus, were very low, the life cycle block appeared to have occurred in embryogenesis.

Development of stages appeared to occur normally except in the case of pure callus where the conversion from fourth stage to adult was appreciably hindered. Clearly, for our *D. dipsaci* biotypes, differentiated tissue is far superior to undifferentiated callus for population growth and development. That is not to say that *D. dipsaci* biotypes of other origin cannot grow and develop on callus, nor that biotypes cannot adapt by selection to callus tissue. It has been shown that one biotype of *D. dipsaci* can be hindered at different steps in the life cycle depending upon the nature of the tissue in culture of either alfalfa or clover; a different kind of host tissue can hinder the life cycle at a different step. Broad bean able to support moderate reproduction of the California Biotype appeared to hinder the transition from third stage to fourth stage. On the other hand the proportion of stages in a dynamically growing population can be plant-tissue dependent, as for example, the California Biotype on tissues of *Vicia faba*. In this case, the population structures were essentially the same, though the population levels differed greatly. The reduced reproduction rates appeared to be the result of a general nonspecific hindrance to all steps of the life cycle.

The population level and population structure of different biotypes of the same race on tissues of the same origin, for example, *Vicia faba,* were as variable as one biotype on different kinds of tissue. The Mexican Biotype with a

very low reproduction rate had a population structure more comparable to the California Biotype, which had up to a twenty-five-fold greater reproductive rate. The French Biotype with an intermediate reproductive rate had a population structure that differed from the other two. When a biotype of the alfalfa race was compared to three biotypes of the onion race on type onion tissue, it manifested many characteristics in reproduction and structure similar to several of the onion race biotypes. There is some indication of hindrance in the transition from second stage to third stage of the alfalfa race biotype on onion, as there

was with the California onion race biotype on broad bean.

These investigations illustrate the "inter-racial" and the "intra-racial" characteristics and differences of only a small sector of the nominal species, *D. dipsaci* host-parasite complex. In view of these studies the concept of race as commonly interpreted has limited usefulness, and its designation on the basis of host preference is not only unwarranted but misleading. It would seem that the commonly accepted views of the nominal species, *D. dipsaci,* merits some skepticism and possibly reexamination and reinterpretation.

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