Development of Heterodera glycines Ichinohe on Soybean, Glycine max (L.) Merr., under Gnotobiotic Conditions[1]

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Abstract: The life cycle of the soybean cyst nematode, Race 3 (SCN 3), *Heterodera glycines* Inchinohe was determined from observations of the developmental stages on soybean *Glycine max* cv. Kent root explants under gnotobiotic conditions at 25 C. Approximately 51% of the second-stage larvae penetrated the root 1 day after inoculation (DAI). Third-stage larvae appeared 4 DAI, became sexually differentiated 5 DAI, and protruded from the root tissues 6 DAI. Fourth-stage males and females were observed 7 DAI. Ensheathed adult males were observed at 9 DAI and exsheathed to free adults at 11 DAI. The fourth-stage female became an adult at 10 DAI. Males entwined around the gelatinous sac of the female at 12 DAI and were assumed to be mating. Some males actually penetrated and were enveloped by the gelatinous sac. The female-to-male sex ratio ranged from 2.3 to 9.5:1. First- and second-stage larvae were observed in the egg 17 and 19 DAI, respectively. The life cycle of the SCN 3 was completed 21 DAI upon hatching of the eggs and emergence of second-stage larvae. The average number of eggs in the cyst body and gelatinous sac, was 210 and 187, respectively. *Key words:* reproduction, soybean cyst nematode, scanning electron microscopy.

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The life history of the soybean cyst nematode (SCN), *Heterodera glycines* Ichinohe (7), has been studied by Ichinohe (8,9) and Skotland (22,23). Originally, Ichinohe (9) reported that the length of one generation (number of days from larval invasion to the first sign of the embryonated egg within the sac) of the SCN was 24 days at temperatures ranging between 19.2 and 26.5 C with an average of 23.3 C. Skotland (23) determined that one generation (from host invasion by second-stage larvae (L2) to emergence of L2) of the SCN was 21 days when reared on soybeans at 23 C. Neither investigator presented a complete account of the SCN life cycle. Although Ichinohe (9) determined the number of days required for the development of each life stage, he did not indicate the molting periods nor the time required for the embryo to complete its development and emerge from the egg as an infective L2. Skotland (23), on the other hand, did not observe the fourth molt (M4) of the female, nor the first molt (M1) of the first-stage larva (L1).

A study was undertaken to determine the life cycle of the SCN through direct observations of developmental stages on axenic soybean root explants under controlled environmental conditions. The methodology, application, and significance of phytonematode gnotobiology was reviewed by Zuckerman (27). In the subfamily Heteroderinae (gen. *Globodera*, *Heterodera*, and *Punctodera*), monoxenic cultures were reported for the following species: *H. avenae* Woll. (2), *G. rostochiensis* (Woll.) Behrens (26), *H. glycines* Ichinohe (13,14), *H. oryzae* Luc and Berdon-Brizuela (20), and *H. schachtii* Schmidt (11,12,17,18). Of these species, only the life cycle of *H. schachtii* has been documented from observations of gnotobiotic cultures (11). In this study, we describe the development of *H. glycines* in monoxenic cultures using light (LM) and scanning electron microscopy (SEM).

MATERIALS AND METHODS

Specimens: Seeds of *Glycine max* (L.) Merr. cv. Kent were obtained from seed stocks at ARS, USDA, Beltsville, Maryland. Axenic L2 of the SCN, Race 3 (SCN 3) were obtained from previously established monoxenic root explant cultures (13,14).

Inoculation of root explants: The L2 inoculum was prepared by aseptically transferring ca. 30 gravid yellow females with egg masses from stock cultures to sterile screw-capped culture vials (15 × 55 mm) containing 4 ml of sterile tap water. After

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incubation for 24 h at 25 C, eggs hatched to yield a concentration of ca. 500 L2/ml. Potato dextrose agar (Difco) plates were inoculated with aliquots of the larval suspension to test for sterility.

Kent axenic root explants were grown in petri dishes (one explant/dish) on holidic agar medium (Table 1) in an environmental growth chamber at 25 C in the dark. Three-day-old root explants were inoculated with a 0.1 ml suspension of L2 (50 ± 2) using a sterile glass pipet. The number and viability of the L2 was confirmed with the aid of a stereomicroscope. After allowing 24 h for L2 penetration, the root systems were removed intact and transferred to petri dishes with fresh medium (Table 1) to prevent further L2 infection. The petri dishes were sealed with Parafilm and incubated as described above.

p*H determination:* The pH of the agar medium was determined from three replicates of SCN 3 infected Kent root cultures using a combination pH surface electrode.

Microscopy: Three replicates of SCN 3 infected root cultures were examined at daily intervals. Roots systems were stained for 5 min in a boiling solution of acid fuchsin in lactophenol (16). Stained nematodes were excised from root tissues and examined with a compound light microscope to determine the sex and stage of development.

For SEM, SCN 3 infected roots were fixed by flooding the petri dishes with 5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 24 h at 25 C. Infected root segments were removed from plates, washed three times for 15 min each and postfixed with 2% OsO_4 in the same buffer for 3 h at 25 C. Fixed tissues were dehydrated in a graded ethanol series and critical point dried using CO_2. Specimens were mounted on aluminum stubs, sputter coated with gold-palladium, and examined with a Hitachi SEM S-430 at 10 or 15 KV.

RESULTS

The described conditions and composi-

Table 1. Composition of the holidic agar medium formulated by Skoog, Tsui, and White (10) and used in this study.

COMPONENTS		COMPONENTS	
Macronutrient salts (Stock A):		Micronutrient salts (Stock C):	
Solution I		$ZnSO_4 \cdot 7H_2O$	0.027 g
$Ca(NO_3)_2 \cdot 4\ H_2O$	1.44 g	$MnSO_4 \cdot H_2O$	0.049
KNO_3	0.80	H_3BO_3	0.016
KCl	0.65	KI	0.0075
KH_2PO_4	0.38	Distilled H_2O q.s.	100 ml
NH_4NO_3	0.40	Store @ 5°C	
Distilled H_2O q.s.	800 ml	Vitamins and Amino Acids (Stock D):	
Solution II		Nicotinic acid	0.050 g
$MgSO_4 \cdot 7H_2O$	0.72 g	Pyridoxine HCl	0.075
Distilled H_2O q.s.	200 ml	Thiamine HCl	0.010
Combine solutions I and II to make 1L of Stock A, store @ 5°C.		Sodium glycinate	0.262
		Distilled H_2O q.s.	100 ml
Iron (Stock B):		Filter sterilize Stock D, store 1.0 ml aliquots @ −20°C.	
$FeSO_4 \cdot 7H_2O$	0.56 g		
Na_2EDTA	0.74		
Distilled H_2O q.s.	100 ml		
Aerate Stock B for 24 hrs., store in dark bottle @ 5°C.			

To make 1L of nutrient agar medium, combine 100 ml of Stock A, 5 ml of Stock B, 10 ml of Stock C, 20 g of sucrose, 15 g of Difco Noble agar and distilled H_2O q.s. 1000 ml. Autoclave medium @ 15 p.s.i. for 15 min. Add 1 ml of Stock D and adjust pH with acid or base to 5.6–5.8 after medium has cooled to about 60 C.

tion of the medium (Table 1) supported prolific growth of the axenic soybean root explants and the complete development of SCN 3 as shown in Fig. 1. The L2-to-L2 life cycle in monoxenic culture was completed 21 days after inoculation (DAI), and the development, which consisted of four molts and five stages, was typical of bisexual endoparasitic phytonematodes. A diagramatic representation of the SCN 3 life cycle under gnotobiotic conditions is shown in Fig. 2.

Growth medium: The pH of the growth medium significantly ($P = 0.01$) decreased during the first 7 DAI, whereas the pH differences during the remainder of the life cycle were not significant (Table 2). The decrease in pH may have resulted from ammonium absorption which would cause an acid drift in the medium (21). However, the low pH did not prevent SCN 3 development in root explants, and reinfection and nematode maturation occurred in cultures in which the pH had decreased to approximately 4.8.

Penetration: In vitro observations of axenic root explants 1 DAI revealed small necrotic lesions along the main and lateral roots. The posterior portion of the L3 were later seen protruding from the root where these lesions occurred. Observations by SEM of intact roots showed that some L2 were still in the process of penetration (Fig. 3). Examination by LM of stained roots 1 DAI indicated that 42–61% ($\bar{x}$ 51%) of the L2 penetrated the root system including the tap root.

Third stage: Observations by LM stained roots revealed that the L2 molted (M2) within the root tissues between 3 and 4 DAI. Third-stage larvae (L3) could not be sexually differentiated on the basis of gonad development until 5 DAI. As shown by SEM (Fig. 4), swollen L3 protruded from the roots 6 DAI causing relatively large root lesions through rupture of the cortical and epidermal layers.

Fourth stage: Molting (M3) of the L3 occurred between 6 and 7 DAI. The sex of

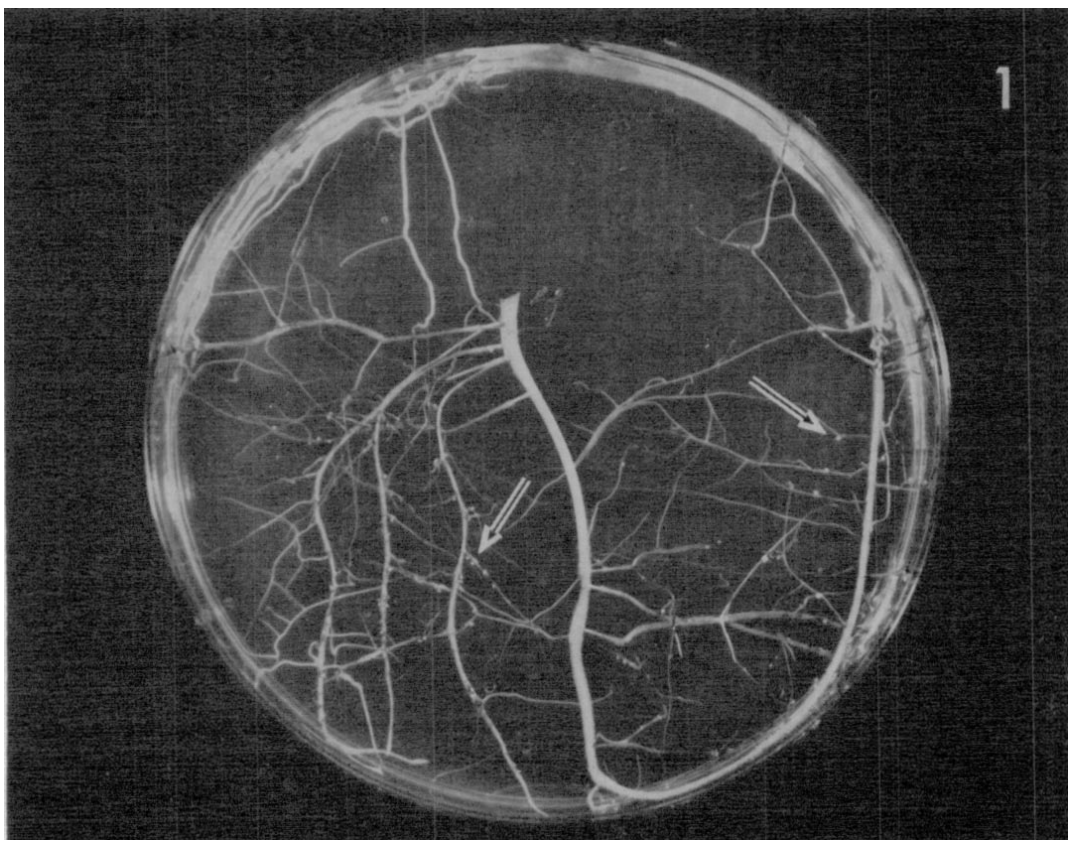


Fig. 1. Monoxenic culture of *Heterodera glycines*, Race 3, on a *Glycine max* cv. Kent root explant showing nematode infected root system (arrows) 21 days after inoculation. × 1.1.

Table 2. Relationship between pH of the holidic agar medium and days of growth of soybean cyst nematode infected 'Kent' root explants incubated in the dark at 25 C.

Days	Average pH
0	5.66 a*
3	5.36 b
7	5.18 c
14	4.91 d
21	4.88 d
28	4.81 d

*Numbers followed by a letter in common are not significantly (P = 0.01) different according to Duncan's multiple-range test.

the fourth-stage larvae (L4) could be determined on the basis of body shape. SEM micrographs showed that the L4 males were more elongated (Fig. 5) than the more oval-shaped L4 females (Fig. 6).

Adult stage: Sexual development diverged with the onset of the fourth molt (M4). The L4 males molted between 8 and 9 DAI but remained ensheathed as adults within the third-stage cuticle, whereas the L4 females molted between 9 and 10 DAI. Adult white females at 10 DAI formed gelatinous sacs at their posterior (Fig. 7), and the sacs continued to enlarge prior to mating (Fig. 8). Evidence of male exsheath-

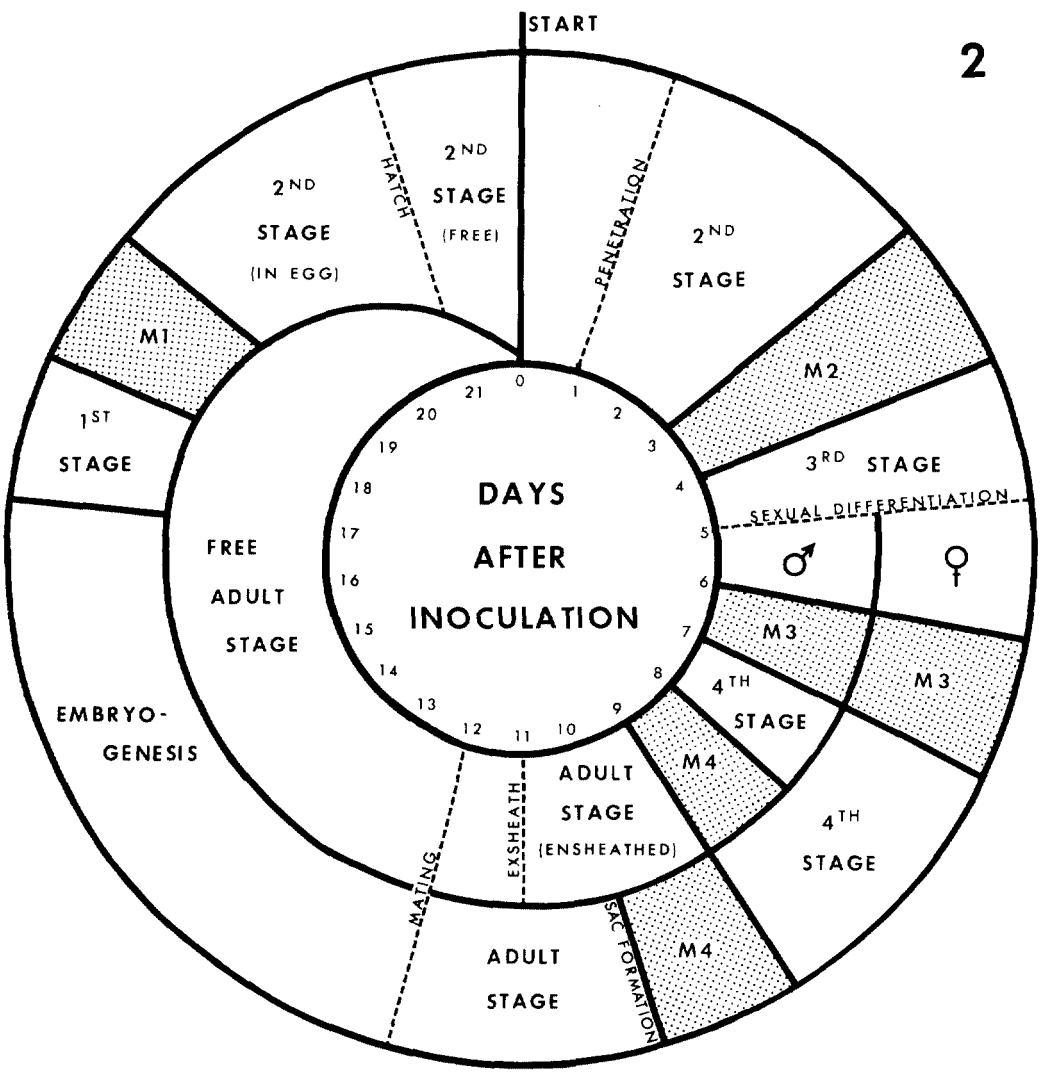
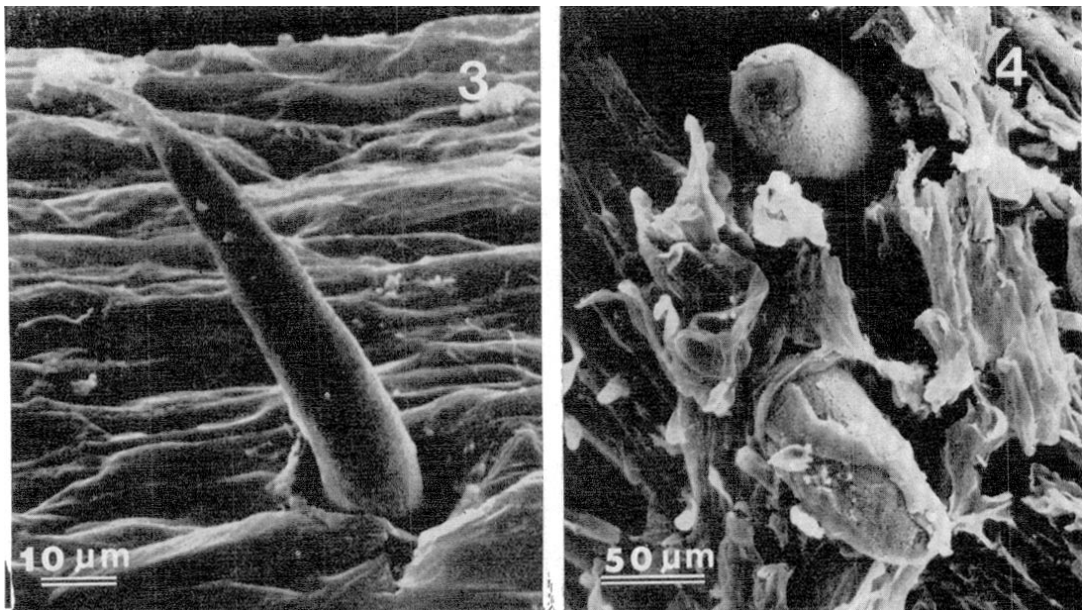


Fig. 2. Schematic representation of the life cycle of *Heterodera glycines*, Race 3, derived from growth and development on *Glycine max* cv. Kent axenic root explants incubated in the dark at 25 C.



Figs. 3–4. SEM micrographs of *H. glycines*, Race 3, on *Glycine max* cv. Kent root explants. 3) Second-stage larva (L2) invading the root 1 day after inoculation (DAI). 4) Swollen third-stage larvae (L3) protruding from the root 6 DAI.

ment was found 11 DAI with the appearance of free adults on the agar surface. The adult female-to-male ration ranged from 2.3:1 to 9.5:1 and averaged 6.3:1. At 12 DAI, males were observed in the process of mating; they were entwined around the gelatinous sac of the female. It was difficult to determine from LM observations whether the male entered the sac; however, SEM observations revealed that some adult males were completely enveloped by the gelatinous sac (Fig. 9). Occasionally, several males were observed around a single adult female.

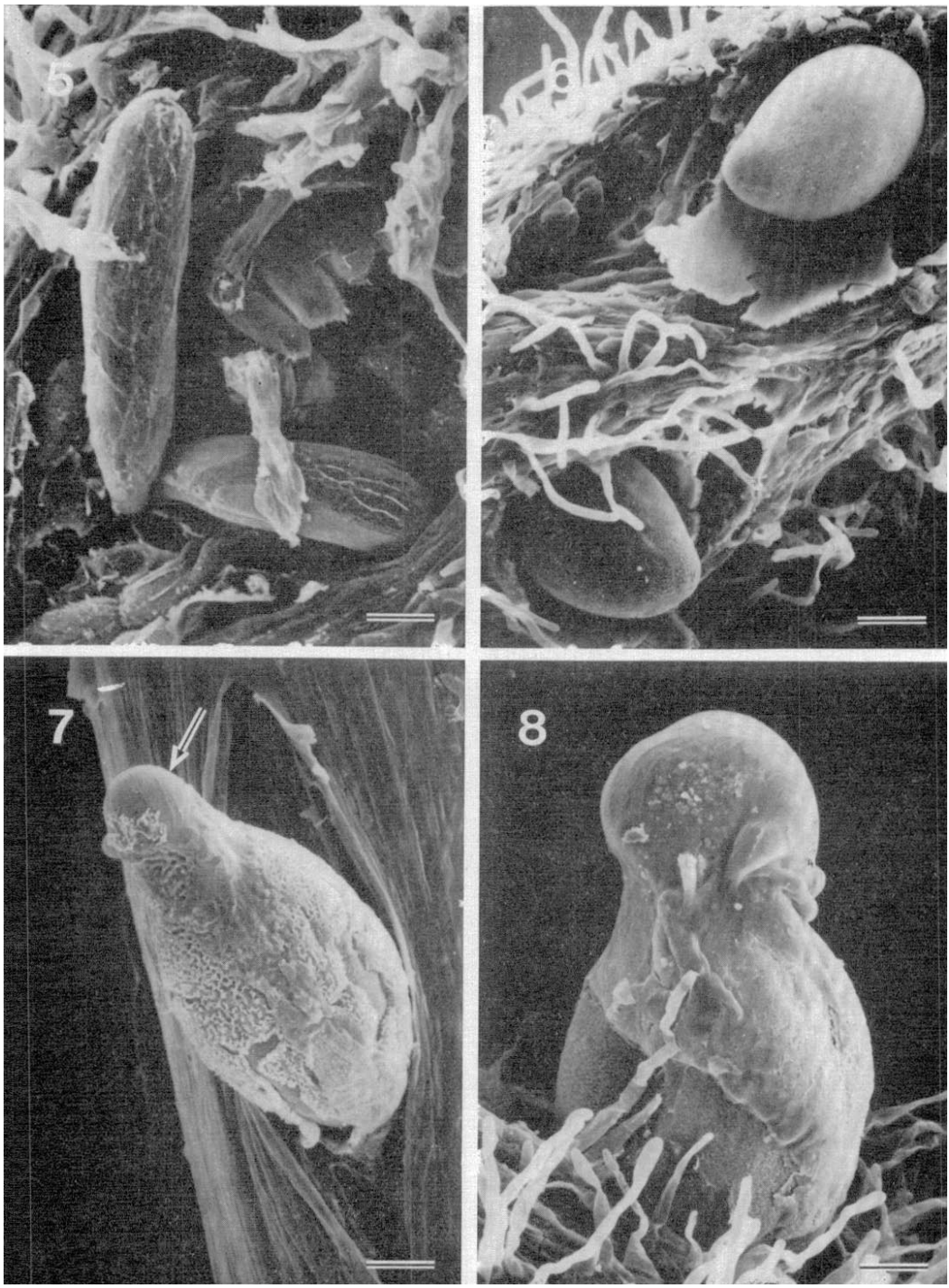
Embryogenesis: Unembryonated eggs were first observed in the body of white females 12 DAI and within the gelatinous sac at 15 DAI. A SEM micrograph of a gravid female at 16 DAI shows eggs within the gelatinous sac (Fig. 10). By 18 DAI, the sac and body were filled with eggs, some of which were fully embryonated. The first molt (M1) of the first-stage larva (L1) was observed 18 DAI. The color of the female during this period was distinctly yellow. Hatching of eggs and emergence of the L2 was observed 21 DAI, thereby completing the L2-to-L2 life cycle.

Cyst stage: The first brown cyst was observed 27 DAI, but the majority of adult females remained yellow for several additional weeks. The total number of eggs within the cyst body ranged from 161 to 291 (average 210), and the total number of eggs within the gelatinous sacs of cysts ranged from 119 to 273 (average 187).

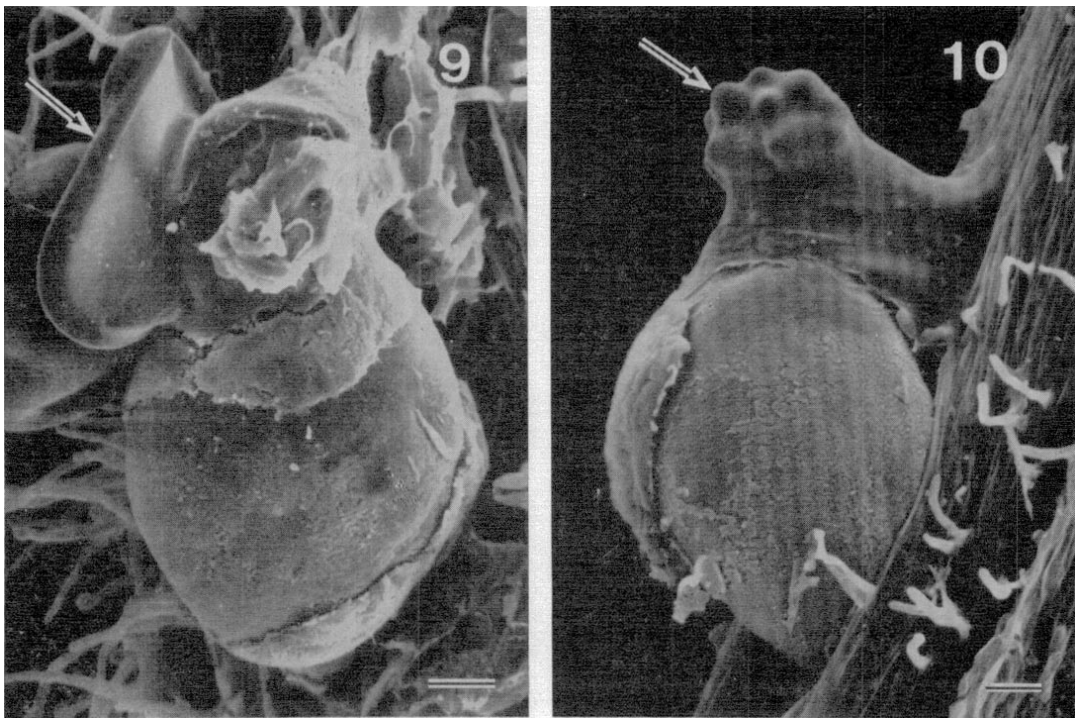
DISCUSSION

The percentile range of L2 that penetrated soybean root explants (42–61%) was slightly higher than reported by Johnson and Viglierchio (11) for L2 of *H. schachtii* penetrating sugar beet root explants (35–45%). Some females which formed within clusters on root explants never developed, possibly a result of competition for plant nutrients. Some positive features that arise from females developing in aggregates are greater capability of sexual reproduction among sparse populations (25) and enhanced attraction of males (6).

The average female-to-male sex ratio of 6.3:1 was considerably higher than the 2.3:1 reported for *H. schachtii* (12). Variation in the numbers of adult females among replicates partly contributed to the wide range of 2.3:1 to 9.5:1. Error in counting free adult males may also have been a factor, since males were easily obscured by the root system. As previously reported (15), the number of adult males does not differ



Figs. 5–8. SEM micrographs of *Heterodera glycines*, Race 3 developing on *Glycine max* cv. Kent axenic root explants. 5) Elongated fourth-stage (L4) males 7 days after inoculated (DAI). 6) Oval-shaped fourth-stage (L4) females 7 DAI. 7) Adult female 10 DAI showing sac formation (arrow) at the vulval end. 8) Adult female with an enlarged sac 12 DAI. Bar equals 50 um.



Figs. 9–10. SEM micrographs of adult females of *Heterodera glycines,* Race 3, on *Glycine max* cv. Kent axenic root explants. 9) Adult male within the female egg sac (arrow) 15 days after inoculation (DAI). 10) Adult female with sac containing eggs (arrow) 16 DAI. Bar equals 50 um.

significantly ($P = 0.01$) among susceptible and resistant soybean cultivars. Johnson and Viglierchio (12) also reported that the percentage of developing males of *H. schachtii* on exercised *B. vulgaris* roots was relatively constant and that the number of adult females fluctuated in relation to host nutrition. Because of the high female-to-male sex ratio, male promiscuity undoubtedly occurs as reported for *H. schachtii* and *G. rostochiensis* in which a male inseminates up to 10 females (5).

The first observation of free adult males and mating was at 11 and 12 DAI, respectively, which was 2 days earlier than previously reported (13,23). Ichinohe (9) first observed SCN males within the gelatinous sac of adult females, and the role of males in sexual reproduction has been well established (4,24). Our results confirm, that the SCN males do enter the female gelatinous sac (Fig. 9), which in its hydrated state has been described as an irregular glycoprotein meshwork (1,3). However, we cannot unequivocally state that the entry of a male into the gelatinous sac is essential for the insemination of the female. It is possible that males inadvertently became enveloped and trapped within the gelatinous sac.

Gravid females in monoxenic cultures contained an average of 210 eggs per cyst, which is comparable to the average of 262 per cyst in pot cultures (9). The average total number of eggs produced by a single female (cyst body and sac) in monoxenic culture was 397, which falls within the range of 228 to 564 total eggs per female reported by Ichinohe (9).

A schematic interpretation of the life cycles of *H. glycines* and *H. schachtii* derived from pot cultures of Ichinohe (9) and Raski (19), respectively, and monoxenic cultures of the authors and Johnson and Viglierchio (11), respectively, is shown in Fig. 11. Evaluations of the life cycles determined from different environmental conditions may not be totally valid; however, some observations seem appropriate. The minimum number of days required for the completion of each developmental stage of *H. glycines* (as reported by Ichinohe [9]) is longer than compared with monoxenic cultures (Fig. 11). Soil temperature affects

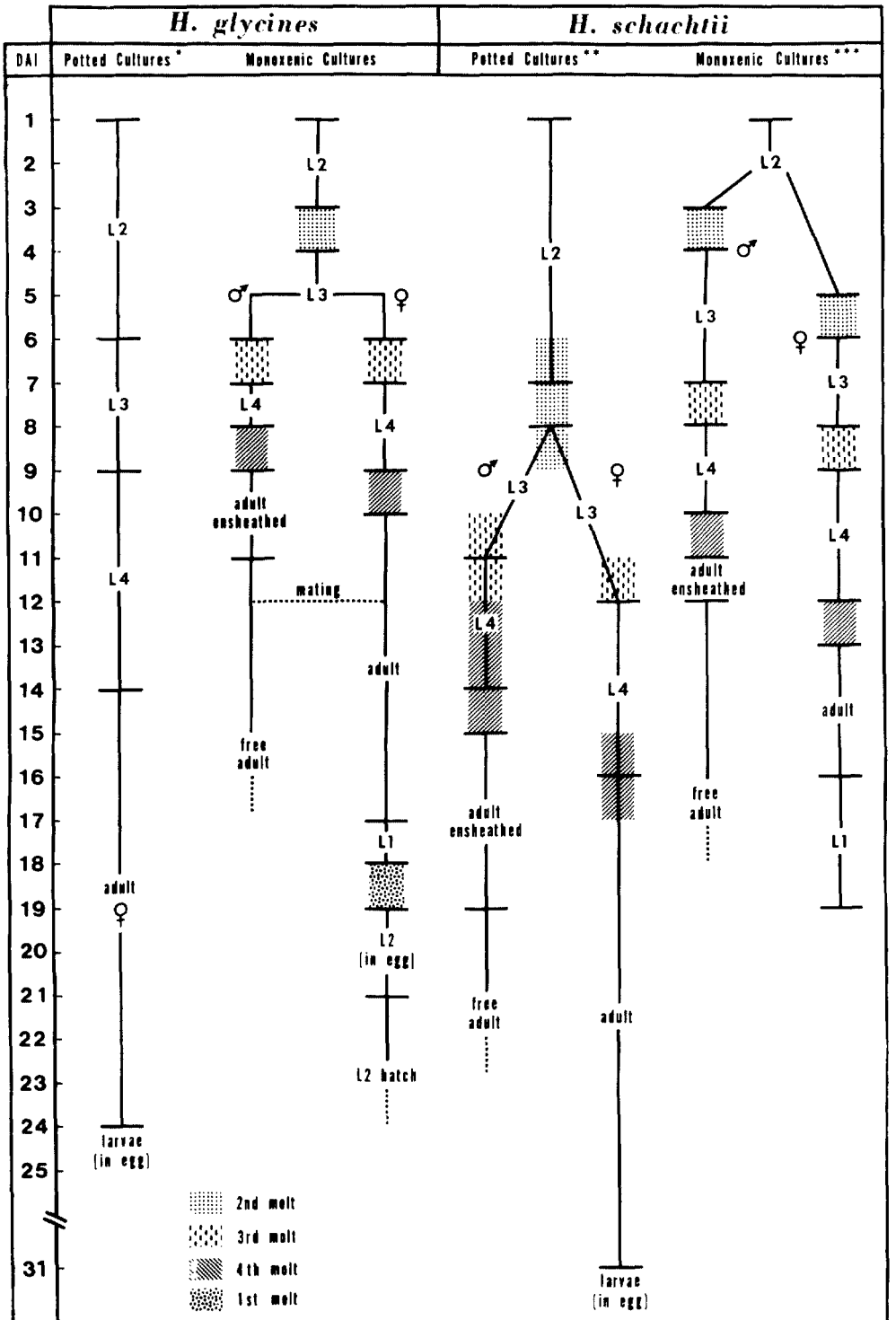


Fig. 11. Schematic interpretation of the development of *Heterodera glycines*, Race 3, and *Heterodera schachtii* under greenhouse and gnotobiotic conditions. *Derived from Ichinohe (9); ** from Raski (19) and *** from Johnson and Viglierchio (11).

the rate of development of the SCN: the lower the temperature the slower the rate of development (9). The low soil temperature recorded by Ichinohe (9) may explain why the generation time was considerably longer than observed in monoxenic cultures. Skotland's (22,23) life history study of the SCN, which was conducted at 24 C, also showed shorter times for development than reported by Ichinohe (9). Although the generation time of 21 days observed by Skotland (23) coincides with the results of the present study, the molting periods were 1 day later than in monoxenic cultures. A similar evaluation of life cycles of *H. schachtii,* determined from sugar beet pot cultures grown at 19.3 C (19) and from monoxenic cultures at 25 C (11), also shows that generation time and duration of each life stage is shorter in monoxenic cultures (Fig. 11). The more rapid nematode development of *H. schachtii* in monoxenic cultures was attributed to the control of environmental factors such as elevated constant temperature and abundance of nutrients (12). In addition, the absence of predators, parasites, and competitors in axenic root cultures would also promote optimal conditions for nematode development.

Comparison of the life cycles of the closely related species *H. glycines* and *H. schachtii,* as determined from monoxenic cultures incubated at 25 C, indicates that there are slight differences in the duration of developmental life stages (Fig. 11). The environmental conditions for phytonematode development were similar except for the host and holidic medium used in each study. Therefore, the differences in the duration of the developmental stages may be attributed to differences in the composition of the environment and/or the esoteric characteristics of the species.

Further studies will be directed toward the isolation and chemical characterization of the SCN female pheromones with the objective of nematode control through interference of pheromone-mediated communication between sexes.

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