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Title: RELATIONSHIPS OF SIX SAPROZOIC NEMATODES AND A NITROGEN-FIXING BACTERIUM, RHIZOBIUM JAPONICUM (KIRCH.) BUCHANAN

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Relationships of six species of saprozoic nematodes, Pristionchus lheriteiri (Maupas, 1919) Paramonov; Panagrolaimus subelongatus (Cobb, 1914) Thorne, 1937; Cephalobus persegnis Bastian 1865; Pelodera (Rhabditis) lambdiensis (Maupas, 1919) Dougherty, 1953; Rhabditis sp. I and Pelodera chitwoodi (Bassen, 1940) Dougherty, 1955 and a nitrogen-fixing bacterium, Rhizobium japonicum (Kirchner, 1895) Buchanan, 1926 were investigated. These nematodes were found reproducing, feeding on cultures of R. japonicum grown on Rhizobium agar medium but the density of the populations varied, depending on the species of nematode. It was demonstrated that these nematodes could carry the bacteria both in the alimentary canal and on the body surface. The average numbers of bacterial cells carried internally, ranging from 12 cells in Rhabditis sp. I to 9,034 cells in

P. lheriteiri, did not correlate with nematode body size. By contrast, the numbers carried externally, ranging from 100 cells on Rhabditis sp. I to 37,776 cells on Pelodera chitwoodi, had a direct relationship.

The relationship between P. lheriteiri and R. japonicum was studied in detail. The saprozoic nematode, P. lheriteiri, could not survive and reproduce on Rhizobium agar medium containing R. japonicum culture broth or an inoculum that were previously autoclaved or sonicated but only could survive if the food source was a living bacterial suspension. Females of P. lheriteiri were found to carry 2,622 bacterial cells in the alimentary tract while only 1,004 cells could be found in males. The bacteria still were viable after they were ingested and defecated by the nematodes.

The nodule-forming ability of the bacteria, after passing through nematode alimentary tract, did not change. Bacteria carried internally by the nematode could develop nodules on Lee soybean, Glycine max (Linn.) Merr., roots in water agar as well as those in suspension. There was no relationship between numbers of nodules and numbers of bacteria-carrying nematodes in any treatment. Nodules were small in size, ranging from 0.5-2.0 mm in diameter, and few in number per plant. Sixty percent of the soybean plants, including 13 percent in the control treatment which were inoculated with a bacterial suspension, formed nodules on roots 30 days after inoculation. Almost 92 percent of nodules were found on or near plant hypocotyls; only

8 percent of the total nodules were seen deep in the agar medium.

This investigation indicates that many kinds of saprozoic nematodes can become carriers and disseminators for an agriculturally beneficial bacterium, R. japonicum. P. lheriteiri, Panagrolaimus subelongatus, Pelodera chitwoodi and Pelodera (Rhabditis) lambdiensis were demonstrated to be efficient bacterial vectors. These nematodes, including Rhabditis sp. I and Cephalobus persegnis could carry the bacteria internally as well as externally but this capability varied with the species of nematode.

Relationships of Six Saprozoic Nematodes and a Nitrogen-Fixing Bacterium, Rhizobium japonicum (Kirch.) Buchanan

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TABLE OF CONTENTS

TABLE OF CONTENTS (Cont.)

LIST OF FIGURES

LIST OF TABLES

RELATIONSHIPS OF SIX SAPROZOIC NEMATODES AND A NITROGEN-FIXING BACTERIUM, RHIZOBIUM JAPONICUM (KIRCH.) BUCHANAN

INTRODUCTION

Nematodes constitute one of the largest and most ubiquitous group of the Animal Kingdom. Macfadyen (53) modified the estimate of Stöckli (81) and claimed that there were approximately 1.8-200 million nematodes per square meter of European grassland. Nematodes can be found in almost every type of the environment. Among them, some are considered important because of their parasitic roles to men, animals and crop plants. Many nematodes are free-living in soils, fresh and marine waters. Major groups of omnivorous nematodes, associated mainly with organic decay, are called saprozoic nematodes.

Terminology for relationships of nematodes and other kinds of microorganisms varies, depending on individual preference. Interactions, relationships, interrelationships, and disease complexes are some examples. There were, in 1972, more than 850 publications found related to these subjects (36). The interrelationships of plant parasitic nematodes and pathogenic fungi, bacteria, viruses and even other nematodes are now widely studied. Investigations on the relationships of saprozoic nematodes, plant parasitic nematodes and nitrogen-fixing bacteria, Rhizobium spp., have already been reported

by many people (6, 7, 8, 39, 83).

Studies of interrelationship of saprozoic nematodes and microorganisms started in 1933 when Steiner (79) reported that a saprozoic nematode, Pelodera (Rhabditis) lambdiensis (Maupas, 1919) Dougherty, 1953, carried a mushroom pathogen in its pharynx and intestine. Since then, a number of reports on saprozoic nematodes and pathogenic fungi, bacteria (16, 40), Streptomyces (42), algae (49), mycoplasma (45) and nitrogen-fixing bacteria (39) appeared. Bacteria, Salmonella spp., were found to be spared from chlorination while ingested and viable after defecation by a saprozoic nematode species (77).

Six kinds of saprozoic nematodes used in this study are usually found associated with organic decay. For years, they have been successfully cultured on agar plates containing bacteria. Pristionchus lheriteiri (Maupas, 1919) Paramonov is the nematode that has been most widely used to study interrelationships with other microorganisms.

This research was designed to: (1) investigate survival of six kinds of saprozoic nematodes on cultures of Rhizobium japonicum (Kirch.) Buchanan, (2) determine the capability of nematodes to multiply on the bacterial culture; (3) to assess the number of bacteria carried, internally and externally, by various kinds of nematodes; (4) study viability of bacteria after ingestion and defecation by nematodes; (5) determine relationships of the number of bacteria carried

by nematodes and number of nodules formed on soybean roots; and (6) study the bacterial food source of P. lheriteiri.

LITERATURE REVIEW

Naturally, coexistence in soil by different microorganisms and other nematodes occurs universally. In the same environment, activity or survival of one organism may be affected by other living organisms. Composition of microflora and microfauna of any habitat is governed by the equilibrium created by associations and interactions of all individuals found in the population (1). First report on relationships of plant parasitic nematodes and another microorganism was made in 1892 by Atkinson (5); a similar report of saprozoic nematodes and other organisms was made by Steiner in 1933 (79). Because of these interesting observations, numerous papers, dealing mostly with nematode-fungus interactions, are developing a new branch of plant pathology. These studies have gained considerable attention and recognition in recent years in terms of understanding of the disease complex. Many terms -- associations, interactions, disease complexes, relationships and interrelationships -- are used interchangeably by many authors and also in this thesis. Literature citations on nematode interactions with other organisms in plant disease complexes has been recently compiled by Jatala and Jensen (36).

In general, the role of nematodes in relationship with other organisms seems to be quite complex. Powell (64, 65) suggests that nematodes, in relation to plants, should no longer be considered

exclusively as single or detached entities, but as members of a complex biotic community. Sasser, Lucas and Powers mentioned that factors other than mechanical injury caused by nematodes appeared to contribute to interactions of black shank fungus and root-knot nematodes. Histologically, nematodes predisposed plant roots to an extent that both black shank-susceptible and resistant varieties became suitable for fungus invasion and colonization (74). Interrelationships of nematodes and other microorganisms are briefly reviewed below.

Nematode-Fungus Relationships

More than half of the literature containing relationships among nematodes and other microorganisms involves nematode-fungus relationships (35). Atkinson (5) was the first investigator who reported that infection by root-knot nematodes increased the incidence and severity of Fusarium wilt in cotton. Another early record made by Young (91) in 1939 was that root-knot nematodes greatly decreased expression of resistance to Fusarium in his tomato breeding lines. He recorded that losses to the fungus in variety Marglobe were much less when it followed root-knot resistant peanuts in a rotation, than when it followed root-knot susceptible cowpeas. Also, nematode populations were directly correlated with wilt severity (28). Gill (24) found that more mimosa seedlings wilted when soil was infested with either Meloidogyne incognita (Kofoid and White) Chitwood, 1949, or

M. javanica (Treub) Chitwood, 1949, and Fusarium oxysporum f. perniciosum (Hepting) Toole than when the fungus was present alone. With the presence of root-knot nematodes, particularly M. incognita acrita Chitwood, 1949, it was concluded that tobacco with high black-shank resistance could be more severely damaged (66, 67). In 1971, Powell and his co-workers (68) suggested the dominant role of root-knot nematodes was as a predisposing agent in root breakdown. Besides these examples, the role of root-knot nematodes in disease severity has been reported with different fungi: Macrophomina phaseoli (Maubl.) Ashby on kenaf root rot (85), on Ligustrum japonicum Linn. (2), and with Rhizoctonia solani Kuhn, 1858, on tobacco (9).

The influence of root-lesion nematodes on many fungous diseases has been recently investigated. Michell and Powell (58) found that a greater percentage of cotton plants wilted when the nematode, Pratylenchus brachyurus (Godfrey, 1929) T. Goodey 1951, and the fungus Fusarium oxysporum f. vasinfectum (Atk.) Snyder and Hansen were applied simultaneously than when the nematode was added two weeks prior to the fungus or when the fungus alone was used. Pratylenchus penetrans (Cobb) Chitwood and Oteifa, 1952, was also reported to increase severity of root-rot of canning peas by Oyekan and Mitchell (62). They claimed that the effect of nematodes was more striking on untreated silt loam soil containing a threshold level of only 25 oospores of Aphanomyces euteiches Drechs., 1925, per gram of soil, but this

effect was reduced when the soil was treated with aerated steam. On sugarcane, Pratylenchus zeae Graham, 1951, was found to increase severity of the root-rot disease caused by the fungus, Pythium graminicola Subr. (73). The role of another plant parasitic nematode, a stunt nematode Tylenchorhynchus dubius (Butschli, 1873) Filipjev, 1936, in development of Fusarium blight of Merion Kentucky bluegrass was studied by Vargas and Laughlin in 1972 (88). This nematode appeared to be the dominant pathogen in the disease complex of Fusarium blight of turfgrass.

Interactions of various nematodes with the club-root organisms of cabbage, Plasmodiophora brassicae Wor., 1877, were studied by Jensen and Vaughan (46). They noted that, when Jersey Wakefield cabbage plants were individually inoculated with P. brassicae and an inoculum containing one of the following nematodes: Helicotylenchus nannus Steiner, 1945, Longidorus elongatus (de Man, 1876) Thorne and Swanger, 1936, Meloidogyne hapla Chitwood, 1949, and Pratylenchus penetrans, the synergistic response was apparent. The comparative percentages of fresh root and dry root weights were significantly increased.

Another way to study the role of nematodes in disease complexes is to control nematodes present in the rhizosphere. This has been successfully done (11, 88). Bird et al. reported that severity of cotton stunt significantly decreased when methyl bromide was used to

fumigate the soil found to contain the nematodes Pratylenchus brachyurus, Trichodorus christiei Allen, 1957, T. porosus Allen, 1957, and soil-borne fungi, Rhizoctonia solani Kühn, 1858, Pythium debaryanum Hesse, P. irregulare Buis, P. ultimum Trow, and Fusarium spp. (11).

Nematode-Bacterium Relationships

A number of research reports also have discussed interactions between nematodes and bacteria; despite a later emphasis on these relationships, the effect of nematodes on many bacterial diseases are widely recognized. Compared to other studies, however, nematode-bacterium relationships are, even now, sparsely investigated. The earliest report seemed to be that of Hunger (33), in 1901, who showed that tomatoes were readily attacked by a bacterial wilt organism, Pseudomonas solanacearum E. F. Smith in nematode-infested soil, but remained healthy in nematode-free soil. Conclusions made by many investigators reveal that nematodes are bacterial vectors, wounding agents or both. A classic example is the "earcockle" disease of wheat caused by the synergistic effect of the bacterium, Corynebacterium tritici (Hutchinson) Bergey et al. and the wheat nematode, Anguina tritici (Steinbuch) Filipjev, 1936. The role of both organisms has been confirmed by many investigators since 1925 (13, 22, 26, 89). Nematodes carried bacteria on the body surface and entered wheat seedlings through the apical meristem, then the bacteria

rapidly multiplied to cause disease symptoms. The bacteria alone were unable to produce the disease. Gupta and Swarup (26) found that surface-sterilization of nematode larvae with 0.1 percent mercuric chloride for 30 minutes was the most effective sterilizing method, and larval development was not affected by the treatment.

A similar type of nematode-bacterium interaction was found in cauliflower disease complex of strawberry. This disease is an association of a bud and leaf nematode, Aphelenchoides ritzema-bosi (Schwartz) Steiner 1932, and the bacterium, Corynebacterium faciens (Tilford) Dowson. To produce the disease, both organisms must be present; inoculation of either organism alone did not cause typical cauliflower symptoms (17, 63). Ditylenchus dipsaci (Kühn) Filipjev, 1936, was reported to carry the bacterial wilt organism, Corynebacterium insidiosum McCull by Hawn (29, 30, 31). Also, Helicotylenchus nannus Steiner, 1945, was found to be involved in wilt symptoms of carnation when the bacterium, Pseudomonas caryophylli Burk. was present (80).

Relationships of root-knot nematodes and plant pathogenic bacteria were studied by a few people. In 1955, Lucas et al. (52) reported the serious effects of the tobacco wilt bacterium, Pseudomonas solanacearum and Meloidogyne incognita acrita Chitwood, 1949, on a wilt resistant variety of tobacco. Similar results were obtained by Stewart and Schindler (80) working with five species of root-knot

nematodes and bacterial wilt organism, P. caryophylli Burk. in carnations; and by Libman, Leach and Adams with M. hapla and the bacterium P. solanacearum in tomato (51).

Several workers have reported the role of plant parasitic nematodes on nitrogen-fixing bacteria, Rhizobium spp. Some reported that root-knot nematodes caused a reduction of nodules on leguminous plants, e.g., Heterodera glycines Ichinohe on soybean (20, 34, 71), H. trifolii Goffart on white clover (90), M. javanica (Treub) Chitwood, 1949, on hairy vetch (54) and on alfalfa (61). Taha and Taski, 1969, claimed that nematodes caused a drastic reduction of the total-N in H. trifolii-infected white clover plants, which reflected stunting of the entire plant (83). Ayala (6) found that bacterial nodules on pigeon peas, Cajanus indicus Spreng., were attacked by females of Rotylenchulus sp. Barker et al. (7, 8) concluded from their greenhouse experiments on H. glycines Ichinohe and R. japonicum (Kirchner) Buchanan that the nematode played a competitive role in reducing the number of nodules even though nodular tissues were unfavorable for development of nematode larvae.

Nematode-Nematode Interactions

Although studies involving interactions of nematodes with fungi, bacteria, viruses and other soil microorganisms have been relatively common, studies on nematode-nematode interactions have received

less attention. In fact, many of the factors which influence dynamics of nematode populations containing more than one species are still unknown. Nevertheless, intergeneric, interspecific inhibition and synergism of one plant nematode by another is known to occur (21, 23, 35, 59, 60, 72, 76, 87).

It was found that populations of Pratylenchus brachyurus on cotton were increased significantly in the presence of either Meloidogyne incognita or M. arenaria (Neal, 1889) Chitwood, 1949 (23). This occurred with either simultaneous inoculation or prior invasion by M. incognita. Turner and Chapman (87) obtained similar results when they worked with M. incognita and P. penetrans. Penetration by M. incognita into alfalfa, but not in red clover, was greatly reduced when both organisms were inoculated simultaneously. In the presence of large numbers of entrant P. penetrans, penetration by M. incognita was greatly reduced. Estores and Chen (21) obtained the same results when greenhouse experiments were conducted with M. incognita and P. penetrans. They found that population densities of both nematodes were depressed when they coexisted in tomato roots. Fifty days after inoculation, the population of P. penetrans monoculture was about four times higher than when M. incognita was present. Conversely, M. incognita reproduced twice as fast alone as in combination. Root entry by P. penetrans was significantly inhibited by the presence of M. incognita. Availability of feeding sites on roots might be the main

factor of inhibition.

In long term greenhouse experiments, Sikora and his co-workers (76), working with three kinds of plant nematodes and creeping bentgrass, concluded that Pratylenchus penetrans and Tylenchorhynchus agri Ferris alone and in combination, inhibited root growth and adversely affected top growth only when the two were co-inoculated. In combination, effects of each species on top growth were additive, with Meloidogyne naasi Franklin as the dominant pathogen. Competition between nematodes was also prominent. They reported that T. agri inhibitied population of M. naasi but neither T. agri nor P. penetrans was affected by any of the combinations.

Jatala and Jensen, 1972, (37) reported three different phenomena in terms of nematode-nematode interactions which they described as amensalism, commensalism and neutralism. They found that there were significant reductions of M. hapla galls in treatments when Heterodera schachtii Schmidt, 1871, preceded M. hapla. Conversely, when M. hapla preceded H. schachtii, there was a significant increase in cyst formation by the latter. In treatments, when both nematodes were inoculated simultaneously, there were no significant changes in population of either parasite. In another experiment, they made comparisons between double and single inoculation treatments receiving the same number of total inocula. The number of galls in treatments receiving 250+250 M. hapla larvae was significantly higher than

the number in treatments receiving 500 larvae at one time. They found general trends of lower mortality rates of plants, Beta vulgaris L. in double-inoculated treatments versus single-inoculated treatments (38).

At the same soil temperature, Kinloch and Allen (47) reported that M. javanica dominated in a mixed species infection of M. javanica and M. hapla. Predominance increased with increasing mixed-species inoculum levels. Invasion by M. hapla seemed to be more density-dependent than M. javanica.

Nematode-Virus Relationships

Relationships of nematodes and viruses present complex phenomena. They were unknown until first demonstrated by Shope (75) in 1941. He found that an animal parasitic nematode -- swine lungworm -- transmitted influenza virus to swine. The nematode also serves as a reservoir and an intermediate host for the virus.

Rathay, in 1882, was the first one who noted that healthy grape-vines became diseased when planted in soils of old vineyards (70). After this observation, many researchers agreed that plant virus diseases were spread by some soil-inhabiting biological agent, possibly nematodes, but failed, experimentally, to prove the theory (3, 57). The research work, which supported the theory that nematodes were vectors of plant viruses, was that a dagger nematode,

Xiphinema index Thorne and Allen, 1950, was the vector of soil-borne grapevine fanleaf virus, according to Hewitt, Raski and Coheen in 1958 (32). They reported that the virus could persist in the nematode vector up to 122 days and that the virus was not carried from one generation to another through eggs (69).

More than 15 species of plant parasitic nematodes have been reported to transmit 23 soil-borne viruses (56). Only three genera -- Xiphinema, Longidorus, and Trichodorus, of the superfamily Dorylaimoidea, class Adenophorea -- are involved in the relationship. Morphologically, nematode-transmitted viruses are divided into two groups, NEPO and NETU viruses, so named by Cadman (12) and Harrison (27), respectively. Species of Longidorus and Xiphenema are vectors of NEPO viruses, while species of Trichodorus are vectors of NETU viruses.

Interactions of Saprozoic Nematodes with Pathogenic and Nonpathogenic Organisms

Interactions of saprozoic nematodes and pathogenic or nonpathogenic microorganisms have recently received attention. A number of reports conclude that saprozoic nematodes feed on various microorganisms and their by-products (15, 79, 84). These nematodes, morphologically, have a large hollow stoma which allows them to swallow viruses, fungi, bacteria, algae, and other microorganisms

(10, 50). First report on the relationship of saprozoic nematodes and bacteria was made by Steiner (79). He reported the association of Rhabditis lambdiensis Maupas, 1919, Dougherty in the brown spot disease of mushroom caused by the bacterium, Pseudomonas tolaasii Paine. Also, he suggested that nematodes fed exclusively on fungi and bacteria and that they carried bacteria in the pharynx or in the intestine and discharged bacteria with the feces.

The role of saprozoic nematodes as carriers and disseminators of plant pathogenic bacteria was first reported by Jensen in 1967 (40), and by Chantanao and Jensen in 1969 (16). They found associations of Pristionchus lheriteiri with Agrobacterium tumefaciens (E. F. Sm. and Towns.) Conn, and Pseudomonas syringae Van Hall. They also reported that Pristionchus lheriteiri Maupas, 1919, Paramonov, Panagrellus redivivus (Linn., 1767) Goodey, 1945, Panagrolaimus subelongatus (Cobb, 1914) Thorne, 1937, and Rhabditis spp. ingested several kinds of fungus spores including those that are plant pathogenic -- Fusarium oxysporum f. lycopersici (Sacc.) Snyd. and Hans., Verticillium dahliae Kleb., Geotrichum sp., Hormodendrum spp., Penicillium spp., Stysanus sp. and several unidentified forms. These spores survived passage through the alimentary canal and were subsequently germinated. Verticillium and Fusarium spores were protected from biocides after ingestion by the nematodes, P. lheriteiri (43, 44). Many species of green algae, Chlorophyceae, Mycoplasma

galliseptum, a Streptomyces phage, and a phage of Agrobacterium tumefaciens (Smith and Townsend) Conn, 1942, were all found to be carried and disseminated after ingestion by nematodes (15, 42, 45, 49).

A nitrogen-fixing bacterium, Rhizobium japonicum (Kirchner) Buchanan was speculated to be carried internally and disseminated by a saprozoic nematode, Pristionchus lheriteiri (39). Experimentally, Jatala and Jensen found that the bacteria in the alimentary tracts of the nematode vector were viable and caused nodulation on soybean roots. This is another important role for saprozoic nematodes such as P. lheriteiri in relation to bacteria.

MATERIALS AND METHODS

General Procedures

Studies on relationships of saprozoic nematodes and the bacterium Rhizobium japonicum made in this thesis were conducted in the Nematological Laboratory, Department of Botany and Plant Pathology, Oregon State University, Corvallis, Oregon. Throughout these studies, the room temperature, including the experiment which was done in a growth chamber, was kept at about 25°C (78-80°F).

Maintaining Cultures of Nematodes

All saprozoic nematodes used in these experiments were obtained from cultures that have been maintained in the Nematological Laboratory by Dr. H. J. Jensen since 1966 (41). The following nematodes were used as test animals for this research.

1. Pristionchus lheriteiri (Maupas, 1919) Paramonov
2. Panagrolaimus subelongatus (Cobb, 1914) Thorne, 1937
3. Cephalobus persegnis Bastian, 1865
4. Pelodera (Rhabditis) lambdiensis (Maupas, 1919) Dougherty, 1953
5. Rhabditis sp. I
6. Pelodera chitwoodi (Bassen, 1940) Dougherty, 1955

They were reared on a bacterium, Pseudomonas sp. growing on nutrient agar medium (4). Formula for the medium is shown in

Appendix A. The cultures were maintained with a weekly transfer to new nutrient agar plates.

Sharply pointed splinters of bamboo and a micropipette attached to a rubber dropper bulb were used to collect and transfer nematodes. To eliminate contamination, these implements were autoclaved before using.

Maintaining Cultures of Bacteria

A nodule-forming bacterium, Rhizobium japonicum (Kirchner, 1895) Buchanan, 1926, Strain 705, used in the studies was obtained from Dr. H. E. Evans of the Department of Botany and Plant Pathology, Oregon State University, Corvallis, Oregon. The bacterium was cultured on Rhizobium agar medium (18); formula is shown in Appendix A. Viable cultures were maintained by transferring the bacterium every 8-10 days to new agar slants and stored in the laboratory.

When broth cultures were used, the bacterium was allowed to grow in Rhizobium culture broth (18) for seven days. Subcultures of the bacterium in broth were made every two weeks. Rhizobium culture broth formula is shown in Appendix A.

Maintaining Cultures of P. lheriteiri Feeding on R. japonicum

In most experiments, combinations of a saprozoic nematode, P. lheriteiri, and a nitrogen fixing bacterium, R. japonicum, were used. A modification of Jatala and Jensen's surface sterilization technique for nematodes (39) was made. Eggs of P. lheriteiri thriving on Pseudomonas sp. were collected by flooding the agar medium in Petri dishes with sterile distilled water. Nematode eggs were collected with a micropipette. Adults or nematode larvae occurring in the egg suspension were removed individually. Eggs were then surface-sterilized with mercuric chloride by mixing equal amounts of egg suspension with 1:500 mercuric chloride solution for three minutes. The contents were immediately poured through a sterile millipore filter holder containing a no. 1 filter paper, followed by sterile distilled water for three minutes to wash off mercuric chloride residues. The filter paper on which the eggs were collected was cut aseptically into small pieces; each piece was placed on seven-day-old Petri dish culture of R. japonicum. The cultures were incubated at approximately 25°C (78-80°F) and periodically checked for contamination. These nematode cultures were maintained throughout the study by weekly transfers. Transfer of nematodes, bacteria and inoculations were made aseptically in a transfer chamber using a dissecting microscope.

SECTION I. TOLERANCE OF RHIZOBIUM JAPONICUM TO MERCURIC CHLORIDE

Preface

One of the serious problems that is always encountered in studying relationships of nematodes and bacteria is the bacterial and fungal contamination, especially on the nematode body surface. Chantanao (14) satisfactorily used 20 ppm chlorine for three minutes to kill six kinds of plant pathogenic bacteria. He also reported that P. lheriteiri was much more tolerant to chlorine than the bacteria. It was the purpose of this experiment to investigate the tolerance of R. japonicum to mercuric chloride, a widely used decontaminant, at three-minute exposure time.

Materials and Methods

Four different concentrations of freshly prepared mercuric chloride solution were used in this experiment. Each concentration, i.e., 1:500, 1:1,000, 1:1,500 and 1:2,000 represented a treatment and a bacterial suspension in sterile distilled water was used as control treatment. Ten ml of R. japonicum in broth culture were diluted with 10 ml of sterile distilled water in a 125 ml flask. Five ml of the bacterial suspension were pipetted into each flask containing 10 ml of one of the four different concentrations of mercuric chloride

solution. Immediately, each flask was vigorously shaken for 15 seconds and the mixture of bacterial suspension and mercuric chloride solution were left for another two minutes and 45 seconds to make the three-minute sterilizing time. Then, three ml of bacterial suspension was pipetted out and diluted in test tubes containing nine ml of sterile 0.1 percent sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$) solution. Each tube was shaken and diluted to 1:1,000. One ml of the final dilution was inoculated into Rhizobium agar medium. Bacterial counting was made after seven days. There were five replications, and the experiment was repeated five times.

Results and Discussion

It is apparent that the bacterium, R. japonicum, is susceptible to mercuric chloride, as shown in Table 1. Bacteria were completely killed with three-minute exposure time at concentrations of 1:500, 1:1,000 and 1:1,500 but some survived at 1:2,000. Numerous bacteria colonies in control treatment were visible four days after inoculation while there were very few colonies in 1:2,000 treatment.

Table 1. Average number of R. japonicum surviving exposure to different concentrations of mercuric chloride solution for three minutes.

	Number of colonies counted at 7 days[a]					
Treatment	Rep. I	Rep. II	Rep. III	Rep. IV	Rep. V	Average
1:500	0	0	0	0	0	0
1:1,000	0	0	0	0	0	0
1:1,500	0	0	0	0	0	0
1:2,000	3	1	1	3	0	1.6
Control	1991.0	2192.0	2152.6	2021.6	2092.0	2089.8

[a]Numbers were means from the experiment repeated five times.

Mercuric chloride has been widely and effectively used as a sterilizing agent for a long time in plant pathology laboratories. Exposure time may vary, depending on kinds of materials to be sterilized; nevertheless, the concentration which is commonly used is 1:1, 000. Thus, R. japonicum contaminated on nematode body cuticle is completely killed in three minutes with freshly prepared 1:1, 000 mercuric chloride solution.

Chantanao, in 1969, obtained successful results when he used chlorine (from Clorox) at three ppm for two minutes to destroy many species of plant pathogenic bacteria -- Agrobacterium tumefaciens (Smith and Townsend) Conn., Erwinia amylovora (Burrill) Winslow et al., E. carotovora (Jones) Holland, Pseudomonas phaseolicola (Burk.) Dows. and Xanthomonas campestris (Pam.) Dows.(14). He also reported an effective method of surface sterilization for nematodes, P. lheriteiri, by using the same material at 20 ppm for 20 minutes to eliminate surface contamination by those plant pathogenic bacteria (16).

SECTION II. NATURE OF BACTERIAL FOOD SOURCES FOR P. LHERITEIRI ON R. JAPONICUM CULTURES

Preface

It has been stated elsewhere that, in general, saprozoic nematodes feed on a number of other microorganisms -- fungi, bacteria and plant residues (15, 25, 79, 84). In most cases, the organisms seem to be unharmed after ingestion by nematodes. Even though there have been many studies to determine synthetic diet requirements for this group of nematodes, basic information is not available about the exact nature of their natural food source. It was the purpose of this experiment to find out whether the nematodes feed on dead or living bacterial cells or by-products of bacterial digestion.

Materials and Methods

P. lheriteiri thriving on Pseudomonas sp. culture in nutrient agar was used in this experiment. Ten eggs were collected with a micropipette, surface-sterilized with a 1:1,000 mercuric chloride solution, as mentioned before in "General Procedures." Eggs were rinsed with sterile distilled water and individually hand-picked. Then they were transferred to a Rhizobium agar medium previously modified for the different five treatments. There were five replications for each of the following treatments.

1. Control. No bacterium was used. One-half ml of sterile distilled water was pipetted directly on the agar medium.
2. Autoclaved bacteria. Seven-day old R. japonicum culture in Rhizobium culture broth was autoclaved at 15 psi for 20 minutes.
3. Sonicated bacteria. Seven-day old bacteria in culture broth was sonicated aseptically for five minutes.
4. Bacterial suspension. A broth suspension of bacteria was used.
5. Supernatant broth from a centrifuged bacterial culture. For this treatment, a bacterial suspension in broth was centrifuged at 10, 000 rpm for 20 minutes, then the decanted fluid was used.

Except for the control treatment, only one-half ml of the modified bacterial suspensions was pipetted onto the agar medium at the center of the dishes. Surface-sterilized nematode eggs were used for all treatment modifications prior to inoculation. Incubation temperature was at about 25°C (78-80°F) and the experiment was repeated five times. Number of nematodes were counted 2, 7, 10 and 14 days after inoculation.

Results and Discussion

Data obtained from this experiment were analyzed statistically by analysis of variance, and the means compared by using the Least Significant Difference test, both at 0. 01 and 0. 05 levels (78). Effect of various treatments on numbers of nematodes recovered at 2, 7, 10

and 14 days is summarized in Table 2. The same effects are also illustrated in Figure 1. Averaged numbers for each day -- and raw data -- are shown in Appendix B.

Table 2. Effects of modified bacterial suspensions on eggs and development of P. lheriteiri at different time intervals.

Treatment	Number of nematodes recovered[a]			
	2 days	7 days	10 days	14 days
1. Control (no bacteria)	1.04	1.44	1.52	1.64
2. Autoclaved bacteria	1.20	1.44	1.52	1.56
3. Sonicated bacteria	1.36	1.68	1.64	1.64
4. Bacterial suspension	2.84**	16.58**	69.04**	141.88**
5. Culture supernatant	1.0	1.52	1.88	2.08
L.S.D. 0.05	0.427	4.929	24.273	30.977
0.01	0.583	6.722	33.104	42.248

**Significantly different, both at 5% and 1% levels, from control and other treatments on the same day.

[a]Averaged from five experiments, each consisting of five replications originally from ten eggs in each replication.

It was evident that the number of nematodes recovered from eggs in treatment 4 (living bacterial suspension) increases as the incubation period is extended. In other treatments, there is no

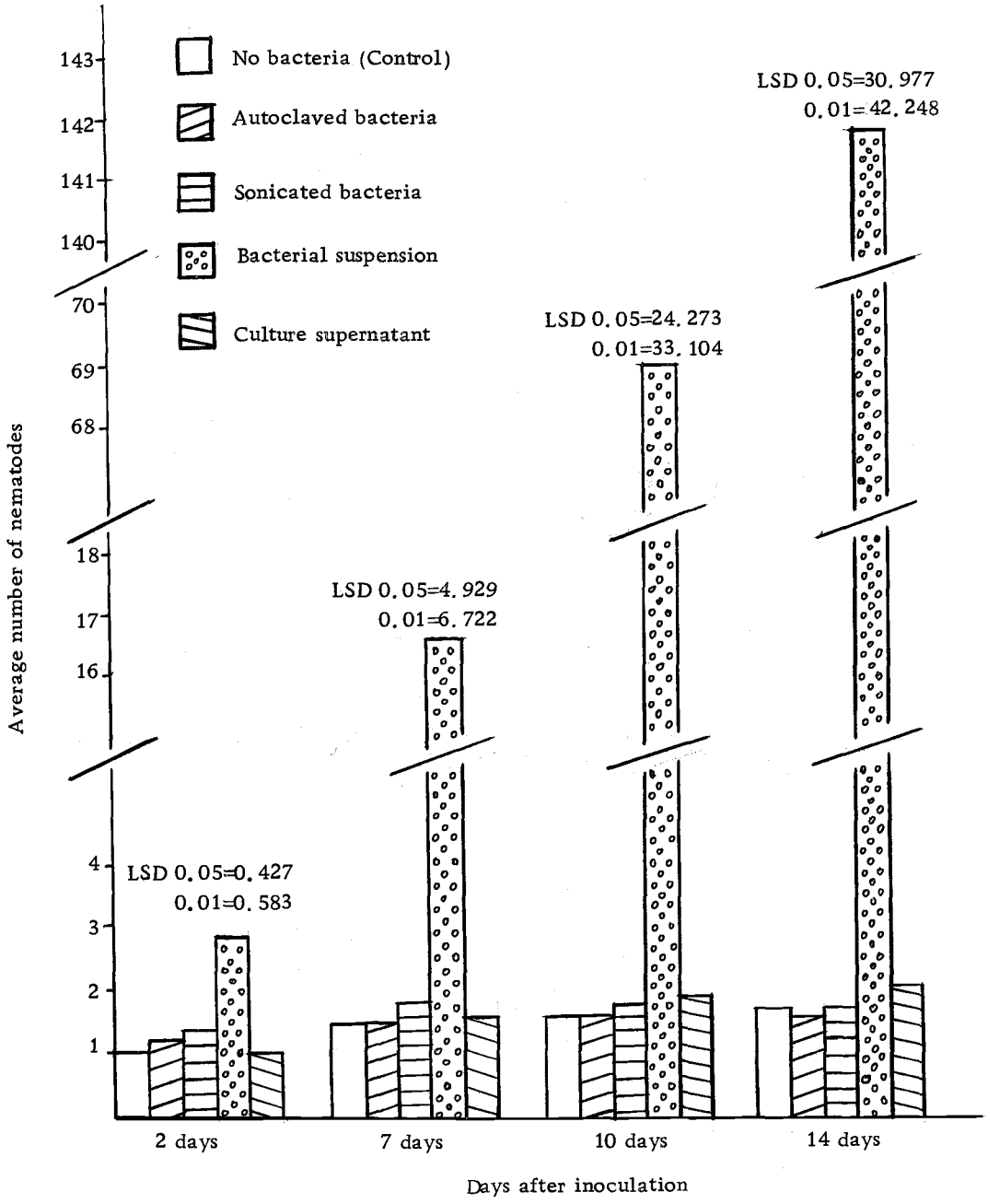

Figure 1. Comparison of effects of various bacterial suspensions on populations of P. lheriteiri.

significant increase at all four time intervals. Obviously, P. lheriteiri cannot thrive and increase on killed bacteria, broken bacterial cells and on culture supernatant. In most instances, they appeared very thin, less active and finally died of starvation. On the contrary, hatched nematode larvae in treatment 4 (which contained the living or active bacterial suspension) survived and increased in numbers very rapidly. Nematode number in this treatment increased with time while in other treatments the number remained almost the same.

An interesting point to note in this experiment is the number of nematode larvae that hatched in every treatment, including those during the first two days from treatment 4, was only about 15 percent of the available eggs. Two important factors that should be considered when working with nematode eggs are: (1) Manipulation. Transferring eggs from culture medium with a micropipette and a bamboo pick might have some effects on low hatching percentage. Even though the experiment was carefully conducted, egg cuticles are easily broken and damaged before they were chemically treated. (2) Immature eggs. Eggs collected contained a mixture of different developing stages. In a small preliminary test, when eggs were fully mature, hatching percentage was about 50 percent. A lower hatching percentage was obtained when these eggs were disturbed, by transferring or moving to other dish. To collect only fully developed eggs is not practical and very time-consuming.

Generally, eggs hatched within two days after inoculation. When a satisfactory food supply was not available, except for treatment 4, many larvae moved out of the dishes, leaving only a zig-zag path on the agar medium surface. This creates problems in counting and keeping track of larvae.

Doncaster (19) reported that Rhabditis sp. ingested whole living microorganisms but failed to mention the name of the particular organisms. The assumption that whole living organisms are ingested has been made by many investigators. Some of them reported the kinds of microorganisms that were ingested as food for saprozoic nematodes but none of them reported whether or not the microorganisms needed to be living. The present experiment shows that whole live bacteria probably are the food source for the nematode, P. lheriteiri. Further investigation on the role of bacteria in relation to the nematode growth should be conducted in the future.

SECTION III. VIABILITY OF R. JAPONICUM AFTER DEFECATION BY P. LHERITEIRI

Preface

It is now obvious (data from the second experiment) that P. lheriteiri can feed on a live bacterium, R. japonicum. Chantanao (16) demonstrated in his experiments that many pathogenic bacteria survived after they were defecated by this nematode. Jatala and Jensen (1974) also speculated on a similar relationship for P. lheriteiri and R. japonicum (39). The purpose of this experiment was to test the speculation made by Jatala and Jensen that R. japonicum were still viable after they were defecated by the nematodes, P. lheriteiri.

Materials and Methods

Females of P. lheriteiri reared on R. japonicum in Rhizobium agar medium culture for seven days, were used for this experiment. Three gravid females were hand-picked, surface-sterilized with 1:1, 000 mercuric chloride solution for two minutes, then rinsed with sterile distilled water. Then they were transferred to the center of a new sterile Petri dish containing the same kind of agar medium. Three hours later they were picked out again, surface-sterilized and transferred to another dish. This procedure was repeated eight times at three hour intervals. There were five replications in each transfer.

The dishes were incubated at about 25°C (78-80°F) for seven days and bacterial counts were made by a dilution plate method at 1:1, 000. The experiment was repeated five times.

Results and Discussion

Bacteria, R. japonicum, were viable after they were defecated by nematodes, P. lheriteiri. Results are summarized in Table 3.

Table 3. Bacterial counts of R. japonicum defecated by female of P. lheriteiri after various periods of starvation.

Time after starvation (hours)	Number of bacteria per nematode[a]					
	Rep. I	Rep. II	Rep. III	Rep. IV	Rep. V	Average
0	56.6	155.0	199.1	42.9	32.9	97.3
3	18.2	54.2	93.0	97.2	45.5	61.6
6	43.6	111.3	120.6	136.0	20.7	86.4
9	24.5	97.4	19.1	34.5	38.2	42.2
12	90.1	168.3	247.3	53.3	150.4	141.9
15	339.0	77.5	29.1	142.7	282.1	174.1
18	273.0	228.9	20.7	212.9	417.7	230.6
21	306.1	238.9	385.7	167.9	521.2	323.9

[a]Averaged from five replications, three nematodes each and repeated five times.

It seems that the number of bacteria recovered increases with time although the rate of increase is not consistent. This phenomenon can be explained by the reproductive activity of the particular bacteria on Rhizobium agar medium. Also the rate of increase is dependent on the initial bacterial inoculum which varied in every individual nematode. Movement of nematodes on agar medium

surface can partially obscure the bacterial population.

It has been shown that bacterial cells were still being defecated and were reproducing 21 hours after starvation began. It is possible that the bacteria will be defecated and still be viable after 30 hours. This is contradictory to the report of Chantanao (16) who found that the number of plant pathogenic bacteria gradually decreased with time. No bacterial cells were found after 33 hours.

From Table 3, the first four transfers of nematodes show a trend of decrease in bacterial counts in almost every replication but these numbers gradually increased again after 12 hours. There are some possible explanations concerning the inconsistency of the rate of bacterial increase in this experiment. First, bacteria ingested are not all defecated at the same time. The nematodes gradually defecated the bacteria taken in. The total numbers of bacteria after 21 hours in every replication are less than the average number of bacterial cells ingested (2, 622 cells in a female), in Section IV. This number might be eventually less and less after 21 hours of starvation. Secondly, the bacteria used in Chantanao's experiment are plant pathogenic, unlike the nitrogen-fixer used in this research. Different bacterial species could account for the fact that bacteria colonies increased after nine hours. Thirdly, it is possible that the increase after nine hours was due to damaged nematodes. Probably due to repeated washes in mercuric chloride the nematodes became more

fragile, so that lysis of some individuals would release additional bacteria.

In conclusion, evidence of defecation of viable nitrogen-fixing bacteria from a saprozoic nematode was found. This means that after ingestion by the nematode, R. japonicum were able to survive in the nematode alimentary canal. This phenomenon confirmed the observations of Jensen (40) and Chantanao (16) who found that many plant pathogenic bacteria survived passage through the alimentary canal of saprozoic nematodes and were viable subsequently.

SECTION IV. NUMBER OF R. JAPONICUM CELLS INGESTED BY P. LHERITEIRI

Preface

In the previous experiment, it was determined that R. japonicum remained viable after defecation by P. lheriteiri. Other investigators also reported survival of several species of plant pathogenic bacteria ingested by saprozoic nematodes (16, 40) but there has been little emphasis on the quantity of bacteria ingested by nematodes. This experiment was designed to determine the number of R. japonicum cells that normally are ingested by P. lheriteiri.

Materials and Methods

Twenty adult males and females of P. lheriteiri were used separately. They were hand-picked from seven-day old R. japonicum cultures, surface-sterilized with 1:1, 000 mercuric chloride solution for three minutes and rinsed with sterile distilled water. Later, the whole nematode bodies were well crushed aseptically in one ml sterile distilled water with a dissecting needle in a BPI watch glass. Nematode body contents were diluted to 1:100, 1:1, 000, 1:10, 000 and 1:100, 000 and one ml of each dilution was poured into a Petri dish with Rhizobium agar medium. There were five replications each for each group (20) of male or female nematodes. Plates were incubated

at about 25°C (78-80°F) for 96 hours before bacterial colonies were counted. The experiment was repeated six times.

Results and Discussion

Apparently, ingested bacteria R. japonicum were viable after they were obtained from well crushed nematodes. Results, as summarized in Table 4, indicate the viability of bacteria and ability of female nematodes to carry more bacterial cells than males. On the average, after treatment with 1:100 dilution, a female nematode carried 2, 622 cells in comparison with only 1, 004 in male.

Difference in size between females and males of P. lheriteiri may explain this variability, because, in general, female nematodes are larger than males. In this case, the average size of females is approximately 1, 400 μ in length and 110 μ in body width while males are 1, 150 μ long and 85 μ wide. Assuming that the nematodes are cylindrical, the females are about 3. 5 times greater in volume than the males.

Results from this study point out that some of the ingested bacteria that passed through nematode's alimentary canal were unharmed in the ingestion process and could survive. When they were defecated out of nematode body, they reproduced, as demonstrated in Section III, again. It is not possible to determine from these studies the percent of bacteria survived ingestion. That some bacteria would

Table 4. Numbers of bacteria ingested per 20 nematodes (P. lheriteiri) calculated from four dilutions.[a]

	Treatment (dilution)							
	1:100		1:1,000		1:10,000		1:100,000	
Replication	Female	Male	Female	Male	Female	Male	Female	Male
1	443.8	221.3	95.8	22.3	17.0	1.6	0.7	0.2
2	534.2	196.3	98.7	26.7	10.7	3.0	2.3	0.3
3	535.2	186.0	137.7	36.3	10.5	5.3	0.7	0.7
4	514.7	260.5	73.7	22.2	8.7	5.3	0.7	0.2
5	594.0	139.7	98.3	26.5	11.5	2.0	0.5	0
Average	524.4	200.8	100.8	26.8	11.7	3.4	0.98	0.28

[a]Averaged from experiment repeated six times, 20 nematodes in each replication.

survive ingestion was anticipated from a study by Mapes (55) of the feeding mechanisms of Rhabditis, Panagrellus and Aplectana. He demonstrated that not all food taken in by saprozoic nematodes, Rhabditis sp., is broken down by the bulb flaps at the posterior of the esophagus because the principal role of the bulb flaps was a valvular rather than a crushing one.

SECTION V. RELATIONSHIPS BETWEEN NUMBER OF P. LHERITEIRI DISSEMINATORS AND NUMBER OF NODULES FORMED ON SOYBEAN ROOTS

Preface

It is evident from other experiments that P. lheriteiri could carry R. japonicum internally ranging from 1,004 cells in males to 2,622 cells in females. Bacteria were still viable after ingestion and defecation. Jatala and Jensen (39) recently reported that R. japonicum, carried by the nematode, was able to form nodules on both intact and excised soybean roots. The purpose of this experiment was to determine relationships of quantity of nematodes that carry Rhizobium bacteria internally and numbers of nodules formed on intact soybean roots in an artificial agar medium.

Materials and Methods

Soybean, Glycine max (Linn.) Merr. variety Lee, seeds were surface-sterilized for three minutes with 1:1,000 mercuric chloride solution, and then washed three times with sterile distilled water. Then each seed was transferred to a nine-inch long test tube containing 10 ml of sterile 0.5 percent water agar (86). Formula for this agar medium is shown in Appendix A. Five days after seedling germination, they were used in the treatments mentioned below. The experiment was repeated five times.

Five different additions to the seedling cultures were made:

1. Control. One ml of sterile distilled water only added to the culture tubes.
2. Bacterial suspension. Half ml of R. japonicum in broth culture was inoculated into culture tubes.
3. Nematode suspension containing 50 gravid females was added to the culture tubes.
4. Nematode suspension containing 100 gravid females was added to the culture tubes.
5. Nematode suspension containing 200 gravid females was added to the culture tubes.

In treatments 3, 4 and 5, gravid females reared on seven-day old Rhizobium culture were removed aseptically, surface-sterilized for three minutes with 1:1, 000 mercuric chloride solution, washed and inoculated onto the seedling roots. These cultures were kept in a growth chamber at about 25°C (78-80°F) for 30 days before counting the nodules. Nitrogen fixation by the bacteria in soybean roots was checked by using Koch and Evans' methods (48) after the plants were harvested.

Results and Discussion

After ingestion by nematodes, the bacteria were viable and caused nodulation on soybean roots in the water agar medium

(Figure 2). Averaged numbers of nodules formed on plant roots do not differ significantly in any treatment except the "water only" control treatment, as summarized in Table 5. Throughout the experiment which was repeated five times, no nodules were found on root system of 25 soybean plants in the "water only" control but a total of 60 nodules were formed on 100 plants in treatments 2, 3, 4 and 5. Among this number, five nodules were seen on roots that had penetrated deeply in the water agar medium. Nodule sizes ranged from 0.5 to 2.0 mm in diameter but there were only few large nodules. Three nodules were visible eight days after inoculation, while most were found between 12-18 days, after the nematodes were inoculated onto the plants. Nodules formed by bacteria in suspension were not different from those formed by the bacteria carried internally by the nematodes.

Obviously, the nitrogen-fixing bacteria carried internally by this saprozoic nematode were able to cause soybean roots to form nodules. With different numbers of surface-sterilized nematodes, there was no statistical difference in numbers of nodules formed among treatments. Different amounts of bacterial inoculum did not have any different effects on numbers of nodules formed under the conditions of this experiment.

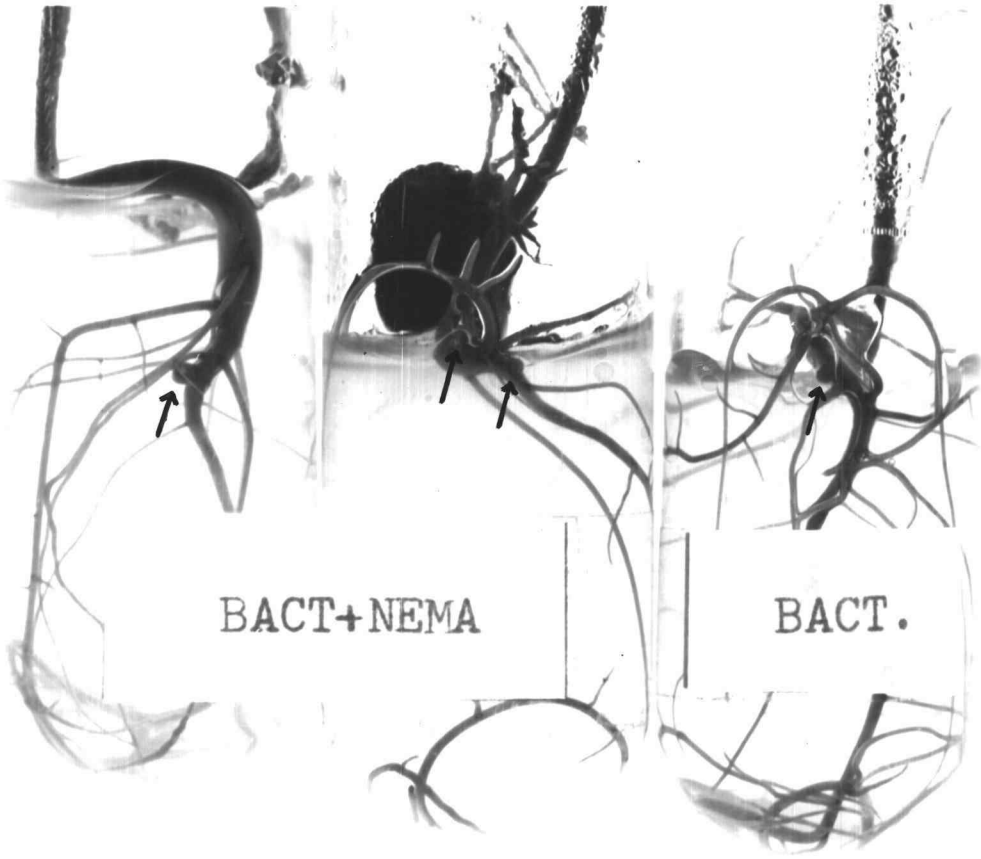

Figure 2. Nodule formation by R. japonicum on soybean roots. Left, nodules were formed by bacteria carried internally by P. lheriteiri; right, a nodule was formed by bacteria from a broth culture.

Table 5. Average number of nodules formed on soybean roots during a 30-day period after inoculation.[a]

Replication	Tr. I (water only)	Tr. II (Bacterial suspension	Tr. III (50 nematodes)	Tr. IV (100 nematodes)	Tr. V (200 nematodes)
1	0	0.6	0.6	0.2	0.4
2	0	0.8	1.0	0.4	0.8
3	0	0	0.6	0.4	0.8
4	0	0.2	1.4	0.8	0.6
5	0	1.0	0.4	0.4	0.6
Average	0^{b*}	0.52^{c}	0.80^{c}	0.44^{c*}	0.64^{c}

L. S. D. 0.05 = 0.379; 0.01 = 0.518

* Significant difference at 5% levels only

Averages with same subscript do not differ both at 5% and 1% levels.

[a]Number averaged from the experiment repeated five times, five replications each and one soybean plant per replication.

Because the number of nodules formed in the bacterial control (Treatment II) was not significantly different from the cultures containing nematodes, it is apparent that nematodes can act as effective disseminators of R. japonicum under culture conditions. The bacterial suspension contained between 2 and 3 x 10^6 cells, whereas the nematodes released several orders of magnitude less. The similarity in number of nodules in Treatments II-V suggests that the limiting factor was not the number of bacteria available but was something else in the system, e.g., culture conditions or the physiology of the soybean plants themselves (1).

It is clear that a saprozoic nematode, P. lheriteiri, can disseminate a nitrogen-fixing bacterium, R. japonicum, which enables a leguminous plant species to form nodules in roots.

SECTION VI. RELATIVE NUMBERS OF VARIOUS NEMATODES OBTAINED ON R. JAPONICUM CULTURE

Preface

In a previous experiment, P. lheriteiri was unable to survive on dead or broken bacterial cells, nor could they survive on centrifuged supernatant medium. In nature, there are many kinds of saprozoic nematodes besides P. lheriteiri. The purpose of this experiment was to determine if other kinds of saprozoic nematodes (available in the laboratory cultures) could survive and multiply on cultures of this nodule-forming bacterium.

Materials and Methods

R. japonicum was streaked on a Rhizobium agar medium 8-10 days before adding surface-sterilized eggs from six nematode species. Later, these cultures were flooded to obtain eggs (which were selected to avoid adults or larvae). They were surface-sterilized by mixing with an equal amount of 1:500 mercuric chloride solution before addition to the culture dish. The rest of the procedure was as mentioned in "Maintaining Cultures of P. lheriteiri feeding on R. japonicum" on page 19. Six kinds of saprozoic nematodes: (1) Pristionchus lheriteiri, (2) Pelodera lambdiensis, (3) Rhabditis sp. I, (4) Panagrolaimus subelongatus, (5) Cephalobus persegnis,

and (6) Pelodera chitwoodi were represented in six treatments. Each treatment had five replications and 20 eggs were used in each replication. The dishes were incubated at about 25°C (78-80°F) for 14 days before nematode counts were made by using Baermann funnel method (82).

Results and Discussion

The six kinds of saprozoic nematodes used in this study survived on R. japonicum culture. Final population size varied from one nematode to another, as summarized in Table 6. P. lheriteiri achieved the highest population within two week's incubation period, while Rhabditis sp. I increased the least. Figure 3 also illustrates the difference in increase of nematode population within the time limit.

Table 6. Numbers of nematodes of various species occurring on R. japonicum cultures 14 days after inoculation.[a]

	Nematode species					
Replication	P. lheriteiri	P. lambdiensis	Rhabditis sp.	P. subelongatus	C. persegnis	P. chitwoodi
1	3.8	0	0	254.2	84.6	24.0
2	1107.8	1.0	0.2	12.4	497.6	628.0
3	435.4	46.0	6.4	16.0	248.4	374.8
4	109.0	5.4	1.0	66.2	142.2	11.2
5	171.0	1.4	4.4	77.4	201.4	3.2
Average	365.4^{b}	10.9^{c*}	2.4^{c**}	85.2^{c*}	234.8^{b}	208.9^{b}

Averages with same subscript do not significantly differ at both 5% and 1% levels.

* Compared to P. lheriteiri, this nematode is statistically different at 5% level only.

** Compared to P. lheriteiri, this nematode is statistically different at 5% and 1% levels.

LSD 0.05 = 266.21, 0.01 = 360.16

[a]Averaged from experiment repeated five times, starting with 20 nematode eggs in each replication.

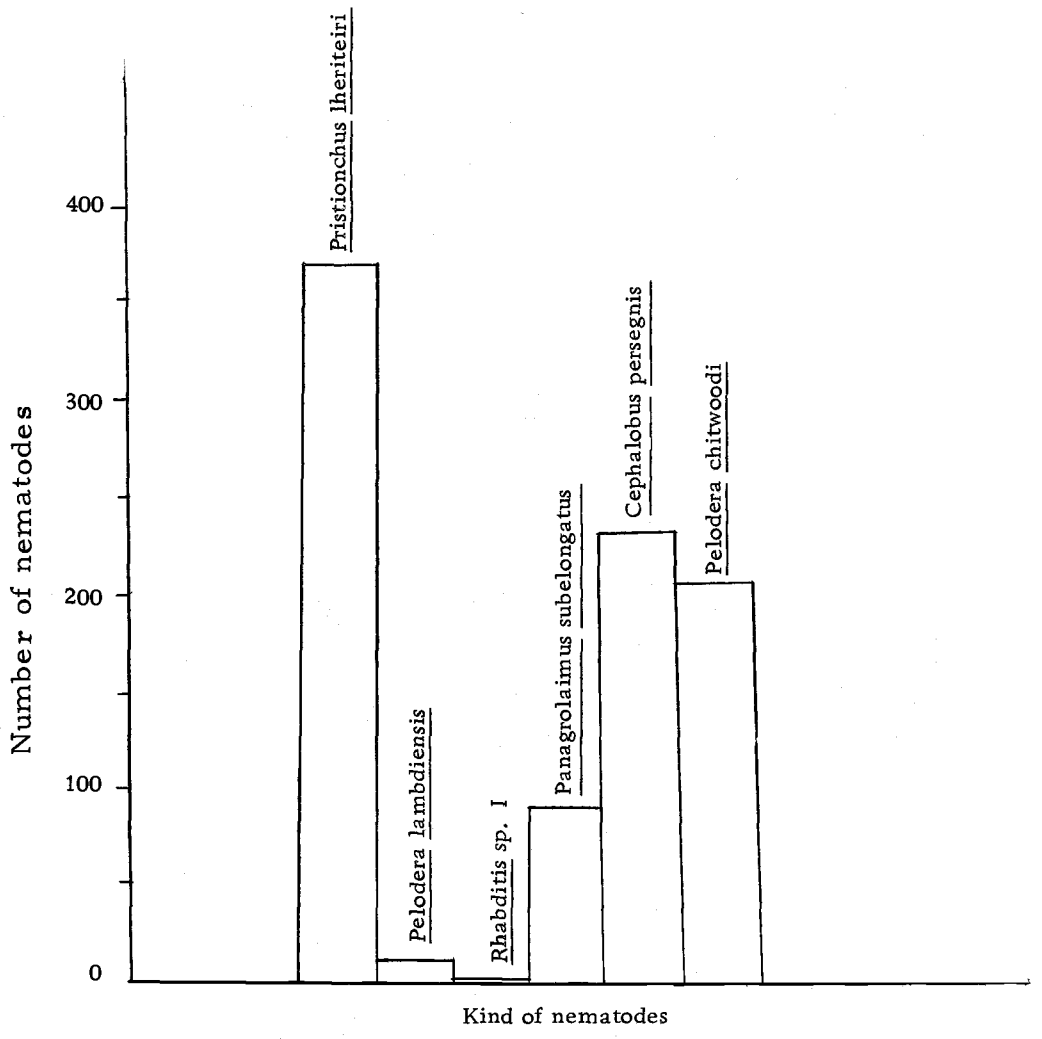

Figure 3. Comparison of populations of various nematodes 14 days after inoculation. (Averaged from experiment repeated five times, beginning with 20 nematode eggs for each species.)

A considerable variation in size of the populations of these six species may be caused by the specific food source. R. japonicum seems to be a desirable food source for P. lheriteiri, under these experimental conditions. It is possible that this food is not optimum for the five other kinds of nematodes to attain the same population size. However, studies of these five other nematodes using various other food sources is necessary in order to determine whether or not their individual growth rates are optimum on R. japonicum.

Evidently, among these six kinds of saprozoic nematodes, P. lheriteiri reproduces at the highest rate. This nematode is, therefore, probably the best of the group to disseminate the bacteria, R. japonicum, under the conditions of this experiment.

SECTION VII. NUMBER OF BACTERIA CARRIED INTERNALLY BY SAPROZOIC NEMATODES

Preface

From experiments in Sections III and IV, it is evident that the nitrogen-fixing bacterium, R. japonicum, was viable and survived nematode ingestion and defecation. It has also been reported that a female of P. lheriteiri could ingest as many as 46,000 bacterial cells and males 10,000 (16). This experiment was designed to assess the number of bacteria carried internally by six kinds of saprozoic nematodes.

Materials and Methods

Each kind of saprozoic nematode, as already mentioned, was reared on R. japonicum cultures for seven days. From each culture, 20 gravid females were hand-picked, surface-sterilized with 1:1,000 mercuric chloride solution for three minutes and rinsed with sterile distilled water. The nematodes were transferred then to a small BPI watch glass containing one ml sterile distilled water and aseptically crushed with a dissecting needle. Crushed nematode suspensions were diluted to 1:1,000 and poured into Petri dishes containing Rhizobium agar medium. The plates were kept at about 25° (78-80°F) for 96 hours before counting the bacterial colonies. Each nematode

species was considered a separate treatment. The experiment consisted of five replications and was repeated five times.

Results and Discussion

Average numbers of bacterial cells carried internally per nematode by each species are summarized in Table 7. It was found that P. lheriteiri female could carry 9, 034 bacterial cells while only 12 cells were carried by Rhabditis sp. I. Statistical differences both at 5% and 1% levels were found among three genera: P. lheriteiri, Pelodera lambdiensis and Panagrolaimus subelongatus but no significant difference among Rhabditis sp. I, Cephalobus persegnis and Pelodera chitwoodi.

Table 7. Numbers of R. japonicum per nematode carried internally by various species.[a]

	Nematode species					
Replication	P. lheriteiri	P. lambdiensis	Rhabditis sp. I	P. subelongatus	C. persegnis	P. chitwoodi
1	15,030	5,120	10	5,840	170	40
2	6,120	7,370	10	3,180	170	0
3	8,260	6,590	10	2,850	200	10
4	8,220	6,750	10	3,400	170	40
5	7,540	6,950	20	3,410	210	20
Average	9,034[b]	6,556[b]	12[c]	3,682[b]	184[c]	22[c]

LSD 0.05 = 1798.00; 0.01 = 2432.55

[a]Averaged from experiment repeated five times, each replication consisted of 20 nematodes.
[b]Significantly different from each other at both 5% and 1% levels.
[c]No significant difference between these three nematode genera.

These six kinds of saprozoic nematodes are commonly found and their major food supply is bacteria and other microorganisms. Ability of these nematodes to carry nitrogen-fixing bacteria internally varies among the species. The nematodes are generally large, except Rhabditis sp. I. There seems to be no relationship between nematode size and number of the bacteria carried internally in this study. Pelodera chitwoodi is a large nematode compared to Rhabditis sp. I, but they are both poor bacterial carriers. Cephalobus persegnis is also a large nematode but carries very few bacteria. There are two possibilities causing this variation. First, the source of food supply. It has been established that Rhabditis sp. I cannot survive and reproduce well on a R. japonicum culture. This may be why the number of bacteria taken internally is low. However, this assumption cannot apply to P. chitwoodi which exhibits a large increase on the bacterial culture but carries only a few bacteria in the body. Secondly, the exposure time. When nematodes were randomly picked out, some species might be slow feeders and had not ingested many bacteria during the short exposure time. Although bacteria were ingested, the result is lower bacterial cell recovery.

It should be pointed out that, although the relationship between saprozoic nematodes and nitrogen fixing bacteria was demonstrated, this relationship seems to be a simple one. Unlike the complex relationship of nematodes and some soil-borne viruses, the virus must

persist for a period of time in the nematode vector (69). Probably, the persistence of bacteria in the nematode body should be one of the relationships for future research workers to study.

SECTION VIII. NUMBER OF BACTERIA CARRIED EXTERNALLY BY VARIOUS SAPROZOIC NEMATODES

Preface

Few reports have been made on the role of plant parasitic nematodes carrying plant pathogenic bacteria on the body cuticle. Respectively, wheat nematode Anguina tritici and Ditylenchus dipsaci have been reported to carry bacteria Corynebacterium tritici and C. insidiosum on their body surface (30, 89). Saprozoic nematodes have not been demonstrated to carry bacteria externally even though many investigators have speculated that this occurs. The purpose of this experiment was to determine the number of bacteria (R. japonicum) carried externally by six different kinds of saprozoic nematodes.

Materials and Methods

Six kinds of saprozoic nematodes used in this experiment were reared separately on Rhizobium cultures for seven days. Ten gravid females were hand-picked near the edge of the dish, away from bacterial colonies, and placed in a small test tube containing one ml of sterile distilled water. The nematode suspension was shaken for 15 seconds to remove bacteria adhered to the body surface, and then the nematodes were removed. The bacterial suspension was diluted to 1:1,000 and one ml added to Rhizobium agar medium. Incubation

temperature was about 25°C (78-80°F) and bacterial counts were made after 96 hours. Each kind of nematode was considered a treatment. There were five replications and the experiment was repeated five times.

Results and Discussion

Results of this investigation on number of R. japonicum cells carried on body surface by various kinds of saprozoic nematodes are summarized in Table 8. Pelodera chitwoodi, the largest nematode, carries the most bacteria on its body surface while, by contrast, Rhabditis sp. I, the smallest, carries only a few bacteria on its cuticle. There is no significant difference between numbers carried by Pristionchus lheriteiri and Panagrolaimus subelongatus.

Table 8. Numbers of R. japonicum per nematode carried on body surface.[a]

	Nematode species					
Replications	P. lheriteiri	P. lambdiensis	Rhabditis sp. I	P. subelongatus	C. persegnis	P. chitwoodi
1	32,280	15,920	220	32,760	8,520	38,240
2	32,360	17,820	0	33,160	5,400	39,440
3	30,640	15,940	280	31,240	6,200	34,820
4	26,300	13,820	0	31,440	8,080	38,880
5	32,380	16,040	0	30,180	7,660	37,500
Average	30,792[b]	15,908	100	31,756[b]	7,172	37,776

LSD at 0.05 = 1688.987; at 0.01 = 2285.052

[a]Averaged from experiment repeated five times, each replication consisted of 10 nematodes.
[b]Averages with same subscript do not significantly differ at both 5% and 1% levels.

At least at the extremes in body size, there seems to be a direct relationship between number of bacteria and size of nematodes. When the area of the body surface is large, the bacterial number is high. More bacteria are found adhered to large nematodes, e.g., P. lheriteiri, Pelodera chitwoodi, P. lambdiensis and C. persegnis have relatively few bacteria even though they are nearly as large as P. lheriteiri. It should be noted that although the bacteria could be all over the agar medium surface, none of these nematodes were taken directly from bacterial colonies. Bacterial populations could be much higher if they were picked from feeding sites inside the colonies.

It is obvious that saprozoic nematodes can carry nitrogen-fixing bacteria on their cuticle. These results are similar to Hawn's report (30). Experimentally, he demonstrated, in 1971, that bacterial wilt organism, Corynebacterium insidiosum, were transmitted by alfalfa stem nematode (Ditylenchus dipsaci) on the external body surface. Conclusively, this experiment showed that the number of bacteria carried by saprozoic nematodes varies according to the kind and size of the carrier nematodes.

The combination of the finding in this experiment and that in Section VII demonstrates the possible role of saprozoic nematodes in transferring pathogenic and non-pathogenic microorganisms from one place to another. Each nematode species, whether it is large or small

in size, can transfer microorganisms like bacteria both internally and externally. Under standardized conditions, the numbers of bacteria carried are dependent mainly on the nematode body size and species.

CONCLUSION

The series of experiments reported in this study were developed to learn more about bacterial-nematode associations. Six kinds of saprozoic nematodes and a nitrogen-fixing bacterium (Rhizobium japonicum) were used as experimental organisms. Specific associations were made by sterilizing the external surfaces of nematodes with mercuric chloride (usually a 1:1, 000 solution for three minutes) and culturing them with R. japonicum for seven days prior to experimentation.

Although previous work indicated that Pristonchus lheriteiri could feed on certain microorganisms, there was no indication that they could survive on R. japonicum for an extended time. In the present work it was demonstrated that this nematode would not only feed but multiply and thrive on this food source. This did not appear to be so for the other five nematodes used in this study. None grew as rapidly or carried as many bacteria internally as P. lheriteiri. P. lheriteiri were found to carry internally 2, 622 colony-forming bacteria in the case of females and 1, 004 in the case of males after 96 hours. Not only were bacteria still viable after they were ingested and defecated by the nematodes, but they retained their capability to form nodules on soybean roots. After 30 days, 60 percent of the total tested plants formed nodules in the presence of bacteria-carrying

P. lheriteiri. This capability was not significantly different from that of bacterial suspensions.

When feeding was terminated, the nematodes defecated various numbers of viable bacteria over a 21-hour period. This number was much less than those ingested, so defecation probably could occur over a longer period of time. Attempts to further characterize the food were undertaken with preparations of autoclaved entire bacterial cells, broken cells, supernatant fluid from a bacterial suspension, and a bacterial suspension of live cells. No continuous growth occurred on anything except the suspension containing live bacteria. The exact reason that live bacteria are required as the nematode food has not been determined. It is not known whether the bacteria provide a metabolic product or whether reproducing colonies are necessary simply to provide a continuous energy source.

Although most attention was given to internal (ingested) bacteria, some attention was directed toward the bacteria which adhere to the surface of the nematode's body. Large numbers of bacteria can be transported in this manner. The number of bacteria was directly related to the size of the nematode.

This study indicates that saprozoic nematodes could perform a beneficial role by transporting nitrogen-fixing bacteria for short distances. The bacteria could be carried externally or internally.

Those borne internally are viable upon defecation and have not lost their ability to nodulate soybeans. Saprozoic nematode species may differ in their ability to transport bacteria.

SUMMARY

Results of experiments in this study are summarized as follows:

1. The nitrogen-fixing bacterium, Rhizobium japonicum, is susceptible to mercuric chloride solutions. Concentration of 1:1, 000 for three minutes can completely destroy the bacterial contamination.
2. Nematode eggs are very fragile and difficult to work with. Injury from handling and sterilization may lower the hatching percentage.
3. A saprozoic nematode, Pristionchus lheriteiri, grows and reproduces well on living cells of the nitrogen-fixing bacterium, Rhizobium japonicum suspension but not on killed bacteria, broken cells of sonicated bacteria or supernatant medium from centrifuged bacterial suspensions. Increase in number with growth begins with the sterilized eggs and is evident at every date of counting.
4. Female saprozoic nematode (P. lheriteiri) carries more bacterial cells in its alimentary canal than the smaller male -- 2, 622 and 1, 004 cells, respectively.
5. After ingestion by P. lheriteiri, R. japonicum survived passage through nematode alimentary tract and still are viable. R. japonicum defecated by P. lheriteiri are viable and reproduce on Rhizobium culture medium.

6. Saprozoic nematodes, Pristionchus lheriteiri, Panagrolaimus subelongatus, Cephalobus persegnis, Pelodera lambdiensis, Pelodera chitwoodi, and Rhabditis sp. I can be established on a nitrogen-fixing bacterial culture (Rhizobium japonicum) with a Rhizobium medium.
7. Among these six kinds of nematodes, Pristionchus lheriteiri grows and multiplies fastest within a two-week period when fed with R. japonicum, while Rhabditis sp. I grows slowest, i.e., P. lheriteiri, C. persegnis, P. chitwoodi, P. subelongatus, P. lambdiensis and Rhabditis sp. I, respectively, show decreasing numbers of individuals after two weeks.
8. Viable R. japonicum after ingestion by the nematode, P. lheriteiri, are carried internally and cause nodulation on soybean roots growing on water agar.
9. Above 50 nematodes at least, there is no significant difference between numbers of nodules formed on soybean roots with an increase in number of nematodes carrying nitrogen-fixing bacteria.
10. There are no statistically significant differences in the number of nodules formed by bacteria carried by P. lheriteiri or by dense bacterial suspension.
11. Numbers of bacteria carried internally by six species of saprozoic nematodes vary from one nematode to another. Nematode body size does not appear to have any clear relation to the number.

12. Numbers of nitrogen-fixing bacteria, adhering to the body surface of various saprozoic nematodes, appear to be dependent in part on body size of the nematode.

13. Saprozoic nematodes could be carriers and disseminators of a beneficial bacterium such as R. japonicum which is still viable after ingestion and defecation by the nematodes and capable of causing nodulation on leguminous host plants.

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APPENDICES

APPENDIX A

Culture Medium Formulae

1. Nutrient agar (4). The culture medium consists of:

Beef extract	3 gm
Bacto peptone	5 gm
Bacto agar	15 gm
Distilled water	1,000 ml

2. Rhizobium agar medium (18). The culture medium consists of two stock solutions.

Stock A	K_2HPO_4	100 gm
	KH_2PO_4	100 gm
	$MgSO_4$	36 gm
	$CaSO_4$	13 gm
	KNO_3	70 gm
	Yeast extract	100 gm
Stock B	$FeCl_3$	4 gm
	Distilled water	1,000 ml

Preparation: Use 4.2 gm stock A
0.1 ml stock B
4.0 ml glycerin (or 1.0 gm arabinose)
15.0 gm agar

Preparation: (Cont.) 1,000.0 ml distilled water

pH was adjusted to 6.3 with 3 N HCl.

3. Rhizobium broth. It was modified from above, except the agar was not included.

4. 0.5% water agar (86). The culture medium consists of:

Bacto agar	5 gm
Distilled water	1,000 ml

APPENDIX B

Table 9. Number of nematodes (P. lheriteiri) recovered at two days from modified bacterial suspension (R. japonicum).[a]

Treatment	Rep. I	Rep. II	Rep. III	Rep. IV	Rep. V	Average
Control	1.2	1.0	1.2	0.6	1.2	1.04
Autoclaved bacteria	0.8	1.6	1.2	1.4	1.0	1.20
Sonicated bacteria	1.2	1.2	1.2	1.8	1.4	1.36
Bacterial suspension	3.6	3.0	2.2	2.6	2.8	2.84
Culture supernatant	0.8	1.0	1.0	1.0	1.2	1.00

[a]Averaged from experiment repeated five times.

Table 10. Number of nematodes (P. lheriteiri) recovered at seven days from modified bacterial suspension (R. japonicum).[a]

Treatment	Rep. I	Rep. II	Rep. III	Rep. IV	Rep. V	Average
Control	1.6	1.6	1.6	0.8	1.6	1.44
Autoclaved bacteria	1.0	1.6	1.4	1.4	1.8	1.44
Sonicated bacteria	1.4	1.2	2.2	2.0	1.6	1.68
Bacterial suspension	16.2	31.0	11.6	12.2	11.8	16.58
Culture supernatant	1.0	1.0	3.0	1.2	1.4	1.52

[a]Averaged from experiment repeated five times.

Table 11. Number of nematodes (P. lheriteiri) recovered at ten days from modified bacterial suspension (R. japonicum).[a]

Treatment	Rep. I	Rep. II	Rep. III	Rep. IV	Rep. V	Average
Control	1.6	1.8	1.8	0.8	1.6	1.52
Autoclaved bacteria	1.2	1.8	1.4	1.4	1.8	1.52
Sonicated bacteria	1.4	2.0	1.2	2.0	1.6	1.64
Bacterial suspension	38.0	132.4	46.2	39.6	89.0	69.04
Culture supernatant	1.4	1.0	3.6	1.4	2.0	1.88

[a]Averaged from experiment repeated five times.

Table 12. Number of nematodes (P. lheriteiri) recovered at 14 days from modified bacterial suspension (R. japonicum).[a]

Treatment	Rep. I	Rep. II	Rep. III	Rep. IV	Rep. V	Average
Control	1.8	1.8	2.2	0.8	1.6	1.64
Autoclaved bacteria	1.2	1.8	1.4	1.4	2.0	1.56
Sonicated bacteria	1.4	2.0	1.2	2.0	1.6	1.64
Bacterial suspension	87.8	217.4	118.8	122.0	163.4	141.88
Culture supernatant	1.6	1.0	4.2	1.6	2.0	2.08

[a]Averaged from experiment repeated five times.