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Harold J. Jensen

Three species of bacteriophagous nematodes, Pristionchus lheritieri (Maupas) Paramanov, Pelodera chitwoodi (Bassen) Dougherty and Rhabditis sp. were studied to determine their potential roles as vectors of selected insect pathogens, Bacillus thuringiensis Berliner, Beauveria bassiana (Balsamo) Vuillemin, Metarrhizium anisopliae (Metchnikoff) Sorokin and a nuclear polyhedrosis virus of Trichoplusia ni Hubner.

Chlorine tolerances were established for P. lheritieri, P. chitwoodi and Rhabditis sp. Investigations established that B. thuringiensis was more susceptible to the action of chlorine than the nematodes. Feeding studies provided evidence that B. thuringiensis is an unsatisfactory monoxenic associate of P. lheritieri. Chlorination treatments following feeding of B. thuringiensis allowed the

development of evidence that all three nematode species do ingest and defecate *B. thuringiensis* in a viable condition. It was demonstrated that *P. lheritieri* and *Rhabditis* sp. retained viable *B. thuringiensis* up to 36 and 42 hr respectively. *P. lheritieri* and *Rhabditis* sp. continued to defecate viable *B. thuringiensis* up to 33 and 36 hr respectively. Attempts to infect *T. ni* directly with *B. thuringiensis* fed nematodes failed. Subcultures of *B. thuringiensis* defecated by *P. lheritieri* retained their pathogenicity to *T. ni*.

The insect pathogenic fungi *B. bassiana* and *M. anisopliae* proved to be more susceptible to the action of chlorine than the bacteriophagous nematodes. The studies indicate that *B. bassiana* and *M. anisopliae* are not a preferred food source for *P. lheritieri*. Evidence for ingestion of *M. anisopliae* by *P. lheritieri* was not obtained. Results failed to demonstrate defecation of viable spores of either fungus. Viable spores of *B. bassiana* were recovered from the gut of *P. lheritieri* and subcultured. The subcultured *B. bassiana* retained pathogenicity to larvae of *T. ni*.

P. lheritieri were positively attracted to and actively fed on the cadavers of nuclear polyhedrosis killed *T. ni* larvae. Single nematodes, after feeding on *T. ni* larvae killed by virus, transmitted the virus to cabbage loopers. The nuclear polyhedrosis virus of *T. ni* proved to be less susceptible to chlorination treatments than the bacteriophagous nematodes. Evidence for active ingestion

of virus particles by the nematode was developed by obtaining inoculum suspensions from nematodes which had been exposed to a virus source for various periods of time. A bioassay of these suspensions revealed an apparently greater virulence in those suspensions produced from nematodes having a longer access to virus.

These studies provided evidence that bacteriophagous nematodes can have an important role in insect disease development in nature. The nematodes acquired viable fungus, bacterial, and virus pathogens by active ingestion. They retained and actively disseminated bacterial pathogens over an adequate period of time. A fungus pathogen retained virulence after ingestion by the nematode and a bacterial pathogen retained viability and pathogenicity after passage through the nematode gut. A single nematode transmitted enough virus inoculum to cause disease in the insect host. Of the nematode-pathogen associations studied, the intensely virulent nematode-virus complex demonstrated potential for biological control utilization. These factors, added to the mobility, habits, and known insect associations of bacteriophagous nematodes, enhance our understanding of the potential roles of these nematodes in insect disease development.

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Professor of Botany
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Redacted for Privacy

Chairman of Department of Botany and Plant Pathology

Redacted for Privacy

Dean of Graduate School

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LABORATORY STUDIES OF BACTERIOPHAGOUS NEMATODES AS POTENTIAL VECTORS OF INSECT PATHOGENS

INTRODUCTION

The importance of nematodes as parasites of animals and plants has long been recognized. Initially these parasites were studied and evaluated only according to their direct effects on the host organism. During the last 35 years, however, much attention has been given to animal and plant parasitic nematodes with respect to their influence on other pathogens and disease development in a common host organism.

Since parasitic nematodes have received the emphasis in research for possible pathogen associations, the ever present and apparently innocuous free-living nematodes have been essentially overlooked. The large majority of free-living terrestrial nematodes are saprophagous, feeding on microorganisms associated with decaying plant and animal matter. Many saprophagous nematodes have been cultured in the laboratory where they feed primarily on bacteria. Recent laboratory investigations utilizing these bacteriophagous nematodes have revealed their potential for carrying pathogens of important diseases. Chang *et al.* (1960) demonstrated their capacity for carrying human enteric bacteria and certain human virus pathogens. Jensen (1967) and Chantanao (1969) have reported that

bacteriophagous nematodes can carry several important fungus and bacterial pathogens of plants.

There are many free-living, bacteriophagous nematodes closely associated with insects. Most have never been studied with respect to their relationships to insect pathogens. The ubiquitous distribution of these nematodes in soil makes them highly important associates of all soil inhabiting insects. Additionally bacteriophagous nematodes are associated with insects in specialized habitats such as insect frass in beetle tunnels, rotting plant parts, animal cadavers, and even sewage. Such nematodes have been associated with dead and dying insects (Weiser, 1966; Fedorko and Stanuszek, 1967); Weiser suggests the possibility of a nematode introduced bacterial infection as the cause of insect death.

A knowledge of the potential roles which free-living bacteriophagous nematodes may have as vectors or reservoirs of insect pathogens could be of importance for practical considerations in biological control and could reveal informational keys to the development of nematode-pathogen symbiosis.

In this investigation several parameters of potential vector-pathogen associations involving free-living bacteriophagous nematodes and insect pathogens were studied. The proposal that these nematodes can actively ingest insect pathogens in the course of feeding on dead insects or laboratory cultures of insect pathogens was investigated.

The viability and virulence of insect pathogens ingested and subsequently defecated by saprophagous nematodes were studied. The ability of a bacterial insect pathogen to support growth and development of certain bacteriophagous nematodes was determined. The capacity of certain saprophagous nematodes to carry sufficient inoculum to cause disease in a host insect was studied.

LITERATURE REVIEW

Parasitic Nematode - Pathogen Relationships

Animal Parasites

Shope (1939) demonstrated that several lungworms of the genus *Metastrongylus* were intermediate hosts for the swine influenza virus (Shope, 1941). Burrows and Swerdlow (1956) suggested that *Dientamoeba* enters its host in the eggs of the pinworm *Enterobius*. Chandler and Read (1961) indicated that species of *Trichinella* are capable of carrying the viruses of lymphocytic choriomeningitis and poliomyelitis into the host's nervous system. *Histomonas meleagridis* Tyzzer, the protozoan which causes infectious enterohepatitis in turkeys, is normally transferred to a new host by becoming enclosed in the egg shells of the nematode cecal worm, *Heterakis* (Chandler and Read, 1961).

Dutky (1937) demonstrated an important symbiotic relationship between a nematode parasite of *Popilla japonica* Newman and a bacterial pathogen of that insect. Dutky reported that an unidentified species of *Neoaplectana* could readily transfer the bacterial pathogen from one lot of *P. japonica* larvae to another.

Interest in such associations was renewed when Dutky and Hough (1955) discovered a disease complex involving again a neoplectanid nematode and an undescribed bacterium in the larvae of

Carpocapsa pomonella L. The complex was known as DD-136; the nematode has since been identified by Poinar (1967) as Neoaplectana carpocapsae Weiser, the bacterium, Achromobacter nematophilus Poinar and Thomas. The wide range of insect species susceptible to this complex has given considerable encouragement that it can be useful as a control agent.

While N. carpocapsae is a well adapted parasite of insects, it is of considerable significance that it is a bacterial feeder and can be cultured in the laboratory without benefit of the host insect. This is true of other species of Neoaplectana as well (Steinhous, 1949). It would appear that N. carpocapsae parasitism could very well represent a type of adaptation in which a free-living bacteriophagous nematode having a close association with insects has become a degree more dependent upon the insect and thus parasitic. N. E. Welch (Steinhous, 1963) suggested that attempts to feed other bacterial pathogens of insects to neoaplectanids might reveal significant information concerning the development of the nematode-bacterial symbiosis.

Plant Parasites

Associations of plant parasitic nematodes with plant pathogens are extensive and well documented. There are plant parasitic nematodes associated with host disease development caused by certain

plant pathogenic fungi, bacteria, and viruses. Atkinson (1892) experimenting with *Fusarium* wilt in cotton noted that root-knot nematode infections appeared to increase the incidence and severity of the wilt disease in cotton. Importance of this nematode pathogen association was better appreciated when Young (1939) reported that root-knot infection of tomatoes decreased the expression of resistance to *Fusarium* wilt in his tomato breeding lines. Increased importance was given to the *Fusarium*-root-knot complex when Schindler *et al.* (1959) reported a synergistic effect on disease expression in carnations when both nematode and fungus pathogen were present in the host.

Carne (1926) and Cheo (1946) incriminated *Anguina tritici* (Steinbuch) Filipjev as an essential vector of *Corynebacterium tritici* (Hutchinson) Bergey *et al.* in the development of the yellow slime disease of wheat. Until Hewitt *et al.* (1958) demonstrated that *Xiphinema index* Thorne and Allen transmitted fan leaf virus in grapevine, the means by which soil borne viruses persist in soil and spread from host to host was essentially unexplained.

Free-Living Nematode - Pathogen Relationships

The ubiquitous, free-living, bacteriophagous nematodes have only recently been considered in any detail with respect to their potential as transmitters or reservoirs of pathogens of any kind.

Ziegler in 1895 observed living flagellates in the gut of *Diplogaster* spp. (Goodey, 1951), thus indicting these nematodes as potential carriers of viable microorganisms. Steiner (1933) associated *Rhabditis lambdiensis* Maupas with a mushroom disease caused by *Pseudomonas tolassii* Paine. He suggested that the nematodes carried the bacteria externally or internally and were active in spreading the pathogens on the mushroom surfaces.

Subsequently Chang *et al*. (1960) found that *Cheilobus quadrilabiatus* Cobb, and *Diplogaster nudicapitatus* Steiner would readily feed on human enteric bacteria. *Shigella sonnei* (Levine) Weldin, *Salmonella typhosa* (Zopf) White and *S. paratyphi* (Kayser) Cantellani all survived two days in the digestive tracts of the nematodes but were not defecated in a viable condition. All three species of bacteria were viable in the gut of the nematode following chlorination treatments sufficiently concentrated to kill the nematodes and exposed bacteria. The same authors demonstrated that the nematodes could ingest Coxackie and Echo viruses and defecate them in a viable infective condition. As a result of this work it is apparent that certain human pathogens can survive routine water chlorination procedures if protected within the gut of bacteriophagous nematodes. The common occurrence of these nematodes in sewage provides an easy access to human enteric bacteria; hence a very real possibility of their becoming carriers or reservoirs of enteric pathogens

does exist.

Bacteriophagous nematodes may be involved in spreading and protecting plant pathogens. Jensen (1967) demonstrated that several plant pathogens could be readily ingested by *Pristionchus (Diplogaster) lheritieri* (Maupas) Paramanov, *Panagrellus redivivus* (Linn.) Goodey, *Panagrolaimus subelongatus* (Cobb) Thorne, and *Rhabditis* spp. Spores of *Fusarium oxysporum* F. *lycopersici* (Sacc.) Synd. and Hans. and *Verticillium dahliae* Kleb. were readily ingested and protected against a surface sterilization of the nematodes designed to destroy any externally adherent fungus spores. The spores were subsequently defecated by the nematodes in a viable condition. The bacterium *Pseudomonas syringae* Van Hall was also ingested and defecated in a viable condition by the same nematodes. Chantanao (1969) working with other plant pathogenic bacteria actually cultured *Pristionchus lheritieri* monoxenically on *Agrobacterium tumefaciens* (E. F. Sm. and Town.) Conn, *Erwinia amylovora* (Burr.) Winsl. *et al.*, and *Erwinia carotovora* (L. R. Jones) Holland. The nematodes readily fed on these bacteria, eventually defecating some in a viable condition.

Further evidence to support the concept of potential virus transmission by bacteriophagous nematodes was given by Jensen and Gilmour (1968). These authors demonstrated that *P. lheritieri* and

P. redivivus can readily ingest and transmit a viable, infective phage of Streptomyces griseus (Krainsky) Waksm. and Schatz. Chantanao (1969) reported the transmission of a phage of Agrobacterium tumefaciens by P. lheritieri.

While previous research has not been aimed directly at revealing the potential role of bacteriophagous nematodes as vectors or transmitters of insect pathogens, reports have been made which bear directly on this issue. Weiser (1966) reported the collection of Pristionchus lheritieri from dead larvae of Saperda carcharias (Linnaeus), Cryptorrynchoides lapathi Linnaeus, Melolontha melolontha Linnaeus, and Hoplia sp. The same author reported laboratory induced infection of Galleria mellonella Linnaeus larvae by P. lheritieri which resulted in insect mortality. Weiser suggested that nematode larvae penetrated the insect gut probably carrying bacteria into the hemocoel with a resultant septicemia causing insect death.

Pristionchus uniformis Fedorko and Stanuszek has been reported to infect and kill as much as 86 percent of natural populations of Leptinotarsa decimlineata Say (Fedorko and Stanuszek, 1967). Sandner and Stanuszek (1967) artificially infected L. decimlineata with P. uniformis, but he considered the insect mortality to be caused by a septicemia. He suggested that bacteria were introduced into the body cavity of the insects by nematodes. Poinar (1969)

followed with laboratory tests of P. uniformis and P. lheritieri on Galleria mellonella to determine the pathogenicity of these nematodes. A very low percentage of wax moths orally infected with nematodes were killed. Poinar considered death to be caused by a septicemia caused in turn by Escherichia coli (Migula) introduced into the body cavity by penetrating nematodes. E. coli did not infect G. melonella when administered orally. Chantanao (1969) in working with bacterial pathogens of plants used Serratia marcescens Bizio to culture P. lheritieri in the laboratory. He demonstrated that this bacterial pathogen of insects could be readily carried by P. lheritieri.

Nematode - Insect Relationships

Nematode-insect associations are extensive in nature. Van Zwaluwenburg (1928) presented a list of 759 insect species which were known to be associated with nematodes. Steinhous (1949) estimated that there were in excess of 1000 species of nematodes associated with insects. Associations range from obligate parasitism to a kind of commensalism in which nematodes and insect larvae merely share a common niche. The degree of variation and gradation between association types makes it impossible to separate them clearly. Christie (1941) divided the entomophilic nematodes into three convenient groups. (1) The first group consists of those parasitic nematodes which live in the digestive tract of the host insect, generally have

simple life cycles, and which do not fall into the second grouping. (2) Christie characterizes the second group of nematodes as novitious parasites and semiparasites. These nematodes are related to the free-living species and may combine saprophagous and parasitic habits. Some members of this group live and reproduce in cadavers of insect hosts while others may alternate parasitic and free-living generations. (3) The third group is limited to the highly specialized obligate parasites. These nematodes parasitize the body cavity and tissues of the host insect, spending only a short time in the digestive tract of the host.

Of Christie's groupings, categories one and two are of interest in this study because of the nematodes' apparent bacteriophagous food habits. Filipjev and Steckhoven (1941) indicate that the group one nematodes are found in insect hosts whose digestive tracts provide for the development of a rich bacterial flora in the rectal area. These nematodes feed and reproduce in this area rarely invading the vital tissues of the host, thus causing little harm to the host insect (Steinhous, 1949). While certain of these nematodes have developed a dependence upon the host organism for completion of their life cycles (Dobrovolny and Ackert, 1934), they probably retain food habits similar to those of the semiparasites of Christie's group two, or free-living bacteriophagous nematodes.

Neoaplectana carpocapsae characterizes Christie's group two

insect parasite. The nematode's dependence upon *Achromobacter nematophilus* to complete its life cycle serves to emphasize the similarity of its food habits to taxonomically related bacteriophagous nematodes. Christie's grouping of entomophilic nematodes fails to include those insect-nematode associations that are not clearly parasitic in nature. It is in this realm that we find a wealth of nematode-insect associations. For the most part these associations are poorly defined but involve bacterial feeding nematodes.

Reports of bacteriophagous nematodes occupying the same ecological niche with insects are too numerous to mention. Bacteriophagous species of the families Rhabditidae and Diplogasteridae frequently inhabit soil and various organic media which are commonly occupied by insects as well. Goodey (1951) lists decaying plant and animal matter such as tubers, bulbs, seeds, roots, animal cadavers, dung and sewage as typical habitats for these nematodes.

Nematode associations with insects in more specialized habitats such as bark beetle tunnels may be more specific (Fuchs, 1915; Steiner, 1932; Massey, 1956, 1964, 1966); here certain nematode species are reported to be found only with certain insect species. Predatory roles have been postulated for the nematodes in these associations, but little experimental evidence has been presented to support this view. These nematode-insect associations remain poorly defined.

Other bacteriophagous nematodes have established phoretic associations with insects. Early reports were made by Maupas (1899) who observed dauer stages of *Diplogaster* spp. under the elytra of several coleoptera. Numerous reports describe diplogasterid larvae recovered from the elytra of beetles (Fuchs, 1915; Steiner, 1930; Bovien, 1937; Massey, 1956). Christie and Crossman (1933) observed larval diplogasterids attaching themselves to roaches. Triffit and Oldham (1927) reported that *Rhabditis coarctata* Leuck. larvae are dependent on dung beetles for transport to fresh dung, without which development to sexual maturity does not occur. Other bacterial feeding species of *Rhabditis* are described as phoretic associates of various insect species (Aubertot, 1923; Bovien, 1937).

More intimate associations have been reported for bacteriophagous nematodes and insects. The nematodes enter external openings and the digestive tract of the insects. Fuchs (1915, 1937) reported that *Rhabditis obtusa* Fuchs, which may pass its entire life cycle in the frass of bark beetle tunnels, has larval forms which occasionally enter the hind gut of the beetle to be transported to new tunnels. Dauer larvae of several diplogasterids, considered to be primarily bacteriophagous, have been found in the genital chambers or in the genital segment of several insect species (Sachs, 1950; Korner, 1954; Ruhm, 1956). *Neodiplogaster wacheki* Korner dauer stages were found by Korner (1954) in the trachea of two cerambycid

beetles. Ackert and Wadley (1921) reported that *Cephalobium microbivorum* Cobb develops in the gut of *Gryllus assimilis* Fabr. The nematode eggs pass out with the feces and new hosts ingest larval nematodes with food. The pharyngeal glands of certain ants house dauer stages of *P. lheritieri* and *Monochoides histophorus* Weingartner (Wahab, 1962).

Several reports have associated diplogasterid nematodes with dead or dying insects. Davis (1919) reported that 90 percent of the *Phyllophaga* grubs in one field were killed by nematodes. The nematodes were identified as *Mikoletzkya aerivora* Cobb and a species of *Cephalobus*. Adult *Mikoletzkya aerivora* were recovered from the heads of sick and dead termites (Banks and Snyder, 1920). Kotlan (1928) reported *Diplogaster brevicauda* Ellinger on dead larvae of *Pyrausta nubilalis* Hubner. Steiner (1929) found *Mesodiplogaster maupasi* Potts in a dead specimen of *Pamphilius stellatus* Christ. *Mikoletzkya aerivora* specimens were recovered from dead pupae of *Nodonota puncticollis* Say and *Heliothis armigera* Hubner (Christie, 1941). None of the above reports offers evidence that the nematodes were the direct cause of insect death. These nematodes would be attracted to the dead insects because of their saprophagous feeding habits.

Evidence has been presented which indicates that saprophagous nematodes may play an important role in causing insect mortality.

Winburn and Painter (1932) reported that larvae of Heliothis obsoleta Fabricus placed on soil infested with Mikoletzkya aerivora died in about eight days. The insect cadavers were consumed by the nematodes, but no details were given on the disease development. Phyllophaga grubs died after being exposed to Diplogaster sp. (Chamberlin, 1944). Nematodes were recovered from the cephalic region of the grubs; however, nematode reproduction occurred only after the grubs had died.

MATERIALS AND METHODS

The investigations in this study progressed from the framework of a model system involving four basic procedures. (1) Tolerances of insect pathogens and bacteriophagous nematodes to certain concentrations of chlorine for various periods of time were established. (2) A nematode-pathogen relationship was established by permitting nematodes to feed on media inoculated with insect pathogens. (3) Ability of the nematode to ingest, retain, and defecate viable pathogens was verified by placing surface sterilized nematodes on appropriate culture media for pathogen growth, subsequent isolation and identification. (4) To determine if the nematode could transfer infective pathogens to an insect host, surface sterilized nematodes previously exposed to pathogens were placed on a common medium with the insects. Host insects were observed for disease development, and isolation of the pathogen was attempted.

The following materials and procedures were in general use during various phases of the study.

Materials

The following preparations were used as culture media in certain investigations in this study.

1. Nutrient agar was prepared by hydrating five grams of Bacto peptone, three grams of beef extract and 15 grams of agar

flakes in one liter of distilled water.

2. Tryptose phosphate agar contained a preparation of five grams of tryptose, five grams of yeast extract, two grams of glucose, five grams of sodium chloride, 2.5 grams of dibasic sodium phosphate, and 15 grams of agar flakes hydrated in one liter of distilled water.

3. Yeast peptone agar was prepared by hydrating 20 grams of glucose, ten grams of peptone, five grams of yeast extract, and 15 grams of agar flakes in a liter of distilled water.

4. An antibiotic agar contained nutrient agar plus Tetracycline hydrochloride. A 1000 ppm Tetracycline medium was prepared by adding one ml of a 10,000 ppm aqueous Tetracycline solution to nine ml of melted sterile nutrient agar at approximately 47° C. The antibiotic solution and nutrient agar mixture was gently swirled to achieve an even distribution of the materials.

5. Martin's medium contained a preparation of 20 grams of agar, one gram of potassium monobasic phosphate, one half gram magnesium sulfate, five grams of peptone, ten grams of dextrose, and 0.033 grams rose bengal hydrated together in one liter of distilled water. Three hundredths gram of streptomycin sulfate was added to the above after autoclaving.

6. A simple artificial medium reported by Shorey and Hale (1965) was used to rear _Trichoplusia ni_. The general composition

included dried pinto beans, yeast, ascorbic acid, agar, water, methyl p-hydroxybenzoate, sorbic acid and formaldehyde.

Except for the insect rearing medium all the media were sterilized in an autoclave maintained at 17 psi for 20 min.

General Procedures

Source and Maintenance of Insect Pathogens

1. *Bacillus thuringiensis* var. *thuringiensis* Berliner was obtained as a commercial dry powder preparation containing approximately 8×10^9 spores per gram. *B. thuringiensis* was subcultured from this preparation on a tryptose phosphate agar. Plate and slant cultures were maintained at room temperature.
2. *Beauveria bassiana* (Balsamo) Vuillemin was subcultured from an isolate obtained by Dr. Clarence Thompson, Entomologist, Forestry Sciences Laboratory, Oregon State University, Corvallis, Oregon. The isolate (Bb-141) was obtained from a European cornborer *Pyrausta nubilalis*, (Hubner). Subcultures for these studies were maintained on yeast peptone agar plates and slants. Sporulation was enhanced by maintaining cultures at 100 percent relative humidity at approximately 23° C. Sporulated slant cultures were stored at room temperature and subcultured every 14 days for the duration of the study.

3. *Metarrhizium anisopliae* (Metchnikoff) Sorokin was also obtained from Dr. Clarence Thompson, Entomologist, Forestry Sciences Laboratory, Oregon State University, Corvallis, Oregon. *M. anisopliae* was subcultured from isolate (M-1) and was maintained for this investigation on yeast peptone agar plates and slants. Sporulation, storage and subculture were handled as with *B. bassiana*.
4. Nuclear polyhedrosis virus inoculum from *Trichoplusia ni* (Hubner) was secured from Dr. Clarence Thompson, Entomologist, Forestry Sciences Laboratory, Oregon State University, Corvallis, Oregon. The inoculum had been stored as air dried smears made from virus killed *T. ni* larvae. Fresh inoculum was secured by infecting five-day old *T. ni* larvae with an aqueous suspension of the virus inclusion bodies in the smears. Inoculum was then readily available from the cadavers of the infected larvae.

Source and Maintenance of Bacteriophagous Nematodes

Three species of saprophagous nematodes were used in these investigations. *Pristionchus lheritieri*, (Maupas) Paramanov, *Pelodera chitwoodi*, (Bassen) Dougherty and an unidentified species of *Rhabditis* were all obtained from cultures maintained in the Nematology Laboratory in the Department of Botany and Plant Pathology,

Oregon State University, Corvallis, Oregon. They had been maintained on an unidentified species of *Pseudomonas* cultured in plates of nutrient agar.

Throughout these investigations the nematodes were reared in a similar fashion. At approximately weekly intervals one cm square sections of agar containing several nematodes were transferred aseptically from old cultures to fresh nutrient agar plates. The cultures were maintained at room temperature.

Source and Maintenance of Insect Larvae

A colony of cabbage loopers, *Trichoplusia ni* Hubner, obtained from Dr. R. E. Berry, Oregon State University Entomology Department, was increased and maintained in the laboratory using the general methods of Shorey and Hale (1965). Mating chambers were made from gallon cans. Heavily waxed paper stapled to blotter paper lined the cans to provide an egg laying surface. Twenty-four adults, 12 males and 12 females, were placed in each chamber. A gauze netting held in place with a rubber band closed the chambers. A four ounce bottle of ten percent glucose solution provided nutrition for the adults. A piece of cellulose sponge placed in the bottle served as a wick and prevented entrapment of the moths.

When the egg sheets were removed from the mating chambers, the sheets were soaked five minutes in 0.1 percent sodium hypochlorite

in a 0.02 percent solution of Triton X-100[1]. The egg sheets were rinsed in sterile water and blotted before being placed in closed one gallon, wide-mouth jars. A 100 ml beaker of sterile water placed in the center of the jar maintained adequate moisture.

The larvae were removed from the egg sheets on a sterile camel hair brush and placed in six ounce paper ice cream cups containing about two ounces of nutrient medium. Larvae were placed 15 to a cup. Each cup was examined at the end of seven days to determine the condition of the larvae. If insufficient food remained the larvae were changed to fresh cups of nutrient medium. Most of the pupae were in cocoons attached to the rearing cup lids. The pupae were removed from the lids with sterile forceps, sexed and placed in separate quart jars for adult emergence.

Standardization and Neutralization of Chlorine Solutions

Sodium hypochlorite (Clorox)[2] was used as a source of chlorine to effect surface sterilization of bacteriophagous nematodes. Various chlorine concentrations were required to establish chlorine tolerances of the insect pathogens and nematodes. The Iodometric

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[1] Registered trademark of Rohm and Haas, Philadelphia, Pennsylvania.

[2] Registered trademark of the Clorox Co., Oakland, California.

Method (American Public Health Association, 1965) was used to determine the concentration of available chlorine in the sodium hypochlorite solutions in parts per million. A stock solution of 200 ppm of chlorine prepared with sterile distilled water served the chlorine requirements for most procedures. New stock solutions were prepared on a regular basis and were repeatedly checked for chlorine content. Working solutions were prepared by diluting the stock solution to the desired concentrations with sterile distilled water.

Since the chlorination processes involved timed periods of exposure to the action of the chlorine, sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$) as a sterile 10 percent solution was used to neutralize the chlorine to halt its sterilizing action.

CHLORINE TOLERANCE OF CERTAIN BACTERIOPHAGOUS NEMATODES

Preface

During the process of feeding, saprophagous nematodes move over and through decaying organic matter laden with microorganisms. As a result microorganisms become surface adherents on the nematode cuticle. In the investigations to follow, those organisms actually ingested and subsequently defecated by nematodes are of primary interest. To prevent confusion, externally borne microorganisms must be eliminated.

Satisfactory surface decontamination of saprophagous nematodes by the use of dilute chlorine solutions has been reported by several workers (Chang *et al.*, 1960; Jensen and Gilmour, 1968; Chantanao, 1969). They submerged nematodes in a chlorine solution sufficiently concentrated and for a time period of adequate duration to destroy surface contaminants without killing the nematodes. In this investigation the tolerances of *Pristionchus lheritieri*, *Pelodera chitwoodi*, and a species of *Rhabditis* to certain concentrations of chlorine for various time periods of exposure were studied. From information gained in this study suitable chlorine concentrations and exposure times were chosen for use in further experimentation.

Procedures

Nematodes maintained on their normal bacterial symbiont and cultured on nutrient agar were used for this study. They were washed from culture plates onto a 325 mesh screen and further rinsed with sterile distilled water. From the screen nematodes were washed into a Syracuse watch glass and individually moved into separate watch glasses with a bamboo pick. Each glass contained five ml distilled water and received ten nematodes. The final concentrations of chlorine were achieved by adding to these glasses five ml of a chlorine solution of the appropriate strength. Stock chlorine solutions were made up in a 0.04 percent solution of Triton X-100 so that final chlorine solutions were in 0.02 percent Triton X-100. The chlorine concentrations evaluated were 10, 20, 30, 40, 50, 100 and 200 ppm. Nematodes were checked for viability with a stereoscopic microscope at five minute intervals for a total of 60 minutes. Nematodes not moving at any time interval were considered to be dead if they failed to respond to tactile stimuli applied with a bamboo pick. The dead nematodes were removed as they were identified. Four replicates of ten nematodes each were done for each concentration. Controls were handled in the same manner except that a sterile distilled water solution of 0.02 percent Triton X-100 was used instead of a chlorine solution.

Results and Discussion

All three species of nematodes *P. lheritieri*, *P. chitwoodi*, and *Rhabditis* sp. tolerated considerable exposure to chlorine. At the two highest concentrations, 100 and 200 ppm of chlorine, the mortality rate was high. However, at least 50 percent of the nematodes in all three species survived a ten minute treatment with 200 ppm of chlorine, Tables 1 to 3. These results appear to be consistent with those of Chang *et al*. (1960) who found that 50 to 60 percent of *Cheilobus quadrilabiatus* Cobb, and *Diplogaster nudicapitatus* Steiner, survived a five minute exposure to 95-100 ppm of chlorine while only 10 to 20 percent survived a 15 minute exposure. Chantanao (1969) reported that *P. lheritieri* tolerated a five minute exposure to 100 ppm of chlorine without mortality. Results indicate that with increasing chlorine concentration there is a decreasing rate of survival. Survival was similarly affected by increased duration of exposure at all levels of chlorine concentration. Figure 1 shows the wide range of concentrations and exposure periods survived by at least 90 percent of the nematodes in three species tested. The three nematode species readily survived chlorine treatments adequate to destroy unprotected bacterial and fungus pathogens of interest in these investigations.

Observations made during this particular study emphasized the

desirability of choosing a shorter exposure period at higher chlorine concentrations over alternate lower concentrations held for a longer period of time to achieve eradication of surface contaminants. The chlorine solutions appeared to irritate nematodes causing them to greatly increase random movements. In nematodes there is some degree of independence in the movement of gut and body wall. However, it appeared that longitudinal contractions of muscle cells located in lateral chords placed pressure on the intestine in such a way as to forcefully expel gut contents through the anal opening. Increased movements and expulsion of gut contents were readily observed with the stereoscopic microscope. Observations revealed that intestines of those nematodes remaining active in lower concentrations of chlorine were nearly empty by the end of the 60 min observation time. Since the amount of inoculum present in the nematode gut is of considerable interest to further studies, reduction of loss from the gut contents during surface sterilization procedure seems important. Regardless of effort to limit this loss, the process of surface sterilization imposes an unavoidable source of error in developing data on total inoculum potential developed through ingestion of pathogens by saprophagous nematodes.

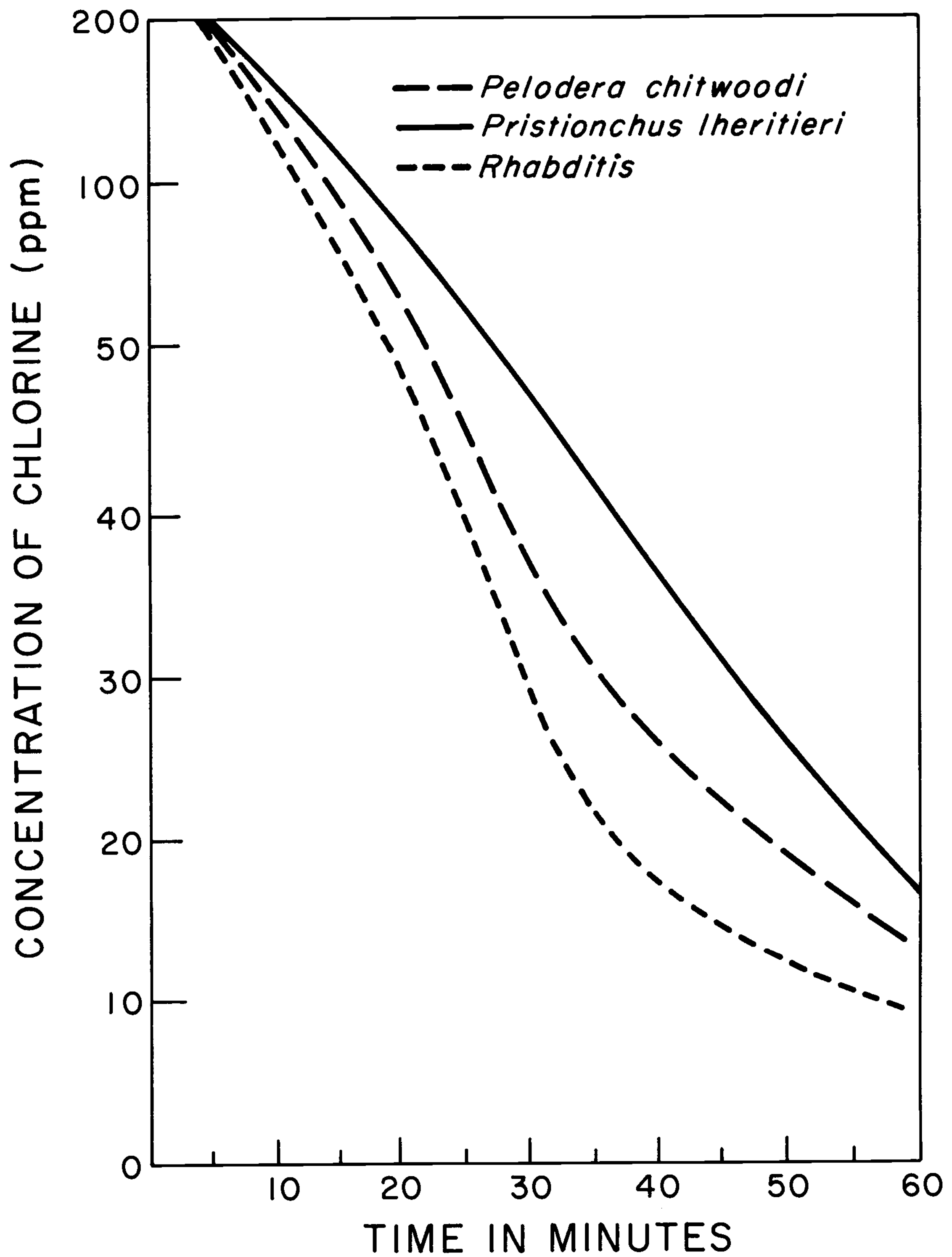

Figure 1. Ninety percent level of survival of three nematode species in various concentrations of chlorine.

Table 1. Percent survival of _Pelodera chitwoodi_ after treatment with various concentrations of chlorine.

Exposure (min)	Concentration of Residual Chlorine and Percent of Surviving Nematodes							
	0 ppm	10 ppm	20 ppm	30 ppm	40 ppm	50 ppm	100 ppm	200 ppm
0	100	100	100	100	100	100	100	100
5	100	100	100	100	100	100	100	65
10	100	100	100	100	100	100	55	15
15	100	100	100	100	97.5	95	20	0
20	100	100	100	100	95	90	10	0
25	100	100	100	100	80	80	0	0
30	100	100	100	100	72.5	70	0	0
35	100	100	100	97.5	60	30	0	0
40	100	100	100	80	50	0	0	0
45	100	100	92.5	67.5	20	0	0	0
50	100	97.5	85	65	0	0	0	0
55	100	95	75	65	0	0	0	0
60	100	95	50	35	0	0	0	0

Table 2. Percent survival of *Rhabditis* sp. after treatment with various concentrations of chlorine.

Exposure (min)	Concentration of Residual Chlorine and Percent of Surviving Nematodes							
	0 ppm	10 ppm	20 ppm	30 ppm	40 ppm	50 ppm	100 ppm	200 ppm
0	100	100	100	100	100	100	100	100
5	100	100	100	100	100	100	100	55
10	100	100	100	100	97.5	95	65	30
15	100	100	100	100	95	90	45	5
20	100	100	100	100	85	90	27.5	0
25	100	100	100	80	70	75	20	0
30	100	100	100	80	70	62.5	0	0
35	100	100	90	80	70	40	0	0
40	100	100	85	70	45	0	0	0
45	100	90	80	65	25	0	0	0
50	100	90	70	65	5	0	0	0
55	100	80	60	40	0	0	0	0
60	100	50	25	20	0	0	0	0

Table 3. Percent survival of *Pristionchus lheritieri* after treatment with various concentrations of chlorine.

Exposure (min)	Concentration of Residual Chlorine and Percent of Surviving Nematodes							
	0 ppm	10 ppm	20 ppm	30 ppm	40 ppm	50 ppm	100 ppm	200 ppm
0	100	100	100	100	100	100	100	100
5	100	100	100	100	100	100	100	90
10	100	100	100	100	100	100	85	50
15	100	100	100	100	100	97.5	40	0
20	100	100	100	100	90	95	35	0
25	100	100	100	100	70	90	20	0
30	100	100	100	97.5	70	82.5	0	0
35	100	100	100	95	70	70	0	0
40	100	100	90	90	50	40	0	0
45	100	100	85	70	50	0	0	0
50	100	100	82.5	70	50	0	0	0
55	100	97.5	60	30	25	0	0	0
60	100	95	60	30	25	0	0	0

CHLORINE TOLERANCE OF *BACILLUS THURINGIENSIS*

Preface

The use of chlorine as a surface decontaminant in studies involving saprophagous nematodes and bacteria is well established (Chang *et al.*, 1960; Jensen and Gilmour, 1968; Chantanao, 1969). However, it is necessary to establish that the bacteria involved are more susceptible to action of chlorine than nematodes. In effect chlorine concentration and duration of exposure required to destroy bacteria must not seriously harm the nematode. In this investigation the minimum chlorine concentrations with corresponding exposure periods required to destroy all exposed *Bacillus thuringiensis* cells and spores were determined.

Procedures

Bacteria for these tests were incubated for 96 hr on tryptose phosphate agar slants. The *B. thuringiensis* slant cultures were washed with sterile distilled water to prepare a suspension of spores and vegetative cells. A Petroff-Hausser bacterial counter was used to make a cell and spore count on a sample of the suspension. The suspension was diluted with sterile distilled water to produce a cell and spore count of 2.5×10^5 per ml of suspension. Ten ml aliquots of this suspension were pipetted into 125 ml flasks to which were

added equal volumes of a chlorine solution (sodium hypochlorite) exactly double the desired testing concentration. The *B. thuringiensis* suspensions were exposed to chlorine concentrations of 10, 20, 30, 40, 50, 100 and 200 ppm. Effects of various concentrations were measured after exposure periods of 2, 4, 8, 16, 32 and 60 min. At the end of a given exposure period a one ml sample was removed from the treatment flask and pipetted into a tube containing nine ml of a sterile 1.0 percent solution of sodium thiosulfate. Sodium thiosulfate neutralized the chlorine and stopped the sterilizing action. Decimal dilutions of each neutralized suspension were plated with approximately 15 ml of melted (47° C) tryptose phosphate agar. Controls were handled in exactly the same manner except that the bacterial suspension received an equal volume of sterile distilled water without any sodium hypochlorite. The plates were incubated at room temperature and the bacteria colonies counted within 48 hr.

Following initial studies the above procedure was repeated with one significant change. A surfactant, Triton X-100, was added to sodium hypochlorite test solutions. Chlorine solutions were made in a 0.04 percent solution of Triton X-100, so that on addition to the bacterial suspension the surfactant concentration was 0.02 percent. Controls were handled as above except that the bacterial suspension was held in 0.02 percent Triton X-100 without any chlorine.

Results and Discussion

Effects of chlorine on viability of *B. thuringiensis* are shown in Tables 4 and 5. The numbers of surviving bacteria are listed with the appropriate chlorine treatment in ppm and the corresponding period of exposure. *B. thuringiensis* was very susceptible to the action of chlorine. Bacteria showed an approximate 50 percent reduction in viability with only four minutes of exposure at the two lowest chlorine concentrations. There was a continued rapid loss of viability in ten and 20 ppm treatments, but some bacteria continued to survive after a full hour of exposure. Results recorded in Table 4 indicate that bacteria were killed by exposure to 30 and 40 ppm of chlorine for 32 and 16 min respectively.

Appearance of very small numbers of viable bacteria from treatments held at the higher concentrations of chlorine for long exposure times, suggested the possibility of escapes from action of the chlorine solution. The rapid rates of decline in viability of bacteria exposed to concentrations above 40 ppm of chlorine were not consistent with apparent tolerances permitting exposure periods greater than one hour to higher chlorine concentrations.

Table 5 shows the results obtained when the surfactant Triton X-100 was added to treatments. Results were similar to those obtained without the surfactant; however, there were no apparent

escapes. The exposure periods required to kill bacteria for given concentrations of chlorine were clearly defined.

It is clear from information shown in Tables 4 and 5 that _B. thuringiensis_ is less tolerant of chlorine in the Triton X-100 solution than are the bacteriophagous nematodes. A choice of several chlorine concentrations and exposure periods are available to effect removal of _B. thuringiensis_ as a surface contaminant on the nematodes.

Use of the surfactant added an apparent source of error in further studies in which this material was used. There was an approximate 50 percent loss of viability in bacteria exposed to the 0.02 percent solution of Triton X-100 for one hour, Table 5. For an eight min exposure the loss in viability was approximately 15 percent. When this material is used the shortest effective exposure possible should be utilized to minimize loss of viability in bacteria exposed subsequent to the surface sterilization process.

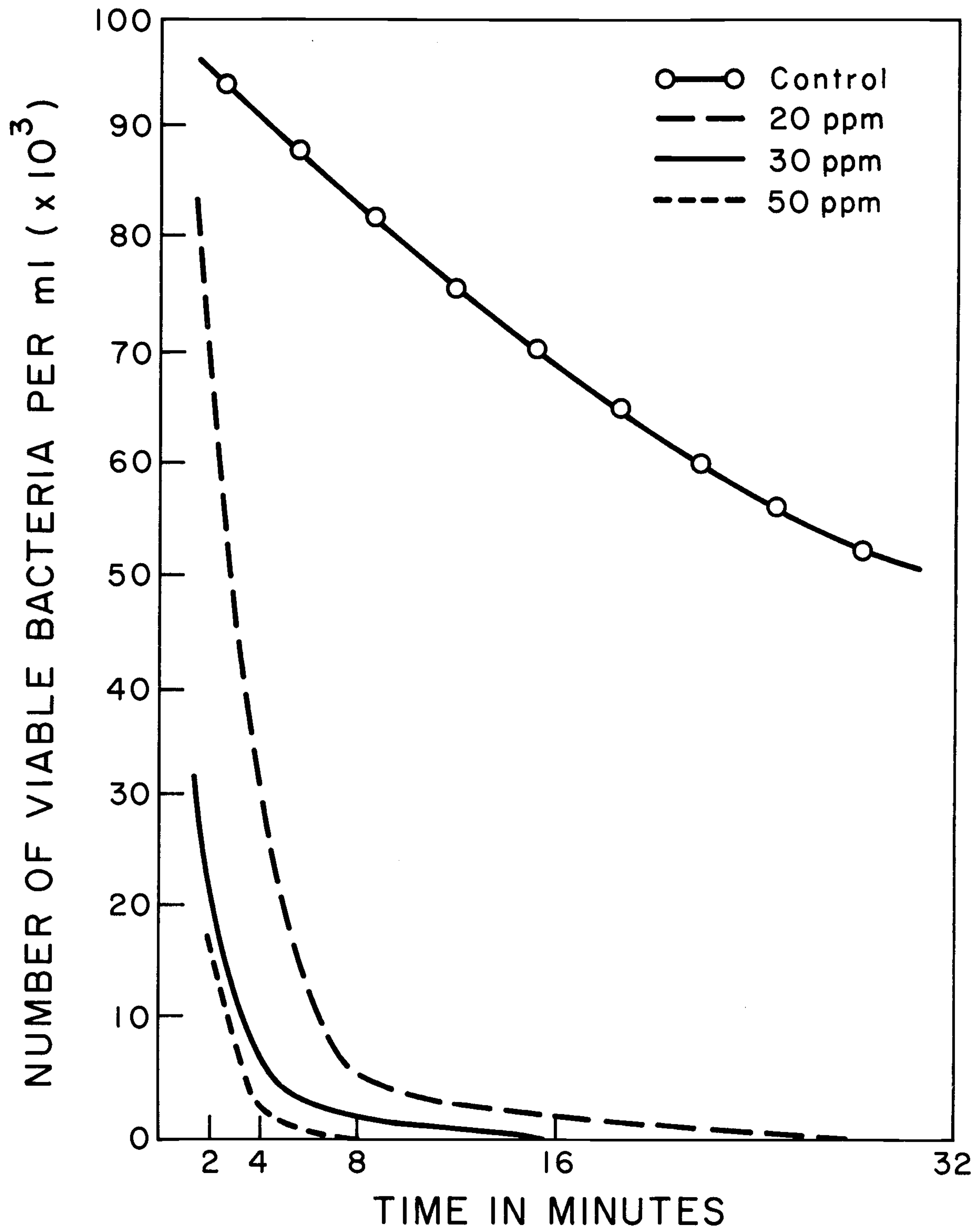

Figure 2. Effect of chlorine on the survival of Bacillus thuringiensis. Chlorine in 0.02 percent Triton X-100.

Table 4. Average number of viable bacteria per ml of original suspension based on plate counts following treatment of *Bacillus thuringiensis* with sodium hypochlorite.

Exposure (min)	Concentration of Residual Chlorine							
	0 ppm	10 ppm	20 ppm	30 ppm	40 ppm	50 ppm	100 ppm	200 ppm
2	101×10^3	92×10^3	91.6×10^3	56×10^3	45.2×10^3	22.3×10^3	4.1×10^3	1.5×10^2
4	97×10^3	39.4×10^3	43.5×10^3	15.3×10^3	7.2×10^3	5×10^2	0	0
8	94×10^3	5×10^3	4.1×10^3	7.2×10^2	1.5×10^2	0	0	0
16	99.8×10^3	2.5×10^3	2.5×10^2	0.25×10^2	0	1.0×10^2	0.25×10^2	0
32	93×10^3	1.5×10^2	0	0	0.5×10^2	0	0	0.25×10^2
60	99×10^3	0.5×10^2	0.25×10^2	0	0	0.25×10^2	0.5×10^2	0

Table 5. Average number of viable bacteria per ml of original suspension based on plate counts following treatment of Bacillus thuringiensis with sodium hypochlorite in 0.02 percent Triton X-100.

Exposure (min.)	Concentration of Residual Chlorine								
	0 ppm	10 ppm	20 ppm	30 ppm	40 ppm	50 ppm	100 ppm	200 ppm	400 ppm
2	92×10^{3}	80.9×10^{3}	89.9×10^{3}	46.6×10^{3}	35×10^{3}	17.6×10^{3}	3.5×10^{3}	8.0×10^{2}	1.5×10^{2}
4	95×10^{3}	43.1×10^{3}	38.9×10^{3}	11.7×10^{3}	5.8×10^{3}	6×10^{2}	0	0	0
8	77.8×10^{3}	6.6×10^{3}	3.8×10^{3}	8.5×10^{2}	0.25×10^{2}	0	0	0	0
16	77.5×10^{3}	3.3×10^{2}	1.0×10^{2}	0	0	0	0	0	0
32	51.5×10^{3}	-	0.25×10^{2}	0	0	0	0	0	0
60	42×10^{3}	0.25×10^{2}	0.25×10^{2}	0	0	0	0	0	0

INGESTION, PERSISTENCE AND DEFECATION OF *BACILLUS THURINGIENSIS* BY CERTAIN BACTERIOPHAGOUS NEMATODES

Preface

Studies by Jensen (1967) and Chantanao (1969) have shown that the bacteriophagous nematode *Pristionchus lheritieri* can ingest plant pathogenic bacteria and defecate them in a viable condition. Indeed, Chantanao (1969) demonstrated that *P. lheritieri* could actually be cultured monoxenically on several plant pathogenic bacteria and an insect pathogen *Serratia marcescens*. On a number of occasions *P. lheritieri* has been associated with dead or dying insects (Weiser, 1966; Poinar, 1969). These investigators suggested that nematodes were not directly responsible for insect mortality but probably carried a bacterial organism which caused death. This investigation was designed to determine whether three bacteriophagous nematodes *P. lheritieri*, *P. chitwoodi* and *Rhabditis* sp. could ingest and subsequently defecate the insect pathogen *Bacillus thuringiensis* in a viable condition. Attempts were made to culture *P. lheritieri* monoxenically on cultures of *B. thuringiensis*, and studies were carried out to determine the persistence of viable *B. thuringiensis* in the intestine of *P. lheritieri* and *Rhabditis* sp.

Procedures for Culturing *Pristionchus lheritieri* with *Bacillus thuringiensis*

Cultures of *P. lheritieri* maintained on its normal bacterial symbiont were used for this study. Adult females bearing eggs showing larval development were aseptically removed from culture plates and placed in ten ml of distilled water. Ten ml of 100 ppm chlorine solution in 0.04 percent Triton X-100 were added to the nematode suspension. After a ten min exposure to 50 ppm of chlorine, 1.0 ml of sterile ten percent sodium thiosulfate was added to the solution to neutralize chlorine. Nematodes were removed singly from this treatment and placed in ten ml of sterile distilled water. These females were carefully dissected with sterile needles releasing intact eggs. The remains of the females were removed and discarded. Eggs were then exposed for ten minutes to a 50 ppm chlorine solution. After chlorination was stopped with sodium thiosulfate, the eggs were aseptically pipetted onto plates of sterile nutrient agar, five eggs per plate, and incubated at room temperature for 24 hr. At the end of 24 hr eggs and emerged larvae on those plates showing no evidence of bacterial contamination were placed on 24 hr streak plates of *B. thuringiensis*. These plates were carefully checked for contaminants as nematode larvae were observed for normal development. Four sets of 20 plates each were prepared as above. The 24 hr nutrient agar plates bearing contaminants following surface

sterilization of eggs served as controls for this study.

Results and Discussion

All attempts to culture P. lheritieri on B. thuringiensis failed. In no case did larvae survive more than three days on B. thuringiensis cultures. None of those larvae surviving three days on B. thuringiensis reached complete adult development. Larvae serving as controls on contaminated nutrient agar plates completed normal development and reproduced. Eighteen plates of a total of eighty prepared remained free of the normal bacterial symbiont on which nematodes were maintained in the laboratory.

Bacillus thuringiensis apparently failed to provide nutritional requirements for normal growth and development of P. lheritieri. However, few Gram-positive bacteria have been reported to successfully support free-living nematodes in monoxenic cultures. Briggs (1946) reported culturing Caenorhabditis briggsae on a spore-forming Gram-positive bacillus, the only one of ten Gram-positive bacteria attempted which provided a satisfactory monoxenic associate. Dougherty (1960) indicated that the culture medium itself may be as important as the bacterial species in determining satisfactory associations. He reported that Staphylococcus pyogenes var. aureus Rosenbach, a Gram-positive bacterium, served as a monoxenic associate for C. briggsae when cultured on tryptose phosphate agar.

Such findings serve to emphasize the complexity of relationships in such monoxenic cultures involving bacteriophagous nematodes. Very importantly, however, they suggest that *B. thuringiensis* could under different cultural conditions serve as an adequate monoxenic associate of *P. lheritieri* and other saprophagous nematodes.

Procedures to Determine Ingestion and Defecation of Viable *Bacillus thuringiensis*

Young adult females from laboratory cultures of *P. lheritieri*, *P. chitwoodi* and *Rhabditis* spp. were picked singly into ten ml of sterile distilled water. An equal volume of 100 ppm chlorine in 0.04 percent Triton X-100 was added for an adjusted 50 ppm of chlorine. After ten min the chlorine was neutralized with 1.0 ml sterile ten percent sodium thiosulfate. The nematodes were aseptically picked onto 24 hr streak plates of *B. thuringiensis* cultured on tryptose phosphate agar. Nematodes were left on *B. thuringiensis* plates for 24 hr and were then removed. A total of ten nematodes was placed into ten ml of distilled water and surface sterilized as before. After the chlorine action was stopped, five nematodes were aseptically placed on tryptose phosphate agar and incubated for 24 hr at room temperature. *B. thuringiensis* colonies appearing on these plates would be produced by viable bacteria defecated by surface sterilized nematodes. The remaining five nematodes were aseptically transferred by means of sterile bamboo splinters to ten ml sterile

distilled water where the intestines were carefully dissected with sterile needles to release gut contents. After a thorough mixing of this suspension, one ml aliquots were aseptically pipetted into 15 ml of melted (47° C) tryptose phosphate agar, four plates per treatment. Plates were incubated at room temperature and examined for *B. thuringiensis* within 48 hr. Ten replicates were prepared for each nematode species.

Presence of the normal bacterial symbiont of laboratory cultured nematodes as contaminants in these studies presented no problems in identification. The *B. thuringiensis* colonies were easily differentiated from the bacterial symbiont colonies. On tryptose phosphate agar *B. thuringiensis* colonies were white and nearly opaque, transmitted light giving a mottled effect; symbiont colonies were light amber to yellow colored and readily transparent. *B. thuringiensis* colonies were rough with irregular whiplike outgrowths on the margins while symbiont colonies were lens shaped with smooth margins.

Results and Discussion

Results of this study are presented in Table 6. Evidence indicates that all three species of bacteriophagous nematodes ingested *Bacillus thuringiensis* and subsequently defecated bacteria in a viable condition. Average numbers of viable bacterial cells

recovered per nematode were surprisingly low when compared to numbers of plant pathogenic bacteria recovered from *P. lheritieri* (Chantanao, 1969). Under similar circumstances Chantanao recovered an average of 46,000 cells of *Agrobacterium tumefaciens* (Smith and Townsend) Conn from each adult female *P. lheritieri*. The same nematode species yielded an average of 511 viable *B. thuringiensis* cells in this study. Several factors discussed in previous sections may explain the apparent variation. *A. tumefaciens* served as a monoxenic associate for *P. lheritieri*; *B. thuringiensis* does not. The general condition of the nematode degenerates when an adequate source of nutrition is not available. The factor of preference in feeding is also important. Just as important as these factors are experimental procedures which tend to reduce the amount of material in the nematode gut. Irritating influence of higher concentrations of chlorine (50 ppm) required to effect surface sterilization of the nematodes stimulates random movement and causes rapid loss of gut contents. Longer periods of chlorine exposure required to eradicate external *B. thuringiensis* contaminants prolonged random movements of nematodes and contributed to a decrease in the number of recoverable bacteria in the nematode gut.

Table 6. Average number of viable B. thuringiensis recovered per nematode based on colony counts from bacteria recovered from dissected nematode intestines.

Replicate	P. lheritieri	Rhabditis	P. chitwoodi
1	252 +	556 +	232 +
2	636 +	696 +	111 +
3	748 +	446 +	107 +
4	686 +	341 +	95 +
5	232 +	540 +	205 +
6	435 +	394 +	175 +
7	216 +	364 +	191 +
8	550 +	299 +	157 +
9	1250 +	156 +	102 +
10	108 +	463 +	36 +
Average	511	399	141

+ Indicates defecation of viable B. thuringiensis.

Procedures to Determine Persistence of Bacillus thuringiensis in Pristionchus lheritieri and Rhabditis sp.

Young adult females of P. lheritieri and Rhabditis sp. were separately removed from laboratory cultures and surface sterilized in 50 ppm chlorine for ten minutes. After the chlorine had been neutralized, nematodes were aseptically moved to 24 hr cultures of B. thuringiensis where they were permitted to feed for 24 hr.

The nematodes were then removed from B. thuringiensis cultures and again surface sterilized as above. Following this treatment adult female nematodes were aseptically transferred to a Tetracycline nutrient agar medium. Neither the normal bacterial symbiont or B. thuringiensis grew on the 1000 ppm Tetracycline medium. Nematodes were placed under essentially starvation conditions in order to determine how long viable B. thuringiensis persist in the nematode gut. At three hr intervals with P. lheritieri and four hr intervals with Rhabditis sp. groups of ten nematodes were removed from the Tetracycline medium and surface sterilized using the same techniques as the previous process. Following surface decontamination, five nematodes were placed on a tryptose phosphate agar plate to reveal any defecation of viable B. thuringiensis. The remaining five were dissected as in the previous experiment to release any viable bacteria from the gut. This suspension was pipetted, one ml per plate, into 15 ml of melted (47° C) tryptose phosphate agar, four plates per treatment. From colonies produced on tryptose phosphate agar the average number of B. thuringiensis cells persisting in nematodes at the end of each time period could be calculated.

Results and Discussion

Results of experiments to determine persistence of viable B. thuringiensis in P. lheritieri and Rhabditis sp. are given in

Tables 7 and 8. The persistence of viable *B. thuringiensis* in the two nematode species studied appears to be essentially similar. During the first eight to nine hr of starvation the number of viable bacteria recovered decreased by nearly 50 percent. Another 50 percent reduction occurred during the next 15 to 16 hr of starvation. Several factors could be involved in this reduction. The results indicated that active defecation of viable bacteria was occurring in this time interval. Observations made with the dissecting microscope revealed that nematodes were very active on the antibiotic medium during this time interval. Assuming that normal physiological activities were on going in the nematode gut during this time, some loss of viability also may be attributed to digestion.

Recoverable viable bacteria were reduced to near zero levels by the end of 36 to 42 hr, at which time failure to defecate viable bacteria was evident. Experiments were concluded at this time interval because the nematodes remaining on antibiotic agar had died. During the last 12 to 18 hr of starvation the nematodes were lethargic and grew decreasingly responsive to tactile stimuli. At this point the nematodes had endured three surface decontamination procedures whose toxic effects must have been debilitating. This, added to effects of starvation, probably resulted in some intrusion of the antibiotic into the nematode gut to influence viability of ingested bacterial cells. Viable bacteria remained in the gut of nematodes

Table 7. Colony counts of viable Bacillus thuringiensis persisting in Pristionchus lhertieri after various periods of starvation.

Time After Starvation (hours)	Average Number of Bacteria per Nematode				Average[1]	Defecation of Bacteria
	Rep. 1	Rep. 2	Rep. 3	Rep. 4		
0	166	178	259	161	191	+
3	108	95	50	72	82	+
6	68	24	18	29	35	+
9	61	45	169	78	88	+
12	53	14	40	29	34	+
15	54	112	40	58	66	+
18	36	20	46	27	32	+
21	190	209	233	341	243	+
24	90	10	13	30	36	+
27	37	5	2	11	14	+
30	0	7	11	18	9	+
33	4	5	9	3	5	+
36	6	3	2	7	5	

[1]Average from four replications of five nematodes each.

Table 8. Colony counts of viable Bacillus thuringiensis persisting in Rhabditis sp. after various periods of starvation.

Time After Starvation (hours)	Average Number of Bacteria per Nematode				Average[1]	Defecation of Bacteria
	Rep. 1	Rep. 2	Rep. 3	Rep. 4		
0	556	195	428	341	380	+
4	107	232	228	175	186	+
8	105	111	205	157	145	+
12	195	107	102	122	134	+
16	191	0	36	50	69	+
20	69	133	145	99	109	+
24	85	78	63	55	71	+
28	11	35	65	19	32	+
32	5	0	28	44	19	+
36	36	0	0	53	22	+
42	4	0	2	3	2	0

[1]Average from four replications of five nematodes each.

failing to defecate bacteria. Where the nematodes died on the tryptose phosphate agar, B. thuringiensis colonies ballooned from anterior and posterior ends of nematode cadavers.

It is apparent from the results of this experiment that free-living bacteriophagous nematodes have the potential to acquire and retain viable insect pathogenic bacteria in such a manner as to permit effective transmission of the pathogen to a host insect. Persistence of viability for up to 42 hr provides adequate time for the nematode to make itself more accessible to host insects. Direct transmission of the pathogen or transportation of nematodes by an insect to a second location to further spread the insect pathogen would be enhanced.

Procedures for Testing the Pathogenicity of Bacillus thuringiensis Subsequent to Ingestion and Defecation by Pristionchus lhertieri

Cultures of Bacillus thuringiensis used for this experiment were subcultured from colonies produced by bacteria defecated by P. lheritieri. The nematodes had been allowed to feed for 24 hr on a 24 hr culture of B. thuringiensis maintained at room temperature on tryptose phosphate agar. Subcultures from defecated bacteria were maintained on tryptose phosphate agar slants for 96 hr. These slants were used for the source of Bacillus thuringiensis cells and spores used in this study. Spores and cells were washed off slants

with sterile distilled water. Samples were taken and spore and cell counts were made using the Petroff-Hausser bacterial counter. Solutions were prepared so that after they had been added to the insect culture medium, there were 25×10^6 and 25×10^5 spores and cells per ml of insect medium. Similar preparations were made from subcultures taken from a commercial preparation of *B. thuringiensis* spores. These preparations served as a basis for comparison with pathogenicity of the spore preparations coming from the nematode defecation subcultures. Five-day old *Trichoplusia ni* larvae were used as host organisms in this pathogenicity test. Larvae were placed, ten to a cup, on the inoculated medium to produce a total of 50 larvae per treatment. The treatments were checked each day for larval mortality and condition of food supply. A control group of fifty larvae was established and maintained in a similar manner except that no *B. thuringiensis* inoculum was added to insect culture medium. The test was concluded when control larvae began to pupate.

Results and Discussion

Results of this study are shown in Table 9. Viable cells or spores of *B. thuringiensis* defecated by *P. lheritieri* retained their pathogenicity to the cabbage looper, *Trichoplusia ni*. This ability to ingest and subsequently defecate a virulent insect pathogenic bacterium further increases the probability that bacteriophagous

nematodes do play important roles in insect disease development.

Attempts to cause disease development in the cabbage loopers by inoculating insect culture medium directly with surface sterilized nematodes that had fed on *B. thuringiensis* failed. No insect mortality occurred as a result of these treatments. Considering the inoculum carrying potential of a single nematode (Table 6) these results are not unexpected. Relatively high concentrations of *B. thuringiensis* inoculum are required to cause death in *T. ni* (Broersma and Buxton, 1967); concentrations of 50,000 viable spores per ml of insect medium failed to show any appreciable effects on *T. ni* larvae.

Table 9. Percent mortality of Trichoplusia ni larvae after inoculation with Bacillus thuringiensis spores and cells.

		PERCENT MORTALITY OF T-NI LARVAE			
		Defecation subculture[a] Spores and Cells/ml		Commercial subculture[a] Spores and Cells/ml	
Days After Feeding	Control	$25x10^5$	$25x10^6$	$25x10^5$	$25x10^6$
1	0	2	4	0	2
2	0	2	8	4	6
3	0	6	24	8	26
4	0	8	50	12	62
5	2	12	64	24	70
6	4	26	76	30	84
7	4	36	80	38	84
8	4	42	80	38	92
9	4	42	82	38	92
10	8	50	82	38	92
11	8	52	82	38	92
12	8	52	84	38	92
13	10	52	84	38	92
14	10	54	84	38	92

[a]Treatment total mortality means different from control at t - 5. 0 percent level of significance.

INGESTION, VIABILITY AND INFECTIVITY OF CERTAIN INSECT PATHOGENIC FUNGI ASSOCIATED WITH *PRISTIONCHUS LHERITIERI*

Preface

Extensive investigations of interrelationships between plant parasitic nematodes and fungus pathogens have revealed the serious impact which certain nematodes have on fungus disease development in plants. Only in recent years (Jensen, 1967) have investigators demonstrated the potential roles of saprozoic nematodes in the spread and protection of fungus pathogens of plants. Jensen showed that *Pristionchus lheritieri* could readily ingest spores and frequently hyphal strands of six plant pathogenic fungi. He also recovered viable spores of two plant pathogenic fungi from excrement of nematodes.

In this study two species of entomophagous fungi, *Beauveria bassiana* and *Metarrhizium anisopliae*, were used in investigations designed to determine whether *P. lheritieri* could serve as a potential vector for these insect pathogens. Feeding experiments were designed to determine the ability of *P. lheritieri* to ingest and defecate spores of insect parasitic fungi in a viable condition. Information as to viability and pathogenicity of spores recovered from the intestine of a saprophagous nematode was sought.

Chlorine Tolerance of *B. bassiana* and *M. anisopliae*

Flask cultures of *B. bassiana* and *M. anisopliae* maintained on yeast peptone agar provided spores for this study. A spore suspension was produced by washing the surface of sporulated cultures with sterile distilled water. The spore suspensions were aseptically pipetted into sterile flasks. Spore counts were determined for the suspensions and aliquots from each were diluted to produce suspensions of approximately 5.2×10^6 spores per ml of *B. bassiana* and 4.4×10^6 spores per ml of *M. anisopliae*. To ten ml of each of the above suspensions ten ml of an appropriate concentration of chlorine solution was added to produce treatment concentrations of 10, 20, 40, 50 and 100 ppm of chlorine. At the end of 2.5, 5, 10, 15 and 20 minutes one ml of spore suspension was removed from each treatment and the chlorine neutralized in 9 ml of 0.1 percent sodium thiosulfate. Five slides per treatment were prepared with yeast peptone agar and each received 0.05 ml of treated spore suspension subsequent to neutralization with sodium thiosulfate. The slides were incubated at room temperature for 30 hr at 100 percent relative humidity. Germination counts were taken from all five slides, at least five fields per slide. Values for percent germination were based on no less than 500 counted spores. Germ tubes at least one half the diameter of the spore indicated positive germination.

Table 10. Average percent germination of _B. bassiana_ spores after treatment with various concentrations of chlorine.

Exposure (min)	Concentration of Residual Chlorine and Percent Germination of _B. bassiana_ Spores					
	0 ppm	10 ppm	20 ppm	40 ppm	50 ppm	100 ppm
2.5	51.7	38.3	41.3	36.4	15.3	10.1
5	32.0	32.3	16.7	10.3	1.5	0.3
10	26.5	28.4	3.6	4.0	0	0
15	36.9	30.3	0.5	1.0	0	0
20	31.0	25.6	1.2	0.6	0	0

Table 11. Average percent germination of _M. anisopliae_ spores after treatment with various concentrations of chlorine.

Exposure (min)	Concentration of Residual Chlorine and Percent Germination of _M. anisopliae_					
	0 ppm	10 ppm	20 ppm	40 ppm	50 ppm	100 ppm
2.5	91.3	65.4	20.1	3.9	0.6	1.1
5	98.1	42.1	8.3	2.5	0	0
10	95.8	15.2	5.1	0.6	0	0
15	94.8	8.0	3.2	1.3	0.1	0
20	92.1	3.0	0.9	0	0	0

Results and Discussion

Results of the chlorine tolerance studies for *B. bassiana* and *M. anisopliae* are summarized in Tables 10 and 11. In spite of the low germination rate of the *B. bassiana* control series, *B. bassiana* spores continued to germinate at a comparatively high rate at the lower concentrations of chlorine and short exposure periods. *M. anisopliae*, however, showed a very rapid decrease in germination rate even at low concentrations of chlorine.

A comparison of the results obtained here with those shown in Tables 1-3 indicates that the fungi are more susceptible than saprophagous nematodes to the action of chlorine. As a result the chlorination process used with the bacterial studies can be used with *B. bassiana* and *M. anisopliae* to effect surface decontamination of saprophagous nematodes.

Procedures Used to Determine the Ingestion and Defecation of Viable Spores of *B. bassiana* and *M. anisopliae*

Laboratory cultures of *P. lheritieri* maintained with its normal bacterial symbiont on nutrient agar plates were used for this study. Young adult female nematodes were aseptically picked out of culture plates into ten ml sterile distilled water. An equal volume of 100 ppm chlorine in 0.04 percent Triton X-100 was added to produce a 50 ppm chlorine solution to effect surface sterilization. After a ten

minute exposure the chlorine was neutralized with one ml of sterile ten percent sodium thiosulfate. Nematodes were then aseptically hand picked with sterile bamboo splinters onto sporulated plate cultures of *M. anisopliae* and *B. bassiana.* The fungi were cultured on yeast peptone agar. Half the nematodes were left on the fungus cultures for an eight hr period and the remainder for a 24 hr period. After the feeding periods the nematodes were removed from the plates in groups of ten and surface sterilized as above. Five surface sterilized nematodes were placed intact on a plate of Martin's medium. The appearance of *M. anisopliae* or *B. bassiana* on these plates could only result from viable spores defecated by active nematodes. The remaining five surface sterilized nematodes were placed in five ml of sterile distilled water and aseptically dissected to release gut contents. The resulting suspension was then pipetted one ml per plate onto four sterile plates of Martin's medium. Appearance of *M. anisopliae* or *B. bassiana* colonies on these plates would result from viable spores ingested by nematodes. All isolation plates of Martin's medium were maintained at room temperature at 100 percent relative humidity. Twenty sets of these treatments were developed for the eight hr and ten sets for the 24 hr feeding times for both fungus species. Fungus isolates resembling either *B. bassiana* or *M. anisopliae* were subcultured on yeast peptone agar, and sterile cover-glass cultures were used to study and verify identities of isolates.

Results and Discussion

No *M. anisopliae* colonies were isolated from defecation test plates or the test plates prepared from gut dissections. Neither were any *B. bassiana* colonies isolated from defecation plates or from the dissections prepared from the 24 hr feeding plates. *B. bassiana* colonies, however, were isolated from gut dissections prepared from nematodes feeding eight hr on fungus cultures. The first ten sets of plates prepared produced *B. bassiana* colonies on three plates, all three plates from separate preparations. Subsequently an additional ten sets were prepared from eight hr fed nematodes from which only one plate produced a *B. bassiana* colony. Only four plates bore colonies of *B. bassiana* from the total of 80 prepared from gut dissections.

The evidence indicates that a saprophagous nematode can ingest spores of an entomophagous fungus and that ingested spores do remain viable in the gut of the nematode. Several factors may have contributed to the low rate of recovery of viable spores from nematodes. Germination tests on *B. bassiana* spores from cultures prepared at the same time and maintained under identical conditions as those used for the feeding experiments yielded a germination rate of only 20 percent. This factor alone could have contributed significantly to the low recovery rate of viable spores from the nematode gut.

Studies by Veen and Ferron (1966) and Walstad et al. (1970) emphasize the difficulty of isolation of these fungi from material contaminated with bacterial and fungus saprophytes. Since the nematodes had been feeding on bacteria prior to being placed on fungus cultures their guts would have contained a ready source of bacteria probably capable of inhibiting germination of fungus spores. The presence of such organisms associated with bacteriophagous nematodes would appear to preclude any role on their part in spreading or protecting infective propagules of these insect parasitic fungi. However, such may not be the case at all since spores of both B. bassiana and M. anisopliae germinate readily on unsterile cuticles of certain insect hosts (Walstad et al., 1970). These investigators found that spores of both fungi germinated at higher rates on unsterile host cuticle than on sterile laboratory media.

Inhibitory effects of bacterial contaminants in nematodes may have been adequate to prevent germination of viable spores defecated by nematodes in both eight hr and 24 hr feeding sets. The build-up of bacteria on yeast peptone agar and preferential feeding of nematodes on bacteria may explain the failure to detect viable fungus spores from gut dissections of nematodes left on fungus cultures for 24 hr.

Procedures to Determine the Pathogenicity of *Beauveria bassiana* Subsequent to Ingestion by *Pristionchus lheritieri*

Subcultures of *B. bassiana* obtained from colonies isolated from gut dissections of *P. lheritieri* from a previous investigation were used for this experiment. The *B. bassiana* cultures were maintained on yeast peptone agar and held at 100 percent relative humidity for sporulation. The culture source of *B. bassiana* used for the feeding experiments with *P. lheritieri* provided comparison cultures for the pathogenicity test.

Trichoplusia ni larvae served as host organisms in the pathogenicity tests. Culture and miantenance of the insects was discussed in the section on Methods and Materials. Five day old larvae were used exclusively in this test. Larvae were removed from their culture cups and dipped in sterile distilled water. Immediately, they were placed on the sporulated mycelial mat of a *B. bassiana* culture. The larvae were carefully rolled on the spore mat and left to crawl on the mycelium for 3 minutes. Larvae were removed in groups of five and placed in a sterile glass culture dish into which a one cm cube of insect culture medium had been placed. Culture dishes were maintained for 24 hr at 100 percent relative humidity and throughout the experiment at room temperature. At the end of the 24 hr period larvae were removed from the plates and placed on

insect culture medium in six ounce paper cups. Cups were then placed in partially closed plastic bags (five cups per bag) to prevent rapid loss of moisture from the cultures. Ten groups of five insect larvae each received this treatment for the B. bassiana cultures isolated from the nematode dissection, and the B. bassiana source culture. A control set of fifty T. ni larvae were treated in the same way except that they were not placed on fungus spores. The cups were checked daily for diseased larvae. After ten days the moribund larvae not showing evidence of mycelial growth were again placed in sterile glass culture dishes and exposed to a 100 percent relative humidity to enhance sporulation of fungi in insect cadavers.

Results and Discussion

Results of the pathogenicity tests are shown in Table 12. Examination of moribund larvae, subsequent to incubation at 100 percent relative humidity, permitted verification of a B. bassiana caused mycosis in ten of eighteen larval cadavers previously exposed to the nematode gut isolate of B. bassiana. The cause of death in eight remaining larvae from that test could not be definitely established.

In the comparative test with B. bassiana source culture, 14 of the 21 moribund larvae developed sporulating hyphae identified as those of B. bassiana. The cause for death in the remaining seven

larvae was not established. The control series produced no *B. bassiana* mycoses; however, a 12 percent mortality among controls did occur.

Results indicate that spores of *B. bassiana* retain viability and pathogenicity after ingestion by the saprophagous nematode *Pristionchus lheritieri*. Such evidence supports the thesis that saprophagous nematodes can have a substantial role in spreading and protecting insect pathogenic fungi in nature.

Table 12. Cumulative percent mortality in groups of 50 *Trichoplusia ni* larvae inoculated with two separate cultures of *B. bassiana*.

Days After Inoculation	Control No Spores	Source Culture *B. bassiana*	Nematode Gut Isolate *B. bassiana*
1	0.0	0.0	0.0
2	0.0	2.0	0.0
3	0.0	6.0	4.0
4	2.0	10.0	12.0
5	2.0	24.0	20.0
6	4.0	28.0	24.0
7	4.0	36.0	28.0
8	8.0	38.0	32.0
9	8.0	38.0	32.0
10	8.0	38.0	34.0
11	10.0	38.0	34.0
12	10.0	42.0	34.0
13	12.0	42.0	36.0

INGESTION AND TRANSMISSION OF THE NUCLEAR POLYHEDROSIS VIRUS OF *TRICHOPLUSIA NI* BY *PRISTIONCHUS LHERITIERI*

Preface

Associations of saprophagous nematodes with several types of viruses have been recently investigated. Chang *et al.* (1960) found that certain bacteriophagous nematodes could readily ingest pathogenic Coxackie and Echo viruses. These viruses were found to survive passage through the nematode and were defecated in a viable infective condition. More recently Jensen and Gilmour (1968) and Chantanao (1969) demonstrated that *Pristionchus lheritieri* could readily transmit infective phages to their hosts.

Among the many virus diseases of insects, the nuclear polyhedrosis virus disease of the cabbage looper, *Trichoplusia ni*, readily lends itself to use in studies of vector capabilities of bacteriophagous nematodes. As described by Semel (1956) behavior of diseased larvae is such that insect cadavers may be easily accessible to saprophagous nematodes feeding on decaying plant material. Diseased larvae may fall to the soil in a moribund condition or they may remain attached by prolegs to a leaf margins of host plants. Drake and McEwen (1959) described such moribund larvae as fragile sacs veritably filled with highly infectious polyhedral bodies, virus particles and saprophytic bacteria.

In this study experiments were designed to determine whether the bacteriophagous nematode, P. lheritieri, would feed on cadavers of T. ni killed by the nuclear polyhedrosis virus disease. Ability of P. lheritieri to ingest and transmit infective nuclear polyhedrosis virus to T. ni was also investigated.

Procedures and Observations to Determine Acceptability of Virus Killed T. ni Larvae as a Food Source and Transmission of the Virus by P. lheritieri

Trichoplusia ni larvae freshly killed by nuclear polyhedrosis virus were placed near the outer edge of a plate of sterile nutrient agar. P. lheritieri females previously exposed to a ten min treatment of 50 ppm chlorine solution in 0.02 percent Triton X-100, were placed in the center of the plate bearing T. ni cadavers. Nematodes were observed over a period of 2 hr. With little random movement the nematodes moved quickly to T. ni cadavers and began feeding. The nematodes were permitted to feed on looper cadavers for a minimum of four hr. Nematodes were then removed aseptically from the plate bearing cadavers and immediately chlorinated as above to remove adherent bacterial contaminants. The nematodes were then aseptically placed on one cm blocks of T. ni medium, one nematode per block. The blocks of insect medium were placed in six oz paper ice cream cups with five four day old T. ni larvae. Ten such cups were prepared and maintained at room temperature.

Daily observations were made for disease development, and fresh food cubes were added as required. Control cups were prepared in the same manner except that nematodes placed on the medium had not fed on insect cadavers.

Results and Discussion

All *P. lheritieri* females located the *T. ni* cadavers within two hours after being placed on the plate with them. All nematodes continued to actively feed on or near dead larvae during the four hr exposure. Apparent positive attraction noted here could only enhance the probability that these bacteriophagous nematodes could serve as vectors for this insect virus disease.

In the treatment series receiving the virus fed nematode on insect nutrient medium, 60 percent of all *T. ni* larvae developed nuclear polyhedrosis and died. None of the control larvae developed nuclear polyhedrosis. It is apparent from these results that one nematode can acquire and transmit enough virus inoculum to cause disease in *T. ni*. The procedures used were very close to the events expected to occur in nature. It could be expected, however, that more inoculum would be transported without the imposed chlorination process to remove part of the surface adherent polyhedral inclusion bodies or to stimulate excessive random movement and thereby loss of part of the gut contents. Evidence does indicate that

P. lheritieri can function as a vector for this intensely virulent insect virus pathogen.

Procedures Used to Determine Active Ingestion of Nuclear Polyhedrosis Virus by *Pristionchus lheritieri*

Attempts to destroy infectivity of nuclear polyhedrosis virus using chlorination treatments which could be survived by *P. lheritieri* failed. As a result an alternative approach to obtain evidence of active ingestion of the pathogen was required. Since the nematodes appeared to actively feed on larval remains, longer feeding periods should result in the nematode accumulating more virus in the gut than during short feeding periods. An inoculum could be obtained from gut dissections of nematodes allowed to feed for various lengths of time on a suspension of nuclear polyhedrosis inclusion bodies.

In order to test virulence of the inoculum acquired from gut dissections, a bioassay procedure developed by Chauthani (1968) was adopted. The procedure made use of a well which received a very small drop of inoculum (0. 02 ml). The larvae had to eat through inoculum placed in the well in order to continue feeding. Individual test chambers were produced by placing eight to ten ml of insect medium in the bottom of a clear four dram plastic pill bottle. A three mm diameter sterile glass tube was inserted about five mm into the surface of the medium. Melted paraffin was then poured over the surface of the medium to about one cm depth. The glass rod

was removed after the paraffin hardened. This left a well which received the virus inoculum. Three time periods for feeding were used: three min, one hr and 24 hr. Each time period and control set was composed of 48 individual test chambers, each receiving one five day old _T. ni_ larva.

Fifty female _Pristionchus lheritieri_ were removed from culture plates with a sterile probe and placed in sterile water. These nematodes were surface sterilized with 50 ppm chlorine solution in 0.02 percent Triton X-100 and placed on the surface of sterile nutrient agar. Immediately the surface of the plate was flooded with a 10 ml suspension of nuclear polyhedrosis inclusion bodies prepared from cadavers of virus killed _T. ni_. As soon as all nematodes were in contact with the suspension of inclusion bodies, fifteen nematodes were removed with a sterile probe, with no nematode receiving more than a three minute exposure to the suspension. Following removal from the virus suspension, nematodes were placed on a sterile 320 mesh screen. The nematodes were then washed with a liter of sterile distilled water to remove surface adherent inclusion bodies. Ten of 15 nematodes were placed in ten ml of sterile distilled H_2O. These ten nematodes were carefully dissected with sterile dissecting needles to release gut contents into water. One ml of this suspension was further diluted to ten ml with sterile tap water. This solution then served as an inoculum, 0.02 ml of which

was placed in wells of insect nutrient medium previously prepared. Subsequently fifteen nematodes were removed after one hr and 24 hr exposures to the polyhedral suspension and given similar treatment to prepare an inoculum suspension. Control inoculum was prepared in the same manner only excluding exposure of nematodes to the polyhedral suspension. After *T. ni* larvae were placed in test chambers, they were observed for a period of ten days for disease development. The larvae were observed daily for development of nuclear polyhedrosis symptoms. Dead larvae were removed from the test chambers and examined for cause of mortality. The experiment was repeated four times.

Results and Discussion

Results of the bioassay procedures are shown in Table 13. There were no occurrences of nuclear polyhedrosis in the control chambers. Unexplained mortalities did occur in both control and treatment groups; however, such low level mortality was common in cabbage loopers cultured in the laboratory. Statistical analysis of data failed to confirm a significant difference between the timed treatments. Analysis of variance using the Fisher distribution indicated a significant difference between the treatment means at the 5.0 percent level. Analysis using the t-distribution failed to confirm the F test at the t-10.0 percent level.

Table 13. *Trichoplusia ni* larval mortality in bioassay for nuclear polyhedrosis virus in *P. lheritieri* gut following timed feedings.

Feeding Time	Mortality in 48 Larvae/Treatment			
	Rep. 1 P-O	Rep. 2 P-O	Rep. 3 P-O	Rep. 4 P-O
Control (0)	0-3	0-2	0-4	0-4
3 min	18-4	16-5	5-3	12-4
1 hr	40-3	24-2	13-3	1-5
24 hr	42-2	27-3	17-2	13-4

P - Death caused by nuclear polyhedrosis virus.

O - Mortality due to causes other than polyhedrosis virus.

A comparison of the results from the three feeding periods indicates an apparent difference between the three min and one hr treatments. The inoculum produced from the one hr feeding periods had an apparently greater virulence than that produced from 3 min exposure. It is reasonable to attribute the greater virulence of one hr treatments to a higher concentration of infectious virus particles and inclusion bodies; hence the longer feeding period could reflect an accumulation of virus and inclusion bodies. Such an accumulation would be possible through active ingestion of the insect pathogen.

There does not appear to be a corresponding increase in virulence in inoculum produced by the 24 hr fed nematodes over the one hr groups. Feeding preference and availabity of bacteria on nutrient agar may account for this occurrence. By the end of 24 hr the nematodes may have already defecated some virus material while selectively feeding on bacteria developing on the nutrient agar.

It is clear from this study and the previous experiments that saprophagous nematodes can effectively carry nuclear polyhedrosis virus of _T. ni._ Results of this study support the concept of active ingestion as a means of acquiring inoculum. Feeding habits of the nematodes enhance the possibility that they will feed on moribund insect larvae and become virulent carriers of this insect pathogen.

DISCUSSION AND CONCLUSIONS

Evidence obtained from this series of studies substantiates the thesis that saprophagous nematodes have an important role in the spread and transmission of insect pathogenic bacteria, fungi and viruses. Except for *M. anisopliae* all pathogens studied were to some degree actively ingested and subsequently recovered in a viable condition. Emphasis in this study has been placed on active ingestion of the insect pathogen. However, the nature of the environment and feeding preferences of the nematode may certainly influence the importance of ingestion as a functional mode of acquisition and transmission of the pathogen. Failure to recover some viable fungus inoculum from intestines of nematodes is a good example. The nematodes appear to prefer bacteria to fungus spores; the spores are probably ingested accidentally. In the process of ingestion the spores are co-mingled with bacterial saprophytes which may inhibit their germination when they are expelled from the nematode gut. In this case those fungus spores which may adhere to the nematode surface are probably more likely to supply viable infectious inoculum than ingested spores. If the bacterial and virus pathogens are considered in a similar light, the inoculum carrying potential of the saprophagous nematode is considerably increased. Attempts to transmit fungus and bacterial disease directly by way of a single or several surface

sterilized nematodes certainly were not enhanced by the removal of pathogens which in nature would adhere to the nematode cuticle.

Of considerable importance in vector-pathogen relationships is the degree to which one influences the normal physiology of the other. Recovery of viable infective pathogens in all of these studies indicate that pathogen physiology has not been drastically altered by the action of the nematode digestive system. That is not to say that some propagules were not destroyed by the digestive process. Nevertheless the failure of the pathogens to lose pathogenicity in passage through the nematodes increases the probability of a vector function by the saprophagous nematode. On the other hand failure of *B. thuringiensis* to support the normal growth and development of bacteriophagous *P. lheritieri* does not enhance the development of a viable pathogen-vector relationship. A more favorable relationship has been reported for the insect pathogenic bacterium *Serratia marcescens* (Chantanao, 1969). *Serratia marcescens* serves as a satisfactory monoxenic associate of *P. lheritieri,* a relationship not far removed from that of the DD-136 complex.

A factor of considerable importance in functional pathogen-vector relationships is the ability of the vector to retain the pathogen in a viable infective condition long enough to effect transfer to a proper host organism. Persistence of *B. thuringiensis* in *P. lheritieri* and *Rhabditis* sp. for 36 and 42 hr respectively appears

to be a short period of time. However, these periods were determined under less than favorable conditions for the nematodes and the bacteria involved. The nematodes were under starvation conditions and the bacteria were subject to action of any antibiotic ingested by the weakened nematodes. With equally short persistence times, however, some vectors such as aphids transmitting stylet born plant viruses play a very important role in plant disease development.

The amount of inoculum which a vector carries is of prime importance where disease development is dependent upon the amount of inoculum received by the host. In these studies direct evidence of the amount of inoculum carried by the potential vector was learned only for B. thuringiensis. Here again these detectable amounts were seriously curtailed by the techniques required to insure that only ingested bacteria were counted (repeated surface sterilization). Where less rigorous treatments were required (Chantanao, 1969), significantly larger numbers of insect pathogenic bacteria were detected. Under less than ideal conditions in this study and others, evidence obtained supports the concept that bacteriophagous nematodes function in nature as effective vectors of insect pathogens.

The ease with which P. lheritieri can become an effective vector of the nuclear-polyhedrosis virus of T. ni is outstanding in this study. A primary factor involved in this association is the virulence of the insect pathogens. A small number of virus particles can be infective

and produce disease; hence, acquisition of sufficient inoculum by the nematode is not difficult. Infectivity of the virus is apparently not inhibited by the normal bacterial symbionts of the nematode, and equally important is the natural attraction of nematodes to feed on moribund *T. ni* larvae. The natural stability of the virus gives the pathogen full advantage of any increased host contact provided by nematode vectors.

As man considers intervention in natural processes to enhance his ability to control insect pests, such relationships as have been established in these studies will become more important. The opposing forces, which on one hand promote maximum agricultural production and on the other limit the use of traditional chemical controls, focus attention on the need to understand, protect and use those natural associations which help to control insect populations.

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