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# The Quest for Plant Nematode Biological Control - Facts and Hypotheses

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**ABSTRACT : The current status of the development of commercial products for the biological control of plant-parasitic nematodes is discussed. An example is given of problems encountered by our program in patenting biocontrol agents in the United States. Two hypothetical approaches to the control of plant nematodes are considered. First, recent experimental results relating to the theory on intervention with host-finding by plant nematodes are reviewed. Second, a newer hypothesis considering the possibilities for genetic approaches to modifying molecular signals between nematodes and their parasites is described.**

The primary means for control of plant-parasitic nematodes are currently the use of chemical nematicides, crop rotation and resistant cultivars. For the most part, there has been a strong reliance on agricultural chemicals as reflected in recent statistics that farmers spend $20 billion world wide and $6~8 billion in the United States on chemicals for crop protection (3). In the United States about 3% of this figure, $172 million is spent on control of nematodes, but this dollar amount does not reflect the fact that most nematicides are no longer available to the farmer.

Nematicides generally must persist in the soil to effectively control plant-parasitic nematodes. Unfortunately, many of these chemicals are proven to be carcinogens, build up residues in food plants, and infiltrate into ground water. These undesirable features have led to a total ban or restricted use of most nematicides by the U.S. Environmental Protection Agency, while usage of the remaining nematicides is currently being re-examined.

For example, the nematicides 1-3-D and aldicarb are under special review and all granular formulations of carbofuran have been prohibited, as has Vorlex. Methyl bromide, widely used for nematode, weed and soil borne disease control, will probably be totally banned by the year 2000 since it has been detected at undesirable levels as an air pollutant. There are the two potentially new nematicides, phosthiazate (ISK Japan) and dazonet (BASF), but current trends indicate that in the near future most chemical nematicides will not be available for use in the industrialized nations. And since many industrialized nations have enacted legislation regulating the levels of pesticide in imported foodstuffs, the use of agricultural chemical in developing nations will concurrently decrease.

It is apparent that alternative to chemical nematicides are urgently needed. Specific alternatives are offered by the development of effective nematode control by bionematicides, nontoxic plant substances such as green manures, or inert materials such as chitin. In addition,

enhanced efforts in developing nematode resistant plant varieties are required. It probably will take a combination of several methods to attain acceptable nematode control. Previous work in these areas in reviewed in the following sections.

## Current Perspectives of Plant Nematode Biological Control

A recent survey by J. Waage, Imperial College, Ascot at Silwood Park, Berks, England indicated that the biotechnology product market amounted to only $100 million as compared to $20 billion for agricultural chemicals. Expansion of the biotechnology segment was estimated at 5% per year to the year 2000.

While studies on plant disease biocontrol have been ongoing for decades, there has been practically no significant development of commercial products available to the farmer. An exception is that provided by *Bacillus thuringienis* (BT) for the control of lepidopteran pests. Biotechnology (18) 1992 lists eight companies with U.S. BT patents. A patent application has been submitted for a nematicidal BT strain developed by our laboratory under license from the University of Massachusetts to Research Corporation Technologies, Tucson, Arizona. This application is considered in detail later in this paper.

Clandosan® represents an example of a non-chemical plant nematode control which has reached the commercial stage (Igene, Biotechnology, Columbia, MD). This product, the most recent commercial formulation being a combination of chitin and urea, was shown to give good control of *Heterodera avenae* and *Tylenchulus semipenetrans* accompanied by significant yield increases (44). Unfortunately at the rates used in this and other experiments which gave similar results, the cost of applications of the Clandosan formulation is too high to be commercially feasible. Also, the product did not give nematode control when tested in the northern, cooler region of the United States (36).

Green manures have been suggested as amendments to various non-chemical nematode control programs (21). Green manures are considered highly economically feasible and the current thinking is that they would provide a substrate for the colonization of the biocontrol microbes in the soil environment in addition to a degree of nematode control (45).

The use of plants which produce substances that depress plant nematode populations, the so-called antagonistic plants, has received increasing attention in tropical and subtropical areas (2). The mode of action of the large group of tropical legumes which have shown antagonistic properties is postulated as being due to the presence of lectins in root exudates which act as confusates to host root finding (38). The broad interest in the potential of tropical legumes is indicated by the increasing number of field tests of legumes such *Mucuna deeringiana* and *Canavalia ensifomis* as nematode biocontrol agents (41, 47).

*Pasteuria penetrans* has given successful root-knot nematode control under greenhouse conditions (46) and field plots. Since this organism is an obligate parasite, a large problem exists in producing the biomass required for commercial exploitation. One attempt in this direction was the development of roller bottles based on a trixenic system (nematode, bacte-

rium, plant root, *P. penetrans*) having roots produced by transformed hairy root bacteria and then inoculated with *P. penetrans* infected J2 root-knot larvae. This system developed by Genetics Inst., Cambridge, MA and later transferred to Ecogen Inc., has thus far been unsuccessful. Likewise other attempts to culture *P. penetrans* axenically have not shown commercial promise.

A number of fungi are being explored for commercialization as biocontrol products but to date without notable success. *Paecilomyces lilacinus*, an egg parasite, has been tested in field experiments with results varying from excellent to poor (1, 10, 45). In a review, Jatala (25) stated that scientists in more than 60 countries were testing *P. lilacinus* under a program which he directed. Asiatic Technologies, Manila, Phillipines markets a product formulated by growing *P. lilacinus* on a coconut substrate (3).

Two species of *Arthrobotrys, A. irregularis* and *A. robusta*, have been produced and marketed in France as nematode biological control products. The *A. irregularis* product, Royal 350, gave inconsistent results in the field against root knot nematode populations (7), suggesting the need for further research. Royal 300, the *A. robusta* strain developed primarily for use against *Ditylenchus myceliophagus* on mushrooms (11), appears to be of limited potential since steam sterilization largely eliminates nematode problems on that crop. However, the fact that yield increases were associated with the use of Royal 300 gives encouragement to further study.

European and American patent applications for the endoparasitic fungus *Drechmeria coniospora* as a nematode biological control agent were made by Uniroyal Corporation, but the original isolate of *Drechmeria* lost pathogenicity to root-knot nematodes and a pathogenic strain has not been recovered (51). Another endoparasite, *Hirsutella rhossiliensis*, is being extensively studied as a candidate biocontrol organism in the United States (24).

Attempts have been made to exploit the natural reduction of plant-parasitic nematodes associated with a range of organisms which prey on nematodes. These so-called nematode suppressive soils have been studied by a number of researchers (37, 50). Two examples are cited here.

The natural decline in damage from *Heterodera avenae* on oats was first reported by Gair *et al.* (20). Kerry *et al.* (9, 35) isolated a number of fungi which parasitized the eggs and cysts of this nematode, and showed that formalin treatment decreased fungal activity and resulted in increases in *H. avenae*. *Nematophthora gynophila* and *Verticillium chlamydosporium* gave successful control in greenhouse studies, but thus far attempts at increasing populations of these natural antagonists in the field have been unsuccessful (33).

The second example, that of studies towards development of nematode antagonists from suppressive soils in Central America is discussed in the following section.

There are many reasons for significant lack of progress in developing biocontrols for nematodes. Certainly the need has been recognized for at least a decade. Significant voids which have impeded development of commercial products are considered here. The creation of the final product requires (to this writers' mind) intimate and intensive interfacing between

industry and academia. Both groups of scientists may have been bred in the same milieu, but there the similarity ends. On the part of academics there is often suspicion that in industry demands for rapid judgements lead to mistakes. Conversely, some scientists in industry express the view that the lack of pressure which often characterizes the academic domain mitigates against economically efficient product development.

## Development of a Biocontrol Product

The stages characterizing the production of a microbial biocontrol are complex and costly. Once the potential nematicidal microbial candidate is identified by laborious screening of isolates, the efficacy of the agent must be proven in greenhouse and microplot field trials. Evaluation on larger field trials follow (a quantum leap forward) at which point significant expenses and labor cost can be anticipated. In the United States until recently federal and state grants have assumed much of the burden. As an example, support for the field and laboratory researches on two biocontrol candidates considered here (the *Bacillus thuringiensis* strains and the *Streptomyces* sp.) has exceeded $500,000, with the funding approximately equally divided between two agencies, the Massachusetts Centers for Excellence and the Corporation for the Technological Development of Tropical Resources (TROPICO, Puerto Rico). Should this work result in a commercial product, each organization is assured of a share in the royalties, which would then be used to fund other research.

Though experiments could represent effects encompassing only a limited number of nematode species, plant hosts, soil types and environmental factors, the field results at this point may be sufficiently promising to suggest seeking commercial interest. Commercial support is predicated upon the ability to attain a proprietory position for the candidate organism, possibly based on a unique way of applying or delivering the organisms, or perhaps unique properties of the organisms. Apparently the criteria for granting a patent associated with the properties of a microorganism are still evolving. For earlier patents, no proof that the organism did what it was said to do was required. More of this later.

Costs for the U. S. Patent process are estimated at as little as $5,000, but supplementary questions from the patent examiner and ensuing revision of the application may significantly increase the required investment. And apparently shifting criteria and guidelines for patentability of living organisms lend no guarantee to success. A salient example of the ambivalence of rules extant in the United States patent system is given by consideration of the current status of a patent for our BT strain active against plant parasitic nematodes.

This BT isolate originated from nematode suppressive soils in Costa Rica (50) and was extensively tested for efficacy in controlling root-knot nematode (*Meloidogyne incognita*) and *Pratylenchus penetrans* (lesion nematode) in greenhouse trials in Massachusetts. The results of these studies and those of large scale field trials over two years in Puerto Rico, showing significant reduction in root knot nematode injury and yield increases, were reported by Zuckerman *et al.* (52).

A major problem in patenting this BT strain centers around a previous claim for nematicidal activity for 5 Mycogen BT strains, as described by the 1992 Mycogen patent (16). The Mycogen patent is based solely on laboratory trials showing nematicidal activity against the free-living nematode *Caenorhabditis elegans*. Those knowledgeable in testing for anthelmintic activity, often utilize *Caenorhabditis* as a preliminary laboratory screen, to indicate potential candidates for *in vivo* testing. In our Massachusetts laboratory, less than 1% of candidate anthelmintics which gave positive results against *Caenorhabditis* reach the field testing stage. Likewise, a European Patent previously granted Mycogen (15) lays claim to BT nematicidal activity against a wide spectrum of animal and plant-parasitic nematodes. However, the efficacy data is limited to *in vitro* tests against *C. elegans* and the sheep nematode *Haemonchus contortus*. Based on the data presented in these patents, the claim for nematicidal activity of the five Mycogen strains against the broad spectrum of animal and plant nematodes listed would appear to be questionable. In contrast, the Massachusetts BT fulfils the criteria of a valid anthelmintic based on the experimental field results (52).

The existance of these prior patents of anti-nematode BT's resulted in an initial rejection of the Massachusetts BT patent application by the U.S. Patent Office examiner. The position of that office was that if 5 BT antinematodal strains were already patented by Mycogen, the granting of another patent would be redundant. The question of efficacy of any of the BT strains against plant-parasitic nematodes was not considered in the initial rejection of the Massachusetts application. Specifically the lack of field data on plant nematode control in the Mycogen patent and the extensive field testing of the Massachusetts BT did not weigh in the Patent Office's negative decision.

To move the patent process forward, we were first obliged to show that the Massachusetts BT was different from the 5 Mycogen BT strains. To do so required an intensive research effort which will be briefly detailed to further underline the difficulties inherent in the patent process.

The approach taken was profile analysis of hydrolyzable fatty acids by gas chromatography-mass spectrometry (GC-MS), since this methodology is widely used in bacterial systematics for differentiating species (43). Our findings successfully discriminated between the 6 BT strains by showing the comparative absence or presence of certain hydrolyzable fatty acids in the six strains as well as differences in the ratios of the hydrolyzable fatty acids as illustrated in Table 1 (17).

The patent process for an antinematodal antifungal *Streptomyces* species developed in our program faced similar problems (12). We have been required by APHIS to compare our isolate with plant pathogenic *Streptomyces*, and by the patent office with the mode of action of *Streptomyces avermitilis*, a species active against animal parasitic nematodes, and finally three *Streptomyces species* which have closely related cultural and physiological characteristics. Differentiation has been accomplished by combining hydrolyzable fatty acid analyses, mode of activity studies, analysis of isozymes and SDS-PAGE studies. In this case the separations were less difficult since species rather than strains were compared. However, the *Streptomyces*

**Table 1.** Ratios of certain diagnostic peaks in chromatograms of hydrolyzable fatty acids from six strains of *Bacillus thuringiensis*

| Peak ratios[a] | CR-371 | NRRL B-18243 | NRRL B-18244 | NRRL B-18245 | NRRL B-18246 | NRRL B-18247 |
|---|---|---|---|---|---|---|
| P12/P9 | >1 | >1 | >1 | <1 | <1 | <1 |
| P17/P12 | <1 | <1 | <1 | >1 | <1 | >1 |
| P22/P17 | >1 | <1 | >1 | <1 | <1 | <1 |
| P25/P12 | >1 | >1 | <1 | >1 | >1 | >1 |
| P25/P19 | >1 | <1 | <1 | <1 | <1 | >1 |
| P25/P22 | >1 | >1 | <1 | >1 | >1 | >1 |
| P33/P12 | <1 | <1 | <1 | <1 | <1 | >1 |

**Table 2.** Nematode interactions with other organisms and associated proved/postulated molecular controls

| Molecular events | Fungus (*Drechmaria*)/ nematode | Interactions plant parasitic nematode/plant root | Nematode/predator (*Mononchus*)/prey (Nematode) |
|---|---|---|---|
| Recognition<br>perception of chemoattractant in the soil environment | Nematode attracted to spore by chemotactic factors | Root exudates containing chemotactic factors fuide nematode to root | Predator/prey find each other in response to chemical signals |
| Contact/attachment<br>lectin/carbohydrate relation indicated in some cases | Sialic acid specific lectin on spore - sialic acid homologue on nematode cuticle | Contact between nematode head and root surface<br>Recognition. No molecular relation known | Contact between predator stoma and prey cuticle<br>Recognition. No molecular relation proven |
| Infection/attack<br>Governed by signals generated by plasma membrane receptors of interacting organisms<br>Concomitant changes in wall/cuticle permeability, etc. | Spore receives infect signal-germinates and penetrates host | Nematode receives feed signal, stylet activated, host penetration | Predator receives feed signal, tooth penetrates prey integument, feeding commences |

patent has not as yet been granted.

## Intervention in Chemical Signals Between Plant Nematodes and Plant Roots

It was proposed earlier that food finding behavior of plant nematodes possibly could be modified to prevent the nematode from finding the plant root (48, 53). This hypothesis discussed two means by which this could be accomplished. First, through blocking of chemotactic signals emanating from plant root which act to attract the nematode to the root zone. The second possible approach was that of intervening in some way with receptors located on the plasma membrane of the dendrites in the nematode cephalic chemosensilla, thereby

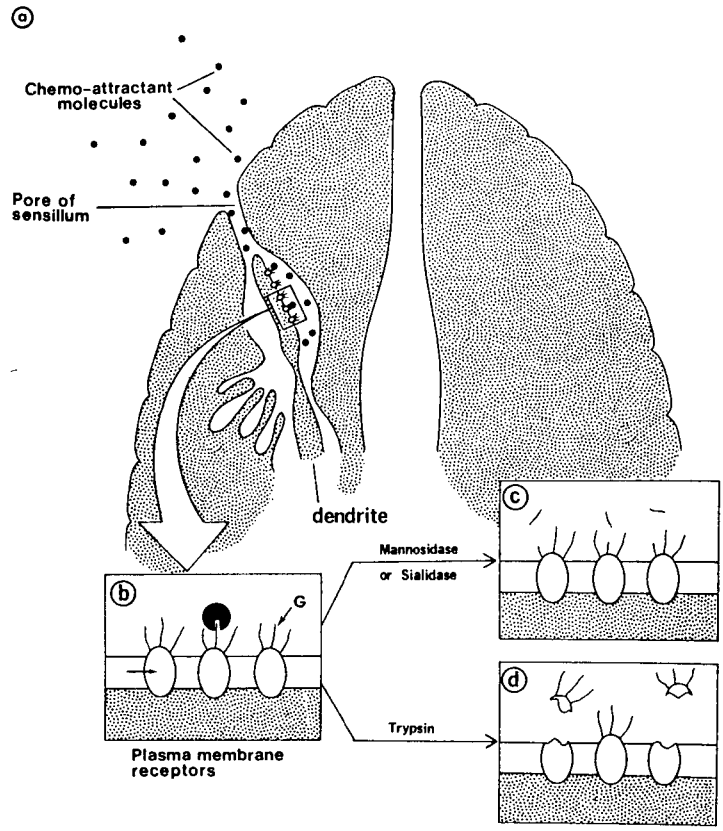


**Fig. 1.** Hypothetical mechanisms for the modification of nematode chemotaxis. (a) The head area of the nematode. Chemoattractant molecules diffuse through the pore of the sensillum and are recognized by receptors located in the plasma membrane of the dendrite. (b) Schematic of plasma membrane receptors (→). Glycans (G) on the receptors are sites of recognition. The black circle represents a lectin molecule blocking receptor function. (c) Enzymatic treatment alters the glycan, resulting in conformational changes and loss of receptor function. (d) Proteolysis also results in loss of receptor function and blocking of chemotaxis.

modifying host-finding behavior (Fig. 1). Research on testing of these two hypotheses is discussed here.

Using *C. elegans* as a model, it was demonstrated that either treatment with certain enzymes (27) or lectins (32) inhibited attraction of the nematode to its food source. These results were interpreted as indicating that membrane receptors on dendrites within the chemosensilla (the site of initial perception of the food signal) had been modified and rendered non-functional (Fig. 1B, C, D). However, Aumann *et al.* (5) reported that a series of lectins, including *Helix pomatia* agglutinin, had no effect on sex pheromone reception. They further note that in electron microscope studies in which lectin were visualized binding to chemosensilla exudates (19), the lectins did not penetrate the exudates. Aumann *et al.* (5) propose that large molecular weight substances such as lectins probably cannot penetrate the chemosensilla exudates, and therefore could have no effect on the level of the chemoreception required

to inhibit the nematode from finding its food source. However, the Forrest and Robertson (19) study does not resolve the question of lectin penetration of amphid exudates. The ferritin-lectin conjugate used to visualize the presence of a specific carbohydrate in the amphid exudate, clearly labels only on the surface of the exudates. Lack of penetration, can be ascribed to the ferritin part of the complex, a molecule which is approximate 50 nm in size. Forrest and Robertson (19) also visualized lectin binding using rhodamine-lectin conjugates. Penetration of the exudates by the lectin as seen by tracing the fluor probably cannot be accurately measured by this method, in view of the very small distance from the exudate surface to the tip of the efferent neuron within the sensillum. The question of lectin permeability to sensilla exudates would benefit from further research. Later Aumann (4) reported that males of *Heterodera schachtii* showed a significant reduction in sex pheromone perception following treatment with the N-acetylgalactosamine specific lectin *Helix pomatia* agglutinin. These conflicting results give further support that the possibility of lectin penetration of sensilla exudates requires elucidation.

Tropical legumes are frequently interplanted with crop plants to reduce nematode damage. Reduction in root-knot nematode damage on tomato was associated with interplanting with the legumes *Pueraria phaseoloides* or *Arachis pintoi* in a study by Marban-Mendoza *et al.* (38). Soluble lectins were demonstrated by ELISA in root exudates from both legumes. These workers attributed the control of root-knot to the flow of lectins into the rhizosphere, this representing a continuous delivery of the nematode blocking agent and accounting for the antinematodal properties of *P. phaseoloides* and *A. pintoi* (Fig. 1B). Here again, the proposed mechanism of action was blocking of nematode chemoreception by the lectin at the site of the nematode chemosensory organs.

## Chemical Signalling Following Contact Between Nematodes and Other Organisms

Advances in knowledge of genetic control of events in cell-cell mechanisms involved in cell-cell recognition (14) suggest possibilities for modification of plant-microbe interactions. Speculations based on the premise that chemical communications between organisms could be blocked there by intervening in infection and the associated physiological processes formed the basis of a working hypothesis presented by the senior author at a section on Biological Control of Nematodes, Fifth International Congress of Plant Pathology, Kyoto, Japan (49).

The illustrative model involves the relation between the endoparasitic, nematophagous fungus *Drechmeria coniospora* and its nematode prey. Background studies provided proof that the initial phases of the relation between this parasite and its host are under molecular control. First, it was shown that nematodes are attracted by chemotactic factors given off by *D. conisopora* spores (30). The spores in turn, produced an adhesive bud which upon contact serves to bond it to the nematode (26, 42). This event may also depend for its initia-

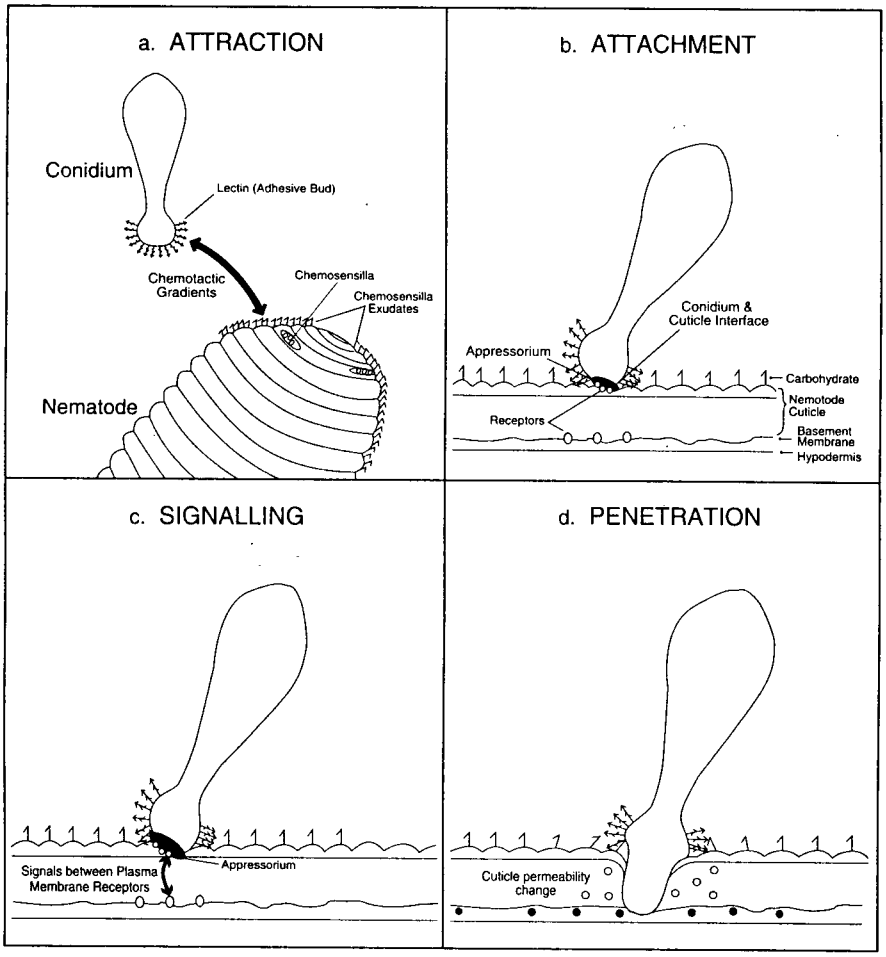


**Fig. 2.** Molecular events associated with cell-cell recognition between *Drechmeria coniospora* and its nematode host-theory and fact. A detailed description appears in the text. (a) Attraction of the nematode to the fungus spore. (b) Lectin on the adhesive bud binds specifically to a carbohydrate on the surface of the nematode cuticle. Receptors located respectively in the basement plasma membranes underlying the cuticle and the appressorium (c) Initiate signalling. (d) Signals preciptate a cascade of events which predispose the cuticle to infection by the fungus.

tion on detection of a chemical signal (Fig. 2A). Attachment of spore to host is under molecular control of a lectin-like substance on the spore with a sialic acid affinity complemented by a sialic acid homologue on the nematode surface (31). The lectin/sugar relation was proven in experiments in which decreased spore attachment followed treatment with the appropriate sialic acid specific lectin or a sialic acid hydrolyzing enzyme (27), although it was later shown that authentic sialic acid is not found in nematodes (6). One conclusion drawn from this latter finding is that the sugar observed on the nematode surface is a homologue capable of mimicking sialic acid. In some nematodes attachment of the spore is limited specifically to the head and tail, and in these cases the cabohydrate appears

to be associated with exudates which emanate from the chemosensilla. Further, the events associated with attachment and infection were demonstrated to be under different sets of molecular control, as shown when following treatment of spores with seven different preparations of sialic acid, significantly different delays in the inception of infection occurred (28). The current working hypothesis considers some of the signalling events which may precede penetration of the cuticle by the infection hypha of the spore. The initial stages of infection by *D. coniospora* have been studied by light and electron microscopy and by cytochemistry (13, 42). Within 30 min of adhesion of the spore to the nematode cuticle, a short appressorial tube developes from the knob. At this stage the adhesive knob, previously was closely appressed to the nematode, is raised and becomes free of the nematode. The appressorium is thin-walled and contains cell organells, including endoplasmic reticulum. Acid phosphatase activity was detected in the cuticle at the point of contact between the appressorium and the cuticle and at the time prior to the penetration of the cuticle by the infection tube. These studies delimit the time frame within which the initial signalling events would occur (Fig. 2C). Plasma membrane receptors are well documented as being central to cell-cell communication (39) and current knowledge dictates that the glycoconjugates must be the focus of the initial signal. Following attachment of the spore to the nematode cuticle, the thory proposes that the spore must receive an "infect" signal. The first perception of spore attachment would be by membrane receptors in the plasma membrane located beneath the nematode cuticle. These are termed primary receptors. The primary receptors would then generate signals which predispose the cuticle to penetration by the spore in several ways. First is the activation of hydrolytic enzymes already present in the cuticle. Evidence for enzymatic activity within the non-living cuticular structure in nematodes is cited by Bird (8) and others. A cascade of events leads to the release of cell wall fragments, increases in the porosity of the cuticle and changes in the gel properties of this structure. Similar events have been postulated as accompanying the penetration of the stigma cell wall by the germinating pollen tube (23), bacteria and plant cells (22). The signals from the primary receptors are concurrently perceived by what is here termed the secondary receptors; the receptors of the plasma membrane which underlie the cell wall of the *Drechmeria* spore (Fig. 2C). It is probable that signalling between primary and secondary receptors will be found to act in concert to bring about changes in the cuticle which immediately precede penetration by the fungus. Transduction of the "infect" signal from the primary to the secondary receptors may involve amplification of the signal via a mechanism similar to that of coupling of cell surface receptors to adenylate cyclase activation via a G protein. Perception of the signal and the accompanying molecular recognition that the cuticle is predisposed to infection mark the advent of the infection process (Fig. 2D). Induction of enzymes by the spore, which function in penetration of the cuticle, would accompany receipt of the message of the secondary receptors.

DNA recombinant technology makes it theoretically possible to isolate the gene for signalling or receptor molecules controlling cell-cell recognition for either of the interacting cells

(23). This approach would require the identification of the relevant gene products of the interaction. Chances for success would be greater if the pertinent molecule was expressed by a single dominant gene. An example of the possible application of this approach derives from the limited number of plant-parasitic nematode species attacked by *D. coniospora* (28). It was noted by Jansson *et al.* (29) that spores of this fungus attach profusely to the cuticle of *Heterodera avenae* second stage larvae, but no infection occurs.

This demonstrates the separation of the molecular requirements for recognition and from those needed for infection. Isolating the gene coding for an "infect" signal molecule and moving this gene to the fungus could lead to the development of a *D. coniospora* strain virulent to a wide range of animal and plant-parasitic nematodes. Interest in *D. coniospora* as a plant nematode was initially high, as shown by the patenting of the fungus for this purpose (Uniroyal Docket No. 6048, Middlebury, CT, Inventors B. M. Zuckerman and H. B. Jansson). However, the finding that many economically important plant nematodes resisted attack by *D. coniospora* and the loss of virulence to root-knot by the patented strain led to a cessation of development of a product. A novel approach, such as described, could stimulate a renewed effort to develop this biocontrol agent.

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