Nematicidal effect of collagen-amended soil and the influence of protease and collagenase (1)

Sergio GALPER*, Eli COHN*, Yitzhak SPIEGEL* and Ilan CHET**

* *Department of Nematology, Agricultural Research Organization, The Volcani Center, Bet Dagan 50250, Israel and* **Dept. of Plant Pathology and Microbiology, The Hebrew University of Jerusalem, Faculty of Agriculture, Rehovot 76100, Israel.*

SUMMARY

Collagen amendments to soil drastically reduced galling of tomato roots by *Meloidogyne javanica* when compared with other protein and chitin amendments. The approximate amount of ammonia released in the decomposition of collagen was inadequately lethal to *M. javanica* larvae. On the other hand proteolytic activity lasting at least 21 days in collagen-amended soil showed harmful effects *in vitro* on larvae and eggs. Soils amended with collagen two weeks prior to planting and inoculation showed a 50 % reduction in galling of tomato roots and an approximate reduction of 90 % in the number of eggs per plant. Collagenase has a stronger lethal effect on the nematode than protease. Collagenase-treated larvae failed to infect the host. The nematicidal action of protease and collagenase is discussed.

RÉSUMÉ

Effet nématicide du collagène utilisé comme amendement du sol et influence de la protéase et de la collagénase

L'utilisation du collagène comme amendement du sol réduit très fortement les galles racinaires de la tomate causées par *Meloidogyne javanica*, en comparaison des amendements à base de protéines ou de chitine. La quantité estimée d'ammoniaque libérée par la décomposition du collagène n'est pas léthale pour les larves de *M. javanica*. D'autres part une activité protéolytique d'une durée d'au moins 21 jours dans les sols amendés en collagène se révèle, *in vitro*, nocive envers les larves et les œufs. Un apport de collagène au sol deux semaines avant la plantation et l'inoculation produit une diminution de 50 % des galles racinaires de la tomate et une réduction d'environ 90 % du nombre des œufs par plante. La collagénase a une plus forte action léthale que la protéase. Les larves traitées avec la collagénase sont incapables d'infester une plante-hôte. L'action nématicide de la protéase et de la collagénase est discutée.

Many organic amendments are known to have adverse effects on populations of plant parasitic nematodes and considerable research has been done on the subject (Muller & Gooch, 1982). The nematicidal action of such compounds has been attributed to the release of toxic substances after bacterial decomposition (Badra, Saleh & Oteifa, 1979; Rodriguez-Kabana, Jordan & Hollis, 1965), the buildup of soil antagonists to parasitic nematodes (Rodriguez-Kabana, Morgan-Jones & Chet, 1987; Sayre, 1980) or the lethal effect of nitrogen buildup in soil, specifically ammonia (Eno, Blue & Good, 1955). Not much is known about the direct effect of soil enzymes on nematodes, but Miller and Sands (1977) working with two plant parasitic nematodes, found that protease, lipase and chitinase killed *Tylenchorhynchus dubius in vitro* and in the soil, and that collagenase was most effective in soil against *Pratylenchus penetrans*.

Since collagen is the main component of the nematode cuticle (Reddigari *et al.*, 1986), it was deemed of interest to study the effect of collagen as a soil amendment in comparison with other protein and chitin amendments, and also to evaluate the role of proteolytic and collagenolytic activity on this system.

Materials and methods

AMENDMENTS

The following amendments were added to pots containing 200 g of sandy soil in a concentration of 0.2 % w/w : collagen (Sigma), egg yolk protein (Sigma), peptone (Difco), chitin (Sigma), and Clandosan (IGI, Columbia, MD, USA) a product composed of roughly 70 % protein and 30 % chitin. Unamended soil was used as control.

Soils were amended and inoculated with approximately 180 *Meloidogyne javanica* eggs per pot on the day of planting. The plants used were 4-week-old tomato seedlings. Amendments with collagen (same concentration as above) at different times prior to planting were given, to observe their influence on the infectivity of the

(1) Contribution from the Agricultural Research Organization, Bet Dagan, Israel No. 2583-2, 1989 series.

nematodes at different stages of decomposition. Here also plants were inoculated on planting day.

The experiment was performed in temperature-controlled chambers (27 ± 3 °C), with each treatment done in eight replicates. After 5 weeks, plants were removed and their root galling index (0 to 50 scale) was determined. Four plants per treatment were sampled for egg counts, which were performed after extracting the eggs by shaking individual roots in an Erlenmeyer flask for 10 min in a solution of 0.3 % sodium hypochloride. Eggs used for inoculation were also obtained in the same way, and thoroughly washed with tap water.

Kinetics of ammonium in decomposing collagen and *Establishment of the ammonia dose lethal to* M. javanica

Collagen was added to pots (0.2 % W/W) containing 200 g of soil and kept in temperature-controlled rooms for different periods. Ammonium was determined in the soil by steam distillation (Keeney & Nelson, 1982).

Sandy soil was sieved, and dried in an oven for 24 h at 80°. 50 g of soil was placed in small plastic containers and wetted until 60 % field capacity. Solutions of ammonium hydroxide (Frutarom, Haifa, Israel) were prepared to give concentrations of 65, 125, 185 and 245 ppm of ammonia and were added to the containers. *M. javanica* larvae, obtained from infested tomato roots in a mistifier, were placed 300 per container, and after 48 h exposure were extracted by the Baermann funnel method and counted.

Effect of collagenase and protease on M. javanica *larvae*

Meloidogyne javanica larvae and eggs were placed in a multi well plate (Beton Dickinson Labware, CA, USA) in a solution of buffer tris, pH 7.6, and collagenase from *Clostridium histoliticum* (Boehringer, Germany) or protease from *Streptomyces griseus* (Sigma), and their motility was measured after 48 h. Larvae were then washed and used to inoculate tomato seedlings that had their roots indexed for galling after five weeks. Eight replicates were used per treatment.

SOIL PROTEOLYTIC ACTIVITY

Five-gram soil samples from pots where collagen had been added at different times were placed in Erlenmeyer flasks containing 50 ml distilled water. Samples were shaken for 20 min, centrifuged, and passed through cellulose triacetate filters (Gelman Sciences, Michigan USA) size 0.45 µm. Samples were tested against Azocoll (Sigma), a substrate for nonspecific proteases, which releases a red dye into the solution when degraded. 25 mg of Azocoll was added to a 5 ml sample and incubated for 8 h at 37 °C. Absorbance was measured at 520 min, following filtration.

Controls consisted of unamended soil and boiled samples of extracts from collagen- amended soils.

Results

All protein and chitin amendments decreased gall formation as compared with untreated plants (Fig. 1); collagen was significantly more effective than the other amendments. In soils amended with protein, there was much less root nematode infection than in chitin-amended soils, as seen from the significantly lower galling index of all protein amendments compared with chitin and clandosan amendments.

All treatments resulted in a markedly lower nematode egg count than with untreated controls (Fig. 1). Protein and chitin amendments yielded a significantly lower egg count than clandosan. Ammonium production by peptone, 300 ppm higher than that by collagen which reached a peak of 80 ppm in the first week and sharply declined at the beginning of the second week (Fig. 2). Sensitivity of *M. javanica* larvae to ammonia was not marked until a concentration of about 90 ppm was reached, when the recovery of nematodes from soil was about 60 % (data not shown). Between levels of 185 and 245 ppm ammonia, almost no nematode larvae survived in the soil. Collagen amendment reduced root gall formation and egg numbers even after two weeks of decomposition in the soil (Fig. 3). The proteolytic activity of a collagen-amended soil could be detected even after three weeks at a higher level than at the start of the measurements (Fig. 4).

Both protease and collagenase had adverse effects on larval motility *in vitro* (Fig. 5), the former being stronger than the latter. However, collagenase had a greater effect on egg hatch (Fig. 6). When larvae were treated with the enzymes prior to inoculation, protease-treated larvae caused a significant decrease of 40 % in galling, while collagenase-treated larvae produced no galling at all (Fig. 7).

Discussion

The idea of amending the soil with a substance similar to the one present on the outer layer of a pathogen was first suggested by Mitchell and Alexander (1961) for controlling pathogenic *Fusarium*. Chitin, which is one of the main components of the cell wall in most fungi was employed. The enhanced chitinolytic microflora as a result of amendment was assumed to be the main factor in pathogen suppression.

Chitin amendments have also been reported to control plant parasitic nematodes (Mankau & Das, 1969; Mian *et al.*, 1982; Spiegel, Cohn & Chet, 1986, 1987, 1988). The mode of action of such amendments is not fully understood. Although chitin was found to be present in the gelatinous matrix of *Meloidogyne* spp. (Spiegel & Cohn, 1985), its amount and precise location have not been determined. Chitin is also present in nematode egg shell, but only in the inner layers, the outer layers being of a proteinaceous nature (Bird, 1971; Lee & Atkinson,

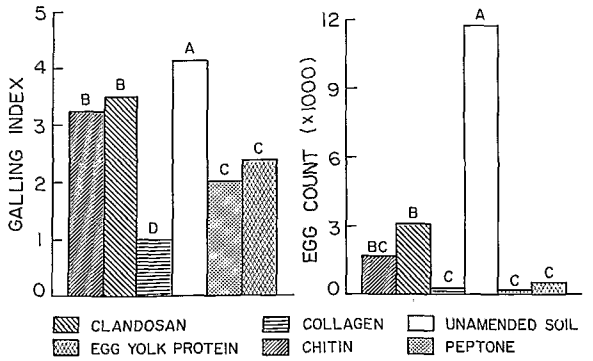

Fig. 1. Effect of protein amendments to soil on tomato root galling and egg count of *Meloidogyne javanica*. Values with the same letter do not differ significantly at P = 0.05 according to Duncan's multiple range test.

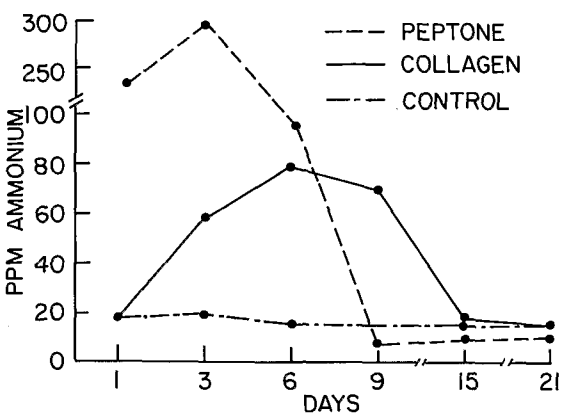

Fig. 2. Kinetics of ammonium content of soils amended with peptone and collagen.

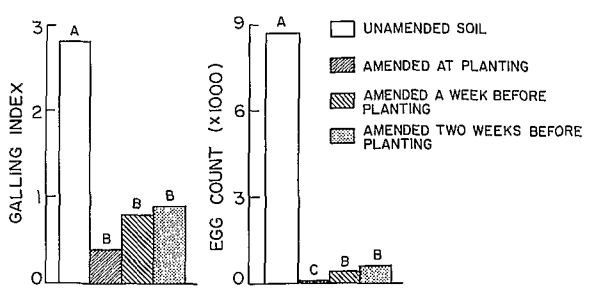

Fig. 3. Effect of collagen amendment at different stages of decomposition in soil on galling index and egg count of *Meloidogyne javanica* on tomato roots. Values with the same letter do not differ significantly at P = 0.05 according to Duncan's multiple range test.

1976). The egg shell of *Heterodera rostochiensis* is reported to contain much less chitin than protein (Clarke, Cox & Shepherd, 1967) with some of the protein being in the form of collagen. Rodriguez-Kabana *et al.* (1983) measured chitinase activity in soil following chitin amendment but did not report on how long chitinase activity persisted in soil. Information on the influence of chitinase on plant parasitic nematodes is lacking. It has been suggested (Godoy *et al.*, 1983) that chitin may increase the population of egg parasites but further experimental evidence is needed to ascertain this possibility.

The rationale of Mitchell and Alexander (1961) would probably be best met by using collagen as a soil amendment to increase collagenolytic and proteolytic microflora. Collagen is a long rod-like molecule consisting of three polypeptide chains wound about each other. The first step in the degradation of collagen is the initial attack by collagenase giving rise to two peptides that can be further digested by proteases (Harper, 1980). These two enzymes are the ones that potentially can harm external structures of nematodes in the egg and cuticle, or in cuticular structures like cysts or stylet due to their protein-collagen nature. This may explain the better results obtained by the protein amendments when compared with chitin amendments.

Ammonium released can explain the lower root galling index found in the protein but not in the collagen treatments. During the first critical days after inoculation, when egg hatch and root penetration occurs, peptone amendment to soil released up to 300 ppm ammonium (Fig. 2), which gives a good indication of the approximate amount of ammonia in the soil. That amount is far above the concentration of ammonia lethal to the nematode larvae, which was found to be between 185-245 ppm. On the other hand, ammonia release does not fully explain the lesser galling index obtained with the collagen amendments because collagen release of ammonium reaches its peak of 80 ppm only after six days (Fig. 2) and the proportional concentration of ammonia is not adequately harmful to larvae.

Proteolytic and collagenolytic activity in collagen-amended soil may explain the lethal effect on larvae even when such an amendment produces low levels of ammonia. Proteolytic activity lasting at least 21 days, can maintain low galling levels, even in soils where collagen was added two weeks before inoculation (Figs 3, 4). Collagenolytic activity in soil was not measured, but we assumed it to be present, since mainly collagenase is necessary for the degradation of collagen (Harper 1980).

Results obtained *in vitro* showed that the two enzymes separately can each affect larval motility and egg hatch. Collagenase proved very effective in reducing larval penetration in roots. The presence of collagenase in addition to protease could explain the stronger effects on *M. javanica* obtained with collagen as compared with other protein amendments.

Enzymes in soil can become stabilized and persist for long periods after their original source has already been destroyed (Ladd, 1978); hence it would be useful to monitor proteolitic and collagenolitic activity, when

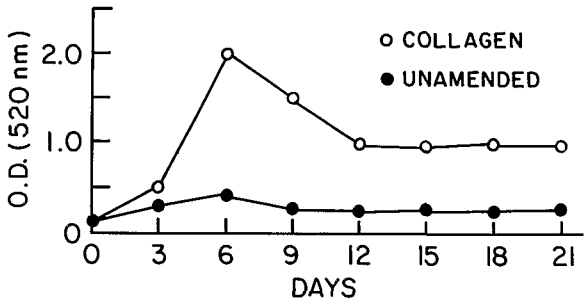

Fig. 4. Proteolytic activity of soil amended with collagen.

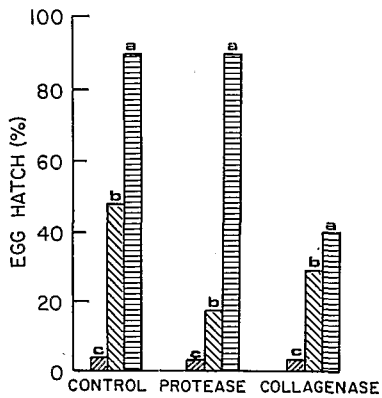

Fig. 6. Effect of protease and collagenase on egg hatch of *Meloidogyne javanica in vitro*. Values A, B, and C in each treatment are significantly different at P = 0.05 according to Duncan's multiple range test (left : 2 days; center : 5 days; right : 9 days).

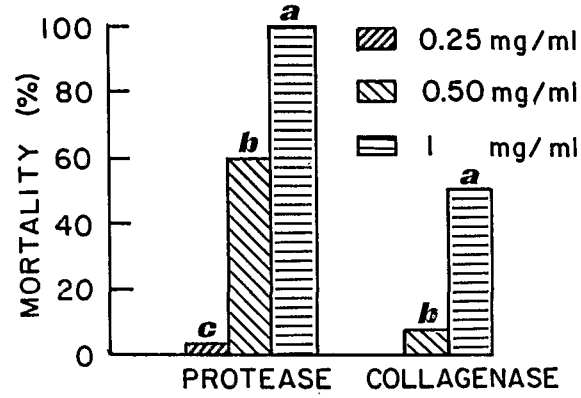

Fig. 5. Effect of protease and collagenase on the mortality of *Meloidogyne javanica* larvae in vitro. Values A, B, and C in each treatment are significantly different at P = 0.05 according to Duncan's multiple range test.

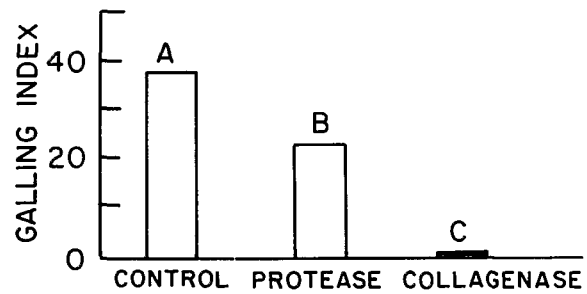

Fig. 7. Effect of protease — and collagenase — treated larvae of *Meloidogyne javanica* on galling index of tomato roots. Values A, B, and C are significantly different at P = 0.05 according to Duncan's multiple range test.

studying the lethal effects of organic amendments on plant-parasitic nematodes. Isolation of microorganisms with strong proteolytic or collagenolytic activity from soil and adding to them a proper substrate is likely to enhance the nematicidal activity of organic amendments to soil.

References

Badra, R., Saleh, M. A. & Oteifa, B. A. (1979). Nematicidal activity and composition of some organic fertilizers and amendments. *Revue Nématol.*, 2 : 29-36.

Bird, A. F. (1971). *The Structure of Nematodes.* New York, Academic Press, 318 p.

Clarke, A. S., Cox, P. M. & Shepherd, A. M. (1967). The chemical composition of the egg shells of the potato cyst nematode *Heterodera rostochiensis* Woll. *Biochem. J.*, 104 : 1056-1060.

Eno, C. F., Blue, W. G. & Good, J. M. (1955). The effect of anhydrous ammonia on nematodes, fungi, bacteria and nitrification in some Florida soils. *Proc. Soil Sci. Soc. Am.*, 19 : 55-58.

Godoy, G., Rodriguez-Kabana, R., Shelby, R. A. & Morgan-Jones, G. (1983). Chitin amendments for control of *Meloidogyne arenaria* in infested soil. II. Effects of microbial population. *Nematropica*, 3 : 63-73.

Harper, E. (1980). Collagenase. *A. Rev. Biochem.*, 49 : 1063-78.

Keeney, D. R. & Nelson, D. W. (1982). Nitrogen-Inorganic forms. In : Page, A. L. (Eds) *Methods of Soil Analysis. Part 2.* Agronomy Monograph no. 9 (2nd ed.) : 643-698.

Ladd, J. N. (1978). Origins and range of enzymes in soil. In : Burns, R. G. (Ed.) *Soil Enzymes.* London, Academic Press : 51-96.

Lee, D. L. & Atkinson, H. S. (1976). *Physiology of Nematodes.* London, Macmillan, 215 p.

Mankau, R. & Das, S. (1969). The influence of chitin amendment on *Meloidogyne javanica*. *J. Nematol.*, 1 : 15-16 [Abstr.].

Mian, I. H., Godoy, G., Shelly, R. A., Rodriguez-Kabana, R. & Morgan-Jones, G. (1982). Chitin amendments for

control of *Meloidogyne arenaria* in infested soil. *Nematropica,* 12 : 71-84.

MILLER, P. M. & SANDS, D. C. (1977). Effects of hydrolytic enzymes on plant-parasitic nematodes. *J. Nematol.,* 9 : 192-197.

MITCHELL, R. & ALEXANDER, M. (1961). Chitin and biological control of *Fusarium* diseases. *Pl. Dis. Reptr,* 45 : 487-490.

MULLER, R. & GOOCH, P. S. (1982). Organic amendments in nematode control. An examination of the literature. *Nematropica,* 12 : 319-325.

REDDIGARI, S. R., JANSMA, P. L., PREMACHANDRAN, D. & HUSSEY, R. S. (1986). Cuticular collagenous proteins of second-stage juvenile and adult females of *Meloidogyne incognita;* Isolation and partial characterization. *J. Nematol.,* 18 : 294-302.

RODRIGUEZ-KABANA, R., GODOY, G., MORGAN-JONES, G. & SHELBY, R. A. (1983). The determination of soil chitinase activity : conditions for assay and ecological studies. *Pl. Soil,* 75 : 95-106.

RODRIGUEZ-KABANA, R., JORDAN, J. W. & HOLLIS, J. P. (1965). Nematode biological control in rice fields : role of hydrogen sulfide. *Science,* 148 : 523-524.

Accepté pour publication le 28 mars 1989.

RODRIGUEZ-KABANA, R., MORGAN-JONES, G. & CHET, I. (1987). Biological control of nematodes : soil amendment and microbial antagonists. *Pl. Soil,* 100 : 237-247.

SAYRE, R. M. (1980). Promising organisms for biocontrol of nematodes. *Pl. Dis.,* 64 : 527-532.

SPIEGEL, Y., CHET, I., COHN, E., GALPER, S. & SHARON, E. (1988). Use of chitin for controlling plant parasitic nematodes. III. Influence of temperature on nematicidal effect, mineralization and microbial population build up. *Pl. Soil,* 109 : 251-256.

SPIEGEL, Y. & COHN, E. (1985). Chitin is present in the gelatinous matrix of *Meloidogyne. Revue Nématol.,* 8 : 184-194.

SPIEGEL, Y., COHN, E. & CHET, I. (1986). Use of chitin for controlling plant parasitic nematodes. I. Direct effects on nematode reproduction and plant performance. *Pl. Soil,* 95 : 87-95.

SPIEGEL, Y., COHN, E. & CHET, I. (1987). Use of chitin for controlling plant parasitic nematodes. II. Mode of action. *Pl. Soil,* 98 : 337-345.