

# The influence of *Pasteuria penetrans* in field soils on the reproduction of root-knot nematodes

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## SUMMARY

Soils from seven localities in vineyards adjacent to the towns of Loxton and Cooltong in South Australia were examined for their ability to suppress reproduction in root-knot nematodes added to these soils. The soils from Cooltong significantly reduced the number of egg masses produced by these nematodes and this inhibition was removed by autoclaving the soil. In all cases where inhibition of nematode reproduction occurred, *Pasteuria penetrans* was found in adult females without egg masses. No inhibitory effects on *Meloidogyne javanica* by various other bacteria isolated from these soils were observed. A comparison of the suppressive effects of the microorganisms in Cooltong soils on several species of root-knot nematodes was made and the results are discussed in relation to biological control of these nematodes.

## RÉSUMÉ

*Influence en plein champ de Pasteuria penetrans sur la reproduction du nématode des racines noueuses*

L'aptitude du sol à inhiber la reproduction des nématodes des racines noueuses a été déterminée en sept endroits de vignobles voisins des villes de Loxton et Cooltong, en Australie du Sud. Les sols de Cooltong ont significativement réduit le nombre de masses d'œufs produites par ces nématodes. Cette inhibition était supprimée lorsque le sol était autoclavé. Dans tous les cas où une réduction de la reproduction du nématode avait lieu, *Pasteuria penetrans* a été trouvé dans les femelles adultes sans masses d'œufs. Aucune autre bactérie isolée de ces sols n'a présenté d'effet inhibiteur sur *Meloidogyne javanica*. Une comparaison des effets suppressifs des sols de Cooltong sur plusieurs espèces de nématodes des racines noueuses a été effectuée, et les résultats sont discutés en relation avec la lutte biologique contre ces nématodes.

Stirling and White (1982) found that *Pasteuria penetrans*, an obligate parasite of root-knot nematodes, was widely distributed in vineyards in South Australia that had been established for 25 years or more. They reported that there was a tendency for the numbers of root-knot nematodes to be lower in these older vineyards than in those that were less than ten years old. They concluded that *P. penetrans* may "have reduced nematode population in old vineyards".

In contrast Spaul (1984) found that root-knot nematode populations were generally larger in sugarcane fields in which they were parasitized by *P. penetrans* than in those fields without this parasite.

In the experiments described below we have tested a number of soils from South Australian vineyards for their capacity to suppress reproduction of root-knot nematodes in tomato plants. Reproduction of larvae added in known numbers to these soils was measured by counting egg masses and the presence of *P. penetrans* was detected by observing its spores in adult females that had not reproduced.

There were three main experiments. The first was designed to detect soils that inhibited nematode reproduction, the second to establish whether the inhibition

had a biological origin and the third to extend the investigation to additional *Meloidogyne* species.

In addition a search was made for an antagonistic bacteria in the soils suppressive to root-knot nematode reproduction using infective larvae of *M. javanica*.

## Materials and methods

### NEMATODES

The species of root-knot nematodes used in these experiments were *Meloidogyne incognita*, originally obtained from Gol Gol (NSW) and grown in capsicums (*Capsicum annuum*). *M. javanica*, originally obtained from Merbein (Victoria) and grown in tobacco (*Nicotiana tabacum*) and an unidentified species of *Meloidogyne* obtained from Loxton (SA) and grown in tomato (*Lycopersicon esculentum*). The *M. incognita* and *M. javanica* had been maintained in the glass house for many years whereas the *Meloidogyne* sp. from Loxton was isolated from one of the soils (4 Ls) collected and tested in the first experiment.

The *M. javanica* used in the last experiment came from a single egg mass derived from the original

Merbein population used in the first two experiments and its descendent, many years ago, from a single egg mass population.

#### SOIL SAMPLES

##### *Experiment I*

Initially, soils were obtained from around the roots of grapevines (*Vitis vinifera*) in seven different localities. These consisted of four vineyards (sultana) in the vicinity of the town of Loxton (1 Ls, 2 Ls, 3 Ls and 4 Ls) and three sites close to the town of Cooltong, each with a different variety of grapevines viz. doradillo, gordo and palomino (5 Cd, 5 Cg and 5 Cp).

These seven soil samples were collected on the 18 October 1984, each sample being made up of six subsamples taken about 10-15 cm beneath the top of the soil and sieved through a 3 mm sieve to remove roots and stones, etc., before mixing uniformly and placing in small pots each containing 500 g of soil.

##### *Experiment II*

This experiment was started on the 23 of May 1985 using soils from Exp. I (4 Ls, 5 Cd and 5 Cp) which had been stored in plastic bags at 5°. These soils were each divided into two equal portions, one of which was autoclaved in bags containing no more than 4 kg of soil at 15 lbs/sq in. for 60 mn prior to placing into small pots as described above.

##### *Experiment III*

Soils from Cooltong vineyards 5 Cd and 5 Cp were collected fresh from the same sites as Exp. I on the 18 February 1986 and sieved and mixed the following day before being divided into two portions, one being autoclaved as described above. Three populations of nematodes (*M. javanica*, *M. incognita* and an unidentified *Meloidogyne* species from Loxton) were used and each treatment was replicated five times. A total of 80 pots was used including four treatments totalling 20 pots to which no nematodes were added. These permitted the background numbers of root-knot nematode egg masses to be determined.

#### INFECTION OF PLANTS

In all experiments tomato seedlings (*Lycopersicon esculentum* cv. Tatura Dwarf) were planted into the soils and were allowed to become established over a one or two week period during which each plant received an equal amount of half strength Hoaglands nutrient solution (Bird, 1960). Infective second stage larvae (L2) of the various populations used were obtained from egg masses dissected from the roots of host plants. These eggs were hatched in shallow distilled water in Petri dishes at 22°. Freshly hatched L2s were collected,

counted and inoculated around the roots of the experimental tomato seedlings in equal numbers in each experiment (1 000 per plant in Exp. I, 500 per plant in Exp. II and 400 per plant in Exp. III). Each treatment was replicated four or five times and the pots were randomized and grown in an air conditioned glass house at temperatures which ranged from 13 to 33°. A constant thermographic record was maintained.

#### HARVESTS

The plants within the different experiments were all harvested at the same time. The mean time for the three experiments was  $19 \times 10^3$  Celsius hours or 444 heat units (Tyler, 1933). Development at this temperature resulted in the formation of large egg masses from the first generation of nematodes without egg masses from the second generation having time to form. At harvest, plants were washed gently in water to remove soil, excess water was removed on paper towels and the plants were then weighed and the roots stored in plastic bags at -18°. When required, the roots were thawed and the egg masses counted with the aid of a dissecting microscope.

#### SPORE COUNTS

Aliquots of root material (0.5 g/5 ml H<sub>2</sub>O) were homogenized with a pestle and mortar and the homogenate passed through a 50 µm mesh and the spores counted in a haemocytometer.

#### USE OF L2 OF *M. JAVANICA* AS A BACTERIAL PROBE

Approximately  $5 \times 10^3$  L2 were added in 15 ml of sterile distilled water (SDW) to 50 g of the Cooltong soil being tested. This soil rested on paper tissue covering plastic mesh suspended over and touching the shallow SDW in a Petri dish. After 18 h the fluid beneath the mesh was removed and centrifuged at 50 g to sediment the nematodes which were examined for spore adhesion after washing several times by centrifugation at 50 g, before plating out for bacterial determination. These L2s were plated onto various media (King B agar, TSA/10 and PDA/2). Bacteria that grew on these media in the tracks of the nematode were replated and isolated populations were grown and tested for their capacity to adhere to L2. They were also tested for their influence on L2 motility, infectivity and capacity to induce galls in the roots of tomato plants.

#### MICROSCOPY

Specimens were observed under coverslips sealed to glass slides using transmitted light and an Olympus Vanox AHB research microscope with either bright field or differential interference contrast optics. Photographs were taken using Ilford Pan F film.

STATISTICAL ANALYSES

The data were transformed by raising them to the 2/3 power in order to give approximately constant variance and consequently valid use of F tables for significance testing. The bar graphs in Figs 1, 2 and 3 show the back transformed means which differ a little from the arithmetic means which are shown as figures at the top of each bar. The use of a non linear ordinate scale allows the single confidence limit to be used for all comparisons.

Results

EXPERIMENT I

Egg mass counts show (Tab. 1) that the soils collected from around the roots of the three varieties of grapevines on the Cooltong vineyard (5 Cd, 5 Cg and 5 Cp) were significantly ( $P < 0.001$ ) more suppressive to nematode reproduction than were the soils taken from around the roots of sultana vines on the four vineyards at Loxton (1 Ls, 2 Ls, 3 Ls and 4 Ls). These differences in the reproductive capacity of *M. javanica* inoculated around the roots of tomatoes grown in soils from two regions (approximately 200 km apart) show (Fig. 1) that the mean egg mass count from Loxton soils was much greater (202) than that from Cooltong soils (10).

The four Loxton samples all contained root-knot nematodes which reproduced in control plants to which

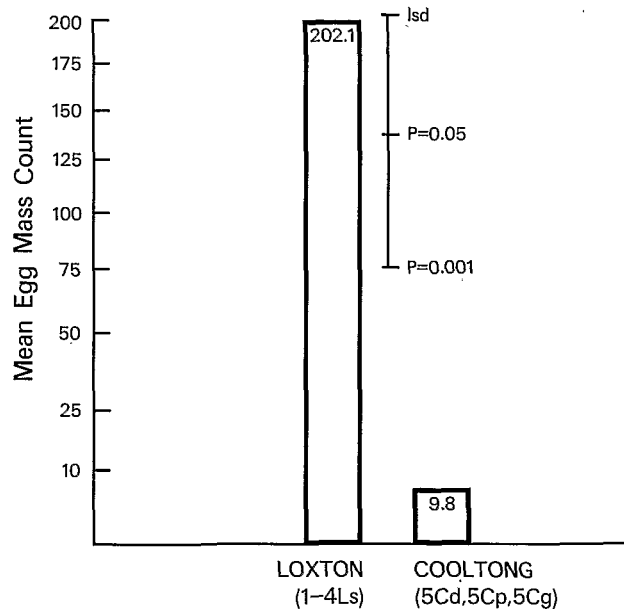


Fig. 1. Bar chart showing back transformed means of egg mass counts of *M. javanica* grown in tomatoes in soil from Loxton (1-4 Ls\*<sup>1</sup>) and Cooltong (5 Cd\*<sup>2</sup>, 5 Cp\*<sup>3</sup>, and 5 Cg\*<sup>4</sup>). The ordinate has been given a two thirds power transformation. The arithmetic means are shown at the top of each bar.

1-4 Ls\*<sup>1</sup> = Vineyards Nos. 1, 2, 3 and 4 from Loxton with grape variety sultana.

5 Cd\*<sup>2</sup> = Vineyard No. 5 from Cooltong with grape variety doradillo.

5 Cp\*<sup>3</sup> = Vineyard No. 5 from Cooltong with grape variety palomino.

5 Cg\*<sup>4</sup> = Vineyard No. 5 from Cooltong with grape variety gordo.

Table 1

Reproduction of *M. javanica* (egg mass counts) in the roots of tomato plants grown in soil from various vineyards in South Australia

Vineyard	Soils	Plant fresh weight (g)			No. of egg masses		
		Range	Mean*	S.D.	Range	Mean*	S.D.
1	L* <sup>1</sup> s* <sup>2</sup>	7.0-7.7	7.2	0.3	176-316	225	60
2	Ls	5.5-9.4	7.2	1.6	41-279	190	106
3	Ls	6.0-7.2	6.6	0.8	178-193	184	11
4	Ls	5.4-7.8	6.6	1.0	84-343	242	114
5	C* <sup>3</sup> d* <sup>4</sup>	4.9-5.5	5.1	0.3	0-35	15	18
5	Cg* <sup>5</sup>	3.6-4.8	4.1	0.6	0-4	2	2
5	Cp* <sup>6</sup>	4.9-6.3	5.3	0.6	0-65	16	32

L\*<sup>1</sup> Loxton, South Australia

s\*<sup>2</sup> Sultana

C\*<sup>3</sup> Cooltong, South Australia

d\*<sup>4</sup> Doradillo

g\*<sup>5</sup> Gordo

p\*<sup>6</sup> Palomino

\* Mean of four replicates

no L2 of *M. javanica* were added (mean of fifteen egg masses per plant), compared with the Cooltong samples in which egg masses were only detected in one sample (mean of one egg mass per plant).

*Fusarium oxysporum* was isolated from tomato roots of 5 Cd and 5 Cg and spores of *P. penetrans* were isolated from the roots of 5 Cp which contained many galls without egg masses.

EXPERIMENT II

The objective of this experiment was to determine whether or not the suppression of nematode reproduction shown in the Cooltong soils was biological in nature. The term suppressive refers to the effects of the microorganisms in these soils on nematode reproduction.

Three of the original seven soils were selected, namely, 4 Ls a non-suppressive soil and 5 Cd and 5 Cp both suppressive soils (Tab. 1). These three soils were

divided into autoclaved and non-autoclaved groups. There were five replicates giving a total of 30 pots.

A single plant grown in each pot was infected with 500 freshly hatched L2 of *M. javanica*. Autoclaving of the non-suppressive soil did not lead to any increase in egg mass production, in fact there was a slight decrease. Autoclaving of the suppressive soils resulted in highly significant ( $P < 0.001$ ) increases in egg mass production by female root-knot nematodes in these soils (Fig. 2). There was a five-fold increase in the mean number of egg masses in autoclaved soil from around the roots of doradillo grapevines (5 Cd) and an eight-fold increase in the mean number of egg masses in autoclaved soil from around the roots of palomino grapevines (5 Cp) as shown (Fig. 2). Thus it is likely that the suppression of egg laying in *M. javanica* in the two Cooltong soils tested is due to a biological factor. We observed galls containing females full of spores of *Pasteuria penetrans* in the two non-autoclaved Cooltong soils but not in the non-autoclaved Loxton (4 Ls) soil.

### EXPERIMENT III

The suppressive effects of these two Cooltong soils on different species of root-knot nematodes were examined using soil samples collected from the original sites in the summer and following recent irrigation.

Preliminary tests of these soils from around the roots of doradillo and palomino varieties of grapevine showed that both contained spores of *Pasteuria penetrans* that

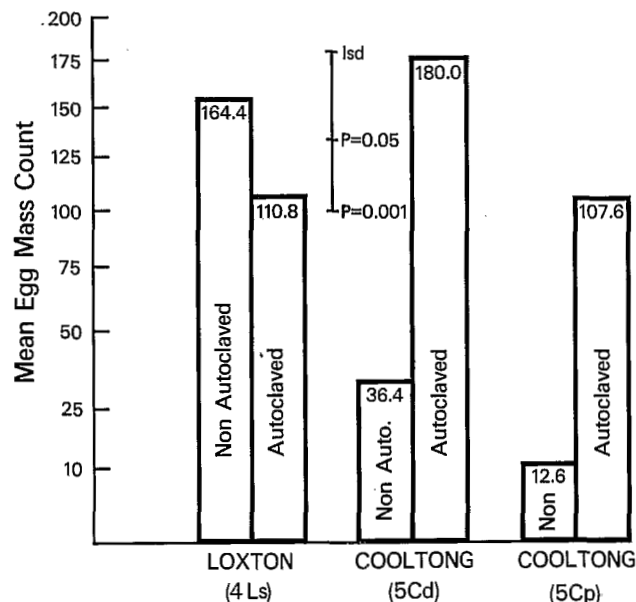


Fig. 2. Bar chart showing back-transformed means of egg mass counts of *M. javanica* grown in autoclaved and non-autoclaved soils from Loxton (4 Ls<sup>\*1</sup>) and Cooltong (5 Cd<sup>\*2</sup> and 5 Cp<sup>\*3</sup>). The ordinate has been given a two thirds power transformation. The arithmetic means are shown at the top of each bar.

4 Ls<sup>\*1</sup> = Vineyard No. 4 from Loxton with grape variety sultana.

5 Cd<sup>\*2</sup> = Vineyard No. 5 from Cooltong with grape variety doradillo.

5 Cp<sup>\*3</sup> = Vineyard No. 5 from Cooltong with grape variety palomino.

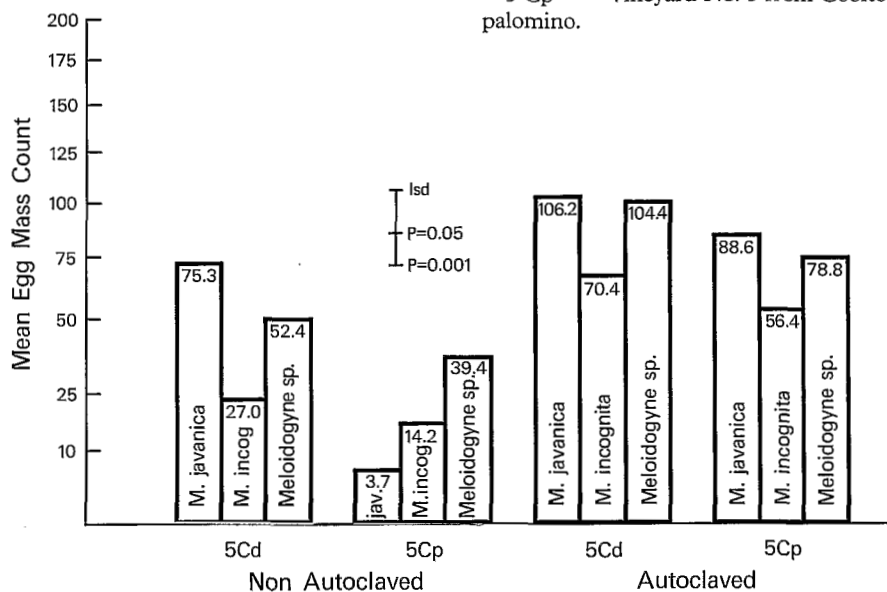


Fig. 3. Bar chart showing back transformed means of egg mass counts of *M. javanica*, *M. incognita* and *Meloidogyne sp.* grown in autoclaved and non-autoclaved soils from Cooltong (5 Cd<sup>\*1</sup> and 5 Cp<sup>\*2</sup>). The ordinate has been given a two thirds power transformation. The arithmetic means are shown at the top of each bar.

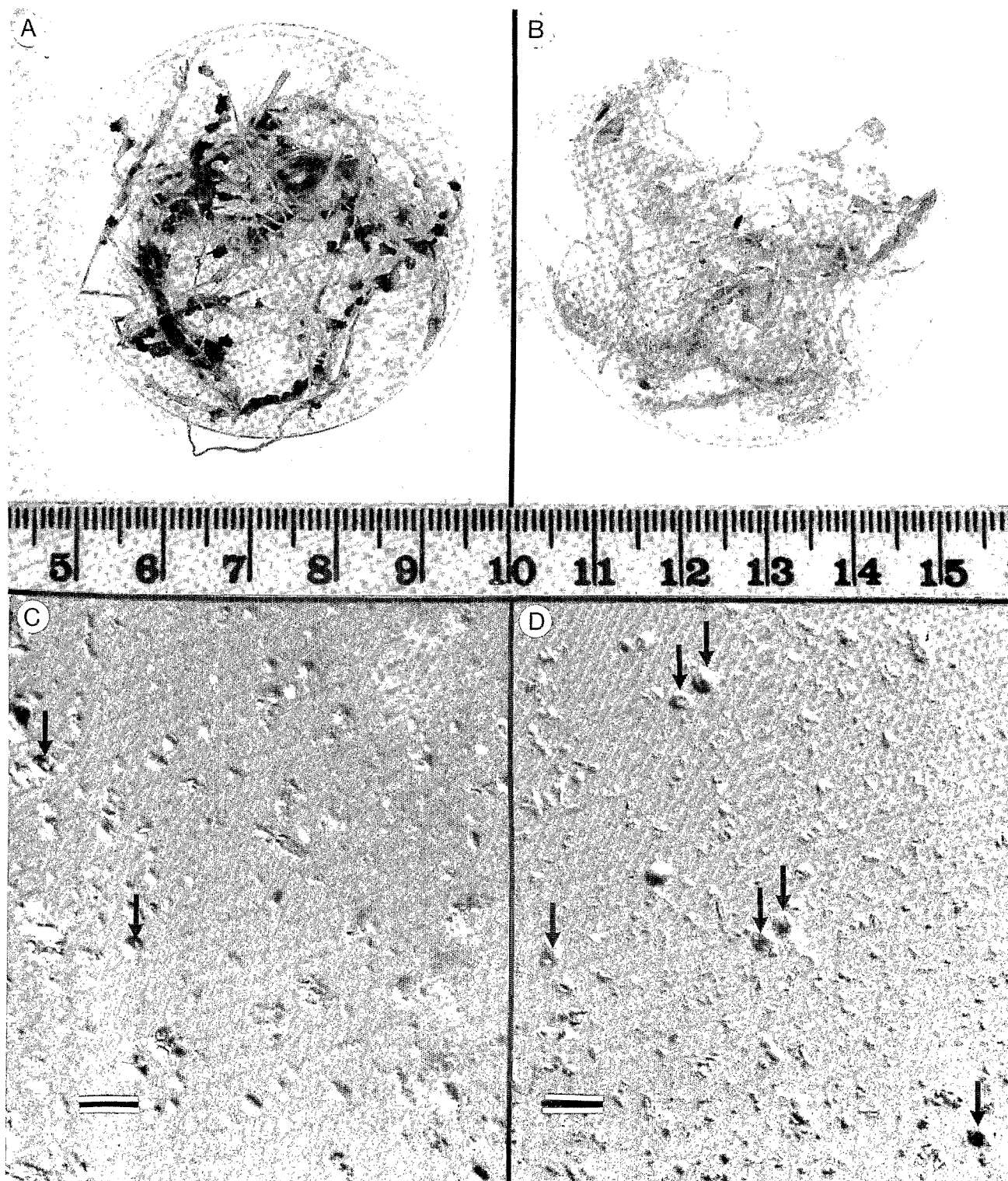


Fig. 4. A-B : Tomato roots grown in soil taken from around the roots of grapevine varieties doradillo (A) and palomino (B). These were infected with *M. javanica* and the nematodes allowed to grow and reproduce. The photographs show that many more egg masses can be seen on roots grown in the doradillo soil than on roots grown in the palomino soil. C-D : Presence of *P. penetrans* spores (arrows) in the ground up roots (A) and (B). More spores occur in ground up palomino roots (B) than in the doradillo (A). These spores originated from females in galls without egg masses (Bar = 1  $\mu$ m).

adhered to L2 of *M. javanica* and *M. incognita* that were made to migrate through them. When roots that had been sieved from these soils were placed in a misting apparatus some L2 of root-knot nematodes were observed but these were greatly outnumbered by males and larvae of the citrus nematode (*Tylenchulus semipenetrans*). These nematodes were free of adhering spores of *P. penetrans*.

The sixteen treatments consisted of two soils (Cd & Cp) which were either autoclaved or non-autoclaved, three nematode species (*M. javanica*, *M. incognita* and *Meloidogyne* sp.) and a control in which no nematode were added; these treatments were replicated five times giving a total of eighty pots.

The same number (400) of freshly hatched L2 were added to a plant in each pot so that a total of twenty pots was inoculated for each nematode species.

The results of this experiment are shown in Figure 3. There was a significant increase ( $P < 0.05$ ) in the reproductive capacities of all nematodes in the autoclaved soils compared with those on non-autoclaved soils. The responses to autoclaving these soils were similar for *M. incognita* and *Meloidogyne* sp. (Fig. 3) but the response of the *M. javanica* population in the two soils was markedly different (Fig. 3). This difference was also apparent when comparisons were made within the non-autoclaved soils. Thus both *M. incognita* and *Meloidogyne* sp. reproduced similarly on doradillo (Cd) and palomino (Cp) soils whilst *M. javanica* females produced significantly ( $P < 0.001$ ) more egg masses on Cd than they did on Cp.

To determine the number of spores of *P. penetrans* infecting the adult females of *M. javanica*, two 0.5 g root samples, taken from one of the five replicates of these two soils, were photographed (Fig. 4 A, B) to illustrate the difference in number of egg masses between A (Cd) and B (Cp). The extent of galling in both these samples was similar. These two samples were each ground and sieved, as described above, and the spores of *P. penetrans* counted. The counts were  $9.1 \times 10^6$  spores/ml for sample Cp (Fig. 4 A) and to  $2.7 \times 10^6$  spores/ml for sample Cd (Fig. 4 B). When drops of the ground and sieved samples were sealed under coverslips and photographed randomly, the number of spores on the photographic enlargements were  $15.4 \pm 4.1$  for Cp compared with  $3.7 \pm 1.0$  for Cd. Sample photographs are shown in Fig. 4 C (Cd) and Fig. 4 D (Cp).

#### FRESH WEIGHTS OF PLANTS

Comparison of the fresh weights of plants grown in unautoclaved and autoclaved soils in both experiments II and III showed that there was a highly significant difference ( $P < 0.001$ ). The plants grown in autoclaved soils were larger, with a mean weight of 14.21 g compared with 10.48 g for the infected soils. This difference was not due to nematodes because it also occurred in

plants grown in soils to which they were not added. The differences may be due to pathological effects caused by other microorganisms such as the fungus *Fusarium oxysporum* which was detected in the Cooltong soils.

#### BACTERIAL ISOLATES

Twenty bacterial populations isolated from Cooltong soils, by using L2 of *M. javanica* as probes, were tested for their effect on the nematodes' motility and capacity to infect plants and induce galls. No reduction in L2 motility or infectivity was observed and we conclude that none of the bacteria tested have potential for biological control of the populations of root-knot nematodes that were examined.

#### SOIL ANALYSES

Analyses of the soils examined in our experiments show only minor differences between the Cp and Cd soils used in Exp. III and soils from the same areas, 5 Cp and 5 Cd used in Exps I and II. These soils had a pH of 8.8, a 10 % clay content, a 2 % silt content, a 34 % fine sand content and a 54 % coarse sand content. The main differences lie in the percentage organic carbon content which was 0.52 (Cp) compared with 0.38 (Cd) in Exp. III and 0.61 (Cp) compared with 0.29 (Cd) in Exps I & II. Because all plants received supplements of Hoaglands nutrient solution during these experiments we do not think that the difference in organic carbon had any influence on plant growth or on the reproduction of the nematode parasites.

#### Discussion

Although it has been shown that there is a widespread incidence of *Pasteuria penetrans* in vineyard soils in South Australia (Stirling & White, 1982) its role in suppressing reproduction of various species of root-knot nematodes in these soils has not been quantified.

In these experiments we have (1) selected and tested soils for their capacity to inhibit egg laying in the nematode (2) demonstrated that this inhibition is biological in origin and (3) shown that it is probably due to *P. penetrans* and not to other microorganisms that we have also isolated and tested.

It has been observed (Stirling, 1985) that adhesion of *P. penetrans* to the L2 of root-knot nematodes can vary within populations of the same species of nematode. Our experiments show that *P. penetrans* from the same vineyard may vary in its capacity to inhibit egg laying in *M. javanica*. Thus although reproduction of the nematode was significantly increased in both palomino and doradillo soils after autoclaving (Fig. 3), in the non-autoclaved condition there was a significant difference in the ability of these two soils to suppress reproduction of the *M. javanica* population compared with the *M.*



*incognita* and the *Meloidogyne* sp. populations which were both similarly suppressed in these soils (Fig. 3).

It is likely that the number of *P. penetrans* spores was similar in doradillo and palomino soils because the reproduction of *M. incognita* and *Meloidogyne* sp. was not significantly different in the unautoclaved soils and both had similar increases with autoclaving which removed the *P. penetrans*. The difference in the *M. javanica* population may represent a change either in the adhesive properties of these spores or a change in their reproductive ability within the nematode.

Increased crop yields following application of *Pasteuria penetrans* to field plots infested with *Meloidogyne incognita* has been demonstrated (Brown, Kepner & Smart, 1985). From the practical point of view our observations may indicate that a particular population of *P. penetrans* can undergo changes in the field that might render it a less effective agent for biological control. It would appear as a result of this work and that of Stirling (1965) that some idea of the effectiveness of the population of *P. penetrans* to be used for biological control should be obtained from tests on the soil populations of *Meloidogyne* in the soil to be treated prior to the addition of *P. penetrans*.

Another factor that has to be considered when using *P. penetrans* as an agent of biological control is that its specificity, a most valuable attribute, can give a competing root parasitic nematode of a different genus a competitive advantage. We have, for instance, observed large numbers of L2 and males of *T. semipenetrans* in the Cooltong soils around vine roots in which the root-knot nematode is being parasitized by *P. penetrans*. Thus the ultimate aim must be to supply a range of *P. penetrans* capable of parasitizing the most common genera of plant parasitic nematodes in a particular area. In order to do this further work on the distribution, isolation and testing of *P. penetrans* from various areas is needed.

The specific chemical and physical components of various soils that are necessary for the optimal spread of *Pasteuria penetrans* are, as yet, unknown. Nor do we know if some soils have non-biological properties that in some way, are inhibitory to the development of *P.*

*penetrans*. These aspects will need to be investigated in future research associated with the commercial application of this parasite in biological control. This application will be enhanced through the selection of natural populations of this parasite that attack the broadest spectrum of plant parasitic nematode populations or through genetic engineering of parasites cultivated *in vitro*, if this becomes a routine procedure, to achieve the same ends.

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