Appendix 2. Methodology for production of *Pochonia chlamydosporia* and *Pasteuria penetrans* (Dudutech K Ltd.)

Evaluation of the mass production of the microbial agents

Production of *Pochonia chlamydosporia*

Such procedures involved the creation of a primary bank and a working bank for the fungus. From the latter, the production inoculum was prepared as needed by setting up 5ml CMA slants, which were stored for a maximum of one month only before use. Liquid inoculum was scaled up by liquid fermentation in orbital shakers and used to inoculate pre-treated, autoclaved rice in polypropylene bags.

Other activities for Pochonia chlamydosporia.

Besides the activities related to the development of a production method for the standard strain of *P. chlamydosporia*, a survey was carried out in the attempt to isolate new and possibly more effective strains. This was done by collecting soil samples from two areas in Kenya (Naivasha and Thika). A total of 50 samples was collected, mostly from small farms and on a variety of crops. Soil samples were taken between 5 and 25 cm deep. Roots were also collected, with care taken not to dislodge the egg masses. Sub samples were collected per block and were mixed thoroughly to obtain a composite sample of 1 kg, which was then analyzed in the laboratory, where isolations were made on semi selective media. A total of 10 sites were positive for P. chlamydosporia var. chlamydosporia, while only one was positive for P. chlamydosporium var catenulata. These were sub-cultured and stored for future reference and comparison with the standard strain. Other egg parasites found during the survey were Verticillium psalliotes (1 isolate) and Paecilomyces lilacinus (2 isolates).

Locality	Crop	Soil type	Isolation
Naivasha	Carnation / rose	sandy	P. c. var. chlamydosporia
Naivasha	Carnation / rose	sandy	Paecilomyces lilacinus
Naivasha	Carnation / rose	sandy	P. c. var. chlamydosporia
Naivasha	Carnation / rose	sandy	Verticillium psalliotes
Naivasha	Carnation / rose	sandy	P. c. var. chlamydosporia
Naivasha	Carnation / rose	sandy	P. c. var. chlamydosporia
Naivasha	Carnation / rose	sandy	P. c. var. chlamydosporia
Naivasha	Carnation / rose	sandy	P. lilacinus
Makuyu	Beans & Courgettes	Light gray loamy	P. c. var. chlamydosporia
Mweiga	Maize	Dark gray loam	P. c. var. chlamydosporia
Mwea	beans	dark brown	P. c. var.

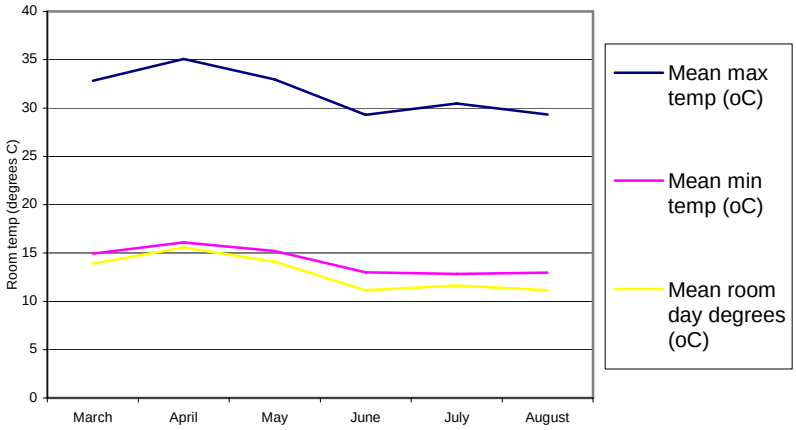
			chlamydosporia
Meru		Brown clay loam	P. c. var. chlamydosporia
Meru		Brown clay loam	P. c. var. catenulata

Finally, an experimental / demonstration tunnel was set up to compare the efficacy of various nematicidal treatments, including Pochonia, as described under 2.1.4. Field trials were also discussed with Homegrown farm managers and set up to assess the performance of this fungus on commercial crops.

Mass production of *Pasteuria penetrans* (Pp)

A weekly planting programme of 100 – 150 tomato plants has been maintained ever since. At the right development stage, plants were inoculated using 2000 Pp endospore encumbered juveniles with a minimum of six spores/juvenile. The plants were maintained in the greenhouse for the time needed by the infected nematodes to reach the adult stage and produce more Pasteuria spores (i.e. a minimum of 40-45 days, depending on climatic conditions). When ready the roots were harvested, air-dried for two weeks and ground to free the Pp spores contained. Temperatures recorded in the tunnel during a period of 6 months of setting up are shown in Fig. 3.

Fig. 3: Mean monthly greenhouse temperatures.



Quality Assurance

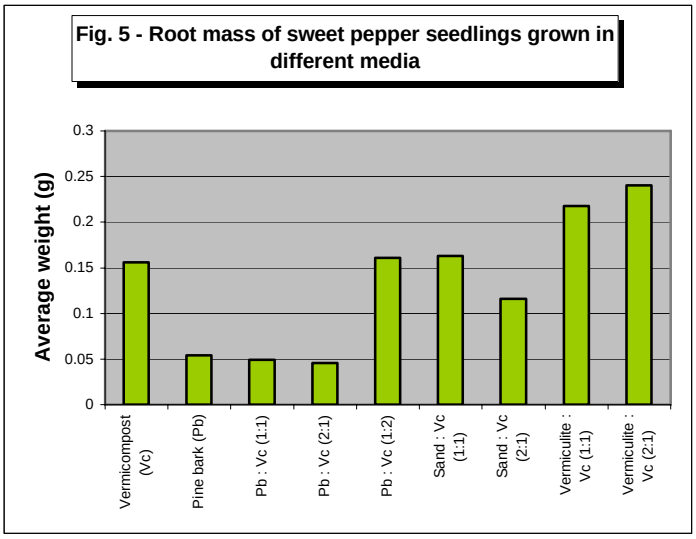
For all production batches a 1 g sample of root powder was suspended in water and the spore suspension calibrated using an improved Neubauer Haemocytometer (0.25-mm depth; 1/16mm^2). A minimum of four counts was done. Spore concentration from propagation house root powder samples were higher (mean of 6.56x10^6) than those obtained from greenhouse samples (mean 3.26x10^6). As part of the QC procedure, the attachment potential was also assessed by exposing 200 freshly hatched juveniles to a suspension of Pp spores during 18 hrs at 25 ^{o}C. The numbers of spores attached on 20 juveniles were counted.

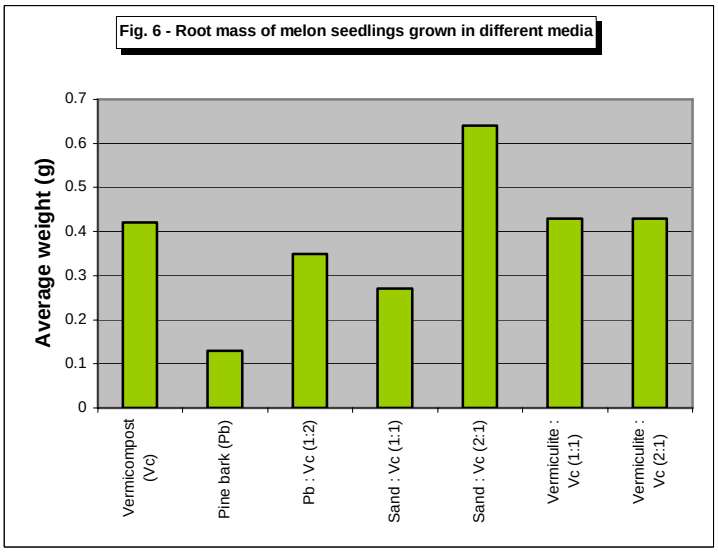
Improvement of the production method

In order to improve the system currently used for the production of Pasteuria penetrans, some experiments were set up to determine suitability of various host plants and also the possibility to increase root mass development through the use of various growth substrates.

Experiment on host suitability

Seedlings of different crop species (egg plant, lettuce, tomato var. Tiny Tim, tomato var. M82, sweet pepper and cucumber) were grown in 5-liter pots and inoculated with 2000 Meloidogyne juveniles/pot. The seedlings were allowed to grow for 1-month post inoculation before harvesting, when the roots were carefully observed for galling, presence of egg masses and scored using the Bridge and Page galling index (1-10). The root fresh weight was also determined.



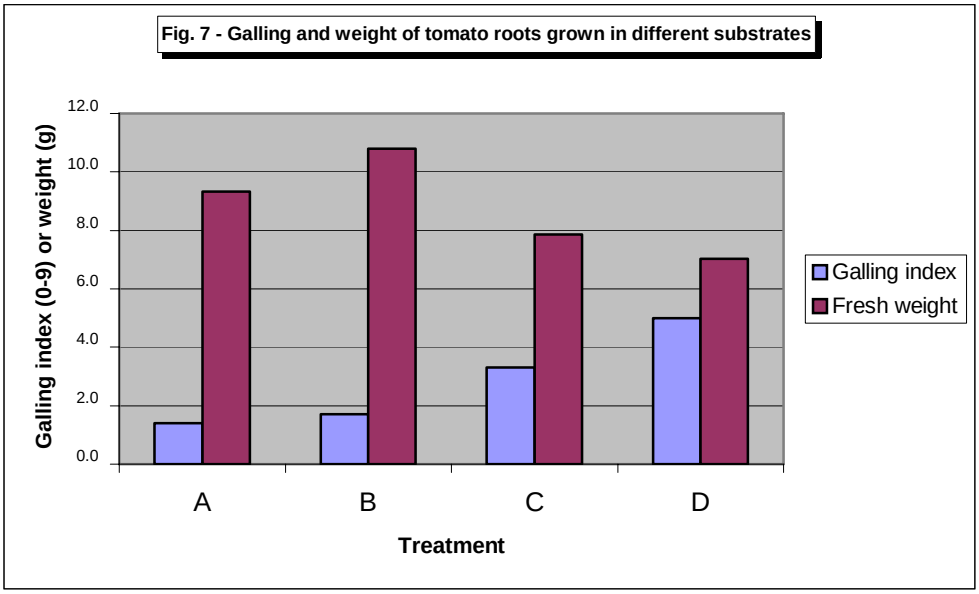


Experiment on production of *P. penetrans.*

Sandy soil was pasteurized and then used to fill 200 ml pots using a mixture of either sandy soil and vermicompost (1:1) or sandy soil and vermiculite (1:1). One tomato seedling (var. Tiny Tim) was grown in each pot during a period of 6 weeks before being inoculated with Pp encumbered juveniles. Two different rates of inoculation were used, to give a total of four different treatments as follows:

Vermicompost & soil-100 pots	50 pots - 250 juveniles/pot 50 pots - 500 juveniles /pot	Treatment A Treatment B
Vermiculite & soil –100 pots	50 pots - 250 juveniles/pot 50 pots - 500 juveniles/pot	Treatment C Treatment D

The plants were allowed to grow for 8 weeks after inoculation before being harvested. At harvest, the soil was gently washed off the roots and 10 roots per treatment scored using the galling index. Fresh root weights of these samples were also taken (Fig. 7).



Set up of an experimental / demonstration tunnel.

A 650 m^2 tunnel was set up at Kingfisher Farm, Naivasha, (Plate 1) and kept during free of any vegetation for 4 months and without watering in an attempt to eliminate the nematodes present in the soil. Forty 2 m^2 plots were then delimited within the tunnel and inoculated with equal numbers of RKN juveniles. In all plots, a planting program which includes rotations with host and non-host crops (in the following succession: carrots, baby corn, tomato, broccoli) was initiated and will be continued during a period of about 18 months. Eight different treatments (replicated 5 times and including an untreated control) were applied before planting the first crop, and will be applied again before each susceptible crop. For each crop, at harvest various parameters are going to be assessed to evaluate the performance of the crop and the effect of the rotation and the different treatments on the nematode populations. So far only the first two crops have been completed and although there appears to be some indication of differences between the treatments, no conclusions can be drown at this stage. See appendix Underneath some preliminary results are reported (Figs. 8-11) with the only purpose of providing an overview of the work that is being carried out.

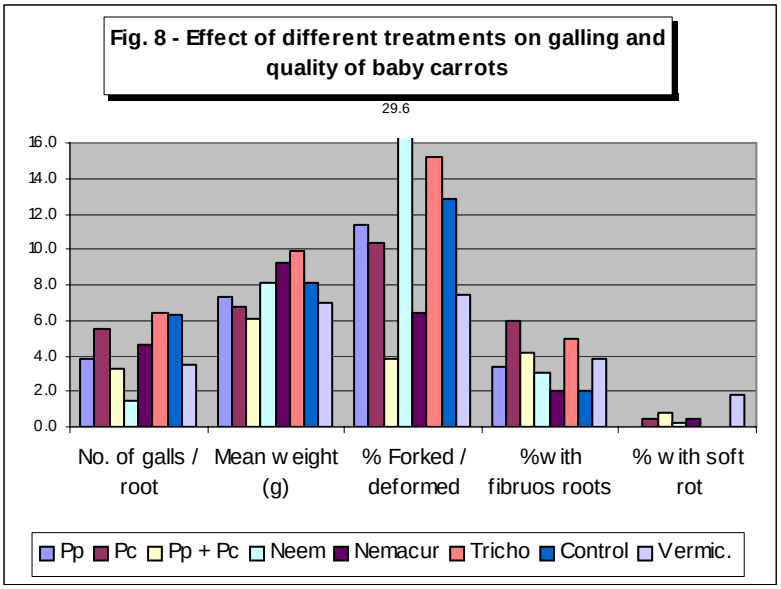


Fig. 8 - Effect of different treatments on galling and quality of baby carrots
29.6
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
No. of galls / root
Mean weight (g)
% Forked / deformed
%with fibruos roots
% with soft rot
Pp
Pc
Pp + Pc
Neem
Nemacur
Tricho
Control
Vermic.

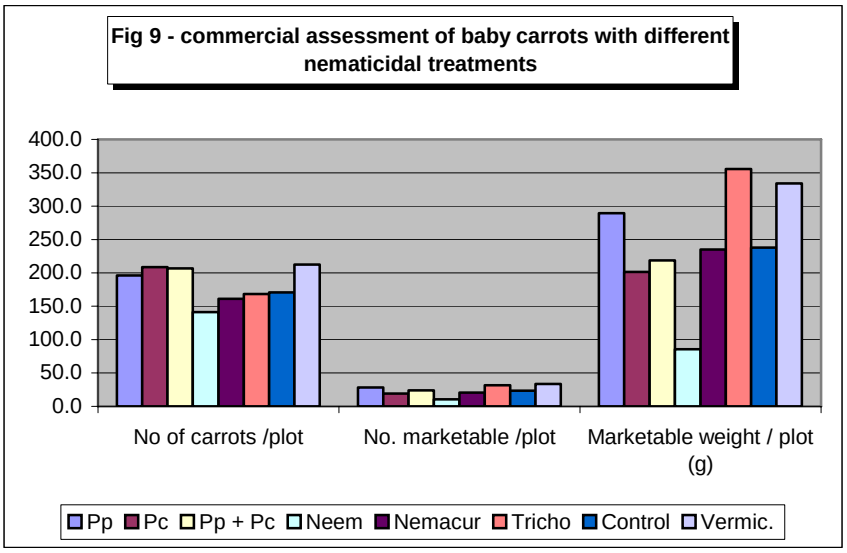


Fig 9 - commercial assessment of baby carrots with different nematicidal treatments
400.0
350.0
300.0
250.0
200.0
150.0
100.0
50.0
0.0
No of carrots /plot
No. marketable /plot
Marketable weight / plot (g)
Pp
Pc
Pp + Pc
Neem
Nemacur
Tricho
Control
Vermic.

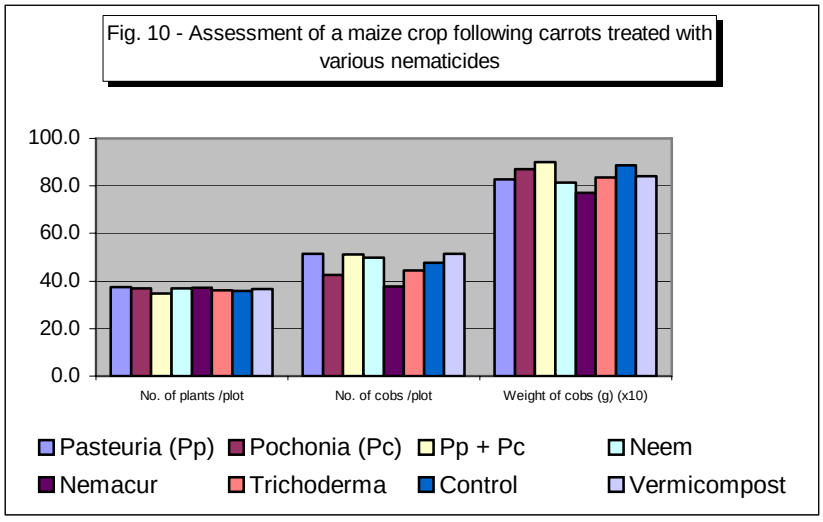
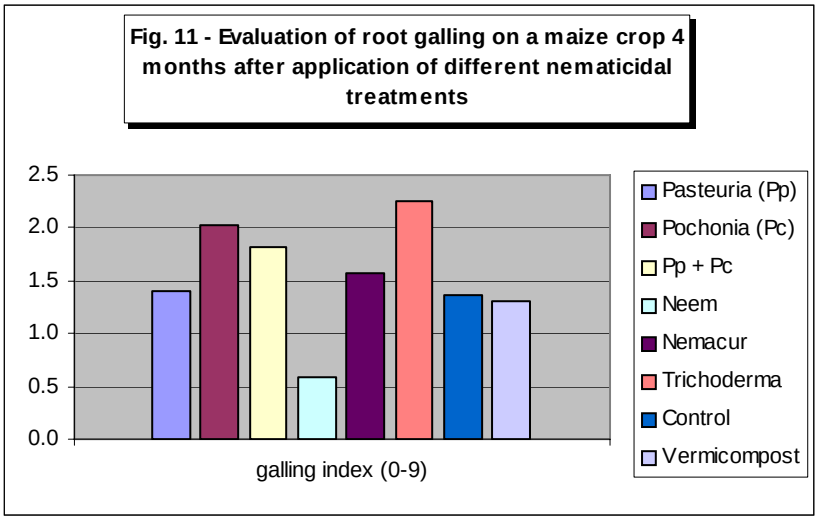


Fig. 10 - Assessment of a maize crop following carrots treated with various nematicides
100.0
80.0
60.0
40.0
20.0
0.0
No. of plants /plot
No. of cobs /plot
Weight of cobs (g) (x10)
Pasteuria (Pp)
Pochonia (Pc)
Pp + Pc
Neem
Nemacur
Trichoderma
Control
Vermicompost



Experimental tunnel at Kingfisher farm, Naivasha