Chapter 37

PRODUCTION OF A BIOCOMPOST BY SOLID STATE FERMENTATION AGAINST THE COFFEE NEMATODES

D. BRAND[1,2], S. ROUSSOS[2], F.A.PROCHMANN[1], J. POHL[3], C.R. SOCCOL[1]

[1]Laboratorio de Processos Biotecnologicos, Departamento Engenharia Quimica, Universidade Federal do Parana (UFPR), 81531-970 Curitiba-PR, Brazil; [2]Laboratoire de Microbiologie IRD, IFR-BAIM;Equipe de Mycologie et des FMS; Université de Provence, Case 925; 163 Avenue de Luminy; 13288 Marseille France; [3] Centro de diagnóstico Marcos Enrietti, Secretaria de Estado da Agricultura e Abastecimento (SEAB), 80040-340 Curitiba-PR, Brazil.

Abstract

The main objective of the present work was to produce a biological nematicide by SSF, with an important number of virulent spores of *Paecilomyces lilacinus* and *Verticillium chlamydosporium* by utilising coffee husk as substrate. Studies of the fermentation physical conditions, such as pH, temperature and initial moisture content were done by using an experimental design 2^{3-1} followed by a complete design 3^2 to obtain the best fermentation conditions. The activity of the biocompost obtained with the fungi *Paecilomyces lilacinus* (strains Pl-1, Pl-2 and Pl-3) and *Verticillium chlamydosporium* (strain Vc-1), were evaluated against the nematode *Meloydogyne incognita* race 1. The measured responses were number of females and root weight. The nematophagous fungi *Paecilomyces lilacinus* Pl-1. showed a reduction in the order of 80%, with the exception of the strain of *Paecilomyces lilacinus* Pl-3, the results obtained with the other strains of fungi propitiated a reduction in the number of nematode females in order of 15 – 25 % .

Key words: Biological nematicide, *Meloydogyne incognita*, *Paecilomyces lilacinus, Verticillium chlamydosporium*, Coffee, Solid State Fermentation.

Introduction

Phytosanitary problems caused by nematodes present an important economic incidence all over the world in different agricultural cultures, mainly in coffee plantations. Nematode combat is difficult due to its extreme resistance and its underground life. The most common species that causes largest damages in agriculture belong to the *Meloidogyne* genera, also called root-knot nematodes (**1**).
The more common practices used in nematode combat have been the use of varieties of resistant cultures, rotation of cultures and application of chemicals, mainly phosphates and carbamates. Even so, the latter method, although very efficient and frequently used, is extremely dangerous to men and animals, due to its

New Horizons in Biotechnology, 403-412, S. Roussos et al. (eds)

wide action spectrum. These chemicals disturb the ecological balance of places in which they are used, they add to competition among plagues, they favor action of parasites and they promote organic matter biodegradation. Besides polluting the environment and nutritious products through the accumulation of residues in the soil and underground water, they affect both the health of animals and men. In light of the present facts, a search is underway to find useful alternatives or substitutes to chemical nematicides. Predatory mushrooms, that capture nematodes or destroy its eggs, mycorrhizal mushrooms, toxins produced by microorganisms and plants, could be used shortly with success in the combat against nematodes of different cultures.(**2-5**).

The term Biological control can be defined as the utilization of living organism populations, parasites, predatory pathogens, antagonistic or competitors in maintaining another organism's population density at a lower average than would occur in their absence (**6**). Commonly, more than one microorganism occurs with plant-parasitic or saprozoic nematodes in a particular rhizosphere. Constant association of these organisms in a given ecological niche undoubtedly has a greater impact on the establishment of such nematodes than would be caused by each microorganism alone. Such association results in a biological balance that may manifest itself in the form of direct parasitism by attachment and penetration by one or more pathogenic microorganism in the eggs, juveniles, or adult nematodes, causing death and possibly allowing subsequent invasion by many or selected saprophytic microorganisms. Egg masses, sedentary females, or cysts may be directly invaded by pathogenic or some opportunistic organisms durind various developmental stages of nematodes. (**7-9**).

The use of solid state fermentation (SSF) may provide the elaboration of efficient formulations with fungi that are employed in the biological control of nematodes. The formulations of the active products could be realized with a natural solid substrates or supports for the development of fungi. The use of agricultural residues or by-products such as cassava bagasse, coffee husk and pulp, sugar cane bagasse, could be even more interesting as it may supply good efficiency and stability of the final product (**10,11**)

The main objective of the present work was to produce a biocompost by SSF, with an important number of virulent spores of *Paecilomyces lilacinus* and *Verticillium chlamydosporium* (nematode eggs parasitic fungi) by utilizing coffee husk as substrate. The biocompost produced was then evaluated against the coffee nematode *Meloidogyne incognita* race 1 in vases containing *Coleus.*

Materials and Methods

Micro-organisms and culture media

The strains of nematophagous fungi utilized in the present work were parasites of nematode eggs *Paecilomyces lilacinus* designated as Pl-1, Pl-2 and Pl-3 and one strain of *Verticillium chlamydosporium*, Vc-1 nematode egg and cyst parasites.

They were maintained in Potato dextrose agar (PDA) and cultivated in coffee husk extract media for the production of inoculum. (**12**)
In an agar medium containing 100 g/L of coffee husk extract (**12**) studies of radial growth and biomass production were conducted in order to verify the velocity of growth and the aspects of each microorganism colony. The test was assayed in Petri dishes of 75 mm diameter with 20 mL of culture media over a 12-day period. The inoculation was made with a droplet of a spore suspension at the center of each dish and incubation was held at 28°C. The diameter of each colony was measured every 24 hours and biomass was weighed after 12 days by the dissolution of agar and separation of mycelia on filter paper and dried at 100°C for 24 hours.

Growth Physiology of nematophagous fungi

Strains belonging to the *Paecilomyces* genera showed similar radial growth velocity, 20 mmh^{-1}, 18 mmh^{-1} and 19 mmh^{-1} respectively for Pl-1, Pl-2 and Pl-3. The fungal parasite of nematodes cysts grows slower than the fungal parasites of eggs and presents a radial growth velocity of 13 mmh^{-1}. The strains Pl-1 and Pl-2 produced more biomass in a 12-day period (128 and 132 mg respectively) than Pl-3 and Vc-1 were less efficient in producing biomass but nevertheless were able to assimilate and metabolize the components present in the coffee husk.

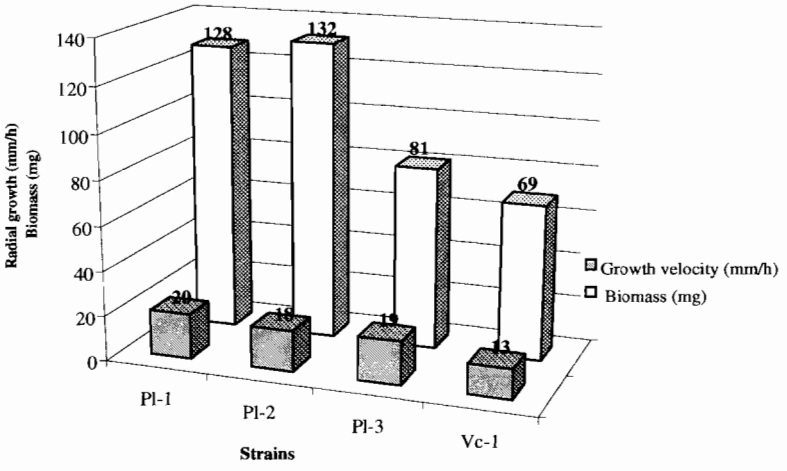

Figure 1. Radial growth of nematophagous fungi.

Solid state fermentation

Experiments were conducted by utilizing coffee husk as substrate. Coffee husk is the major residue of coffee processing in Brazil. The husk was dried, milled and classified. The granulometric fraction comprised between 0.8 and 2.0 mm in diameter was employed. The sterilization of this material must be done without the addition of water otherwise occurs the formation of toxic products to the fungi metabolism mainly to its growth and sporulation.

Fermentation was carried out in glass flasks covered with filter paper for allowing gas exchanges; each flask was filled with 20 g of dry husk. To prepare the inoculum, the strains were grown on coffee husk extract agar and incubated for 10 days at 28° C and spores were counted in a Neubauer cell. Studies of the fermentation's physical conditions, such as pH, temperature and initial moisture content were done initially by using an experimental design $2^{1,3}$. The optimization of this culture conditions was realized in order to obtain better concentration of spores (the response variable). Table 1 shows the real and coded values for all strains tested.

The inoculation rate employed was always 2 E+06 spores/g of substrate in dry weight basis and the incubation period was 7 days at 28°C for each strain studied. After incubation the spores were counted in a Neubauer cell. A total of 5 grams of substrate (wet basis) was vigorously homogenized for 30 minutes with 50 mL of water containing tween 80 and glass beads; the proper dilutions were made.

Table 1. Real and coded values of experimental design 1.

Coded values / Factors	- 1	0	+ 1
Initial moisture (%)	60	65	70
Temperature (° C)	26	28	30
pH	3,5	4,5	5,5

In this first step of optimization, the response variable was evaluated by Pareto chart of effects for each strain employed. The optimized conditions according to the results obtained in this experiment will be done by using a complete experimental plan $3^{2.}$ The results are shown in figures 2 to 5 for each strain employed. By analyzing the figures for the strains of *Paecilomyces lilacinus* Pl-1 and Pl-2 the only significant factor at level of 5% was the pH, and it had a negative effect. This meant that the range employed was above ideal conditions for spore production. Variables such as temperature and initial moisture content were not significant at the 5% level. For the fungus *Verticillium chlamydosporium* the most important factor in spore production was the temperature, the range employed was above ideal.

All strains of *Paecilomyces lilacinus* produced more than 3.5 E+08 spores/g coffee husk (dry weight). The best strain Pl-2 was the one that showed best spore production reaching 6.4 E+08 spores/g. The best conditions of spore production were achieved with the plan 3^2. However, the number of spores didn't increase in a decimal order, due to the fact that conditions employed for each strain were established as initial moisture of 65%, natural pH of the substrate and incubation

temperature of 28°C. With these conditions, fermentations were carried out in order to utilize the biocompost obtained in pot experiments as described below.

Pot experiments

Experiments were based on the activity of the compost obtained by solid state fermentation of coffee husk with the fungus *Paecilomyces lilacinus* (strains Pl-1, Pl-2 and Pl-3) and *Verticillium chlamydosporium* (strain Vc-1), against the nematode *Meloydogyne incognita* race 1.

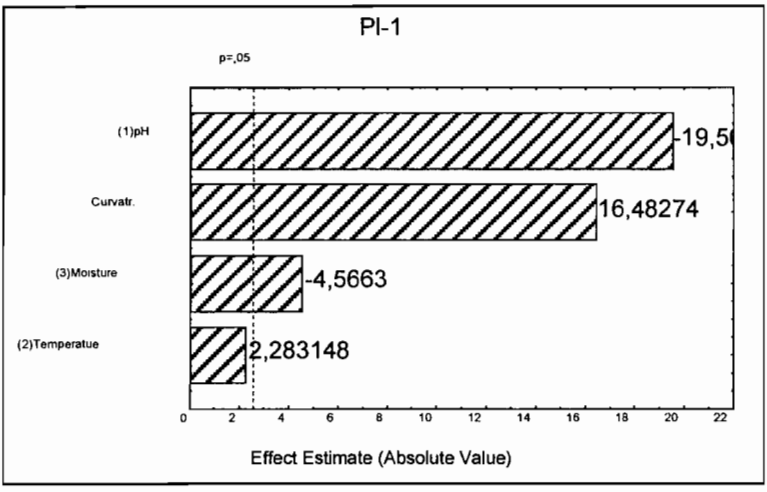

Figure 2: Paredo chart of effect for strain Pl-1.

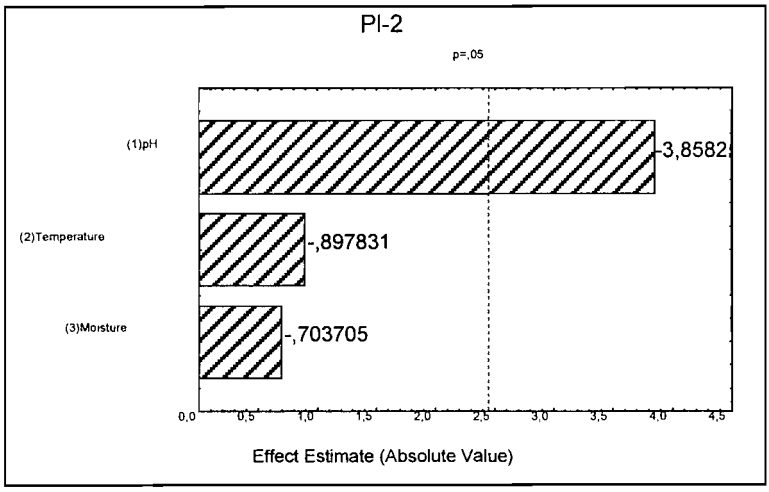

Figure 3: Paredo chart of effcets for strain PI-2.

The nematodes (isolated from a coffee plantation) were reared on tomato as well as in *Coleus*. The experiments were undertaken in a glasshouse without thermal, or illumination controls, therefore subjected to environmental conditions. Two pots containing good quality sterilized soil were prepared for the tests with selected fungi and two more were utilized as control treatments (in which no fungus was inoculated). All experiment were done with two replicates.

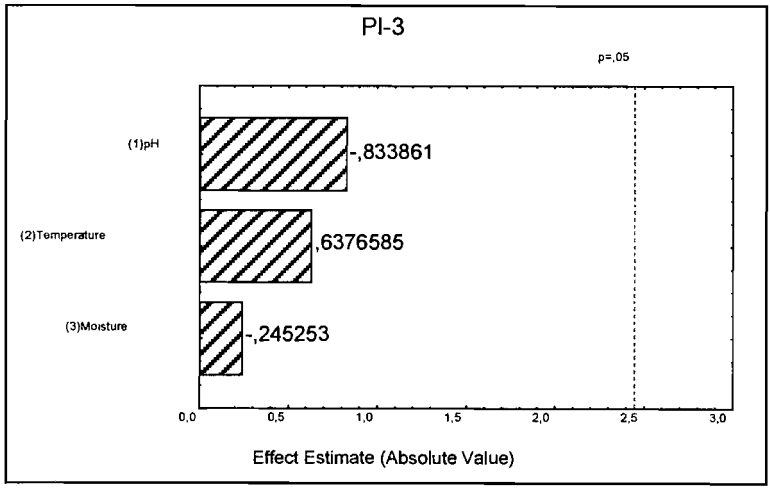

Figure 4. Pareto chart of effects for strain PI-3.

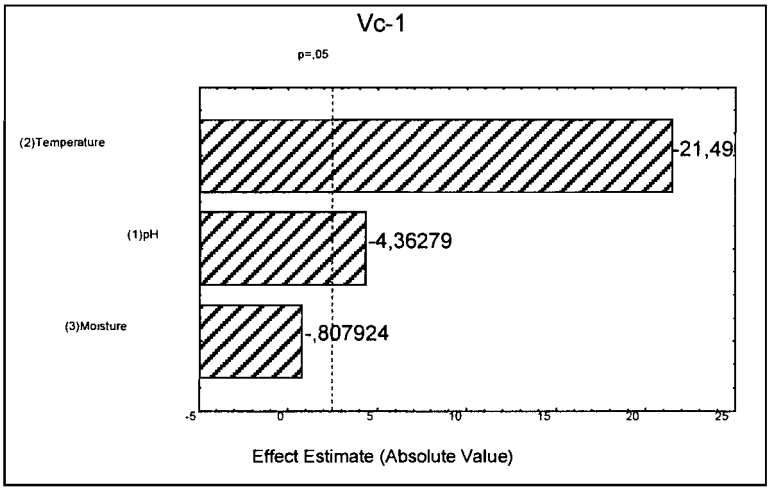

Figure 5. Pareto chart of effects for strain Vc-1.

Each pot received one seedling of the plant *Coleus*, which was chosen for being susceptible to nematode action and for its resistance to other plagues. This experiment was conducted from August 2001 to January 2002. Initially, the analysis of the experiment was stipulated for October 2001, but the low temperatures registered at Curitiba (minimum of 2°C) retarded the development of the nematodes as well as its parasitic action. Temperatures below 18°C reduce the activity of the nematodes as well as the action of nematophagous fungi, amplifying the period of plant infection. Medium temperatures inferior to 5°C may paralyze nematode action. A suspension of nematodes (*Meloydogyne incognita* race 1) was prepared from roots of *Coleus* visually infected, with a great number of galls. Approximately 25 g of the compost obtained by solid state fermentation were homogenized with the soil and inoculated with 100 mL of nematode suspension. Each pot had the same disposition, with layers of soil, biocompost and nematodes alternated around the root of each plant. The concentration of spores was of 10^8 spores/g in wet basis (65 % humidity) and the concentration of the nematodes was of 10000 eggs and juveniles per pot.

The results were analyzed by sampling, due to the impossibility of doing a total nematodes female count present in the galls of plant roots. After removing the plants from pots, roots were isolated, washed and dried at room temperature. The roots were weighed and 5% were evaluated. The results obtained indicated a significant reduction in nematodes count in pots containing the nematophagous fungi Pl-1. The other pots showed great similarity for the gall indexes. The values obtained are demonstrated in Table 2.

Table 2 – Evaluation of nematophagous fungi efficacy.

Fungi	Root weight (g)		Count (female/g root)	
	Pot 1	Pot 2	Pot 1	Pot 2
Vc-1	43,02	33,72	390	345
Pl-3	39,19	13,24	320	462
Pl-1	18,22	19,29	85	89
Pl-2	53,20	34,22	370	360
Control	22,84	29,88	450	433

The values obtained were characterized by a reduction in the number of females in the pots treated with the biocompost containing the strain of nematophagous fungus *Paecilomyces lilacinus* Pl-1. A reduction in the order of 80% was reached when compared to the control pots. With the exception of the strain of *Paecilomyces lilacinus* Pl-3, the results obtained with the others strains of fungi showed a reduction in the number of nematode females in the order of 15 – 25 % inferior to the values observed for the control pots (Fig. 6).

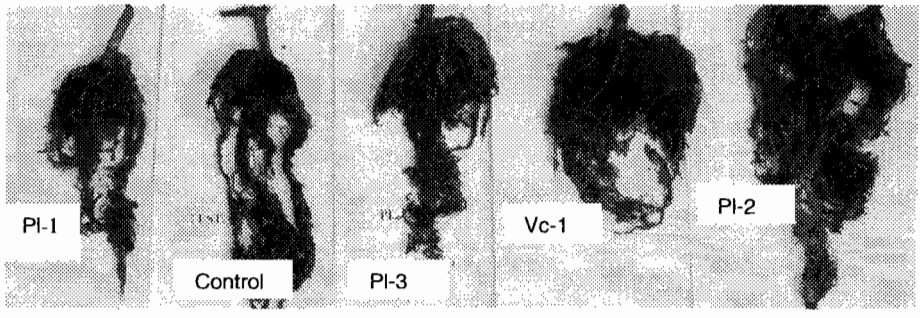

Figure 6. Comparison of roots evaluated in the experiment.

In spite of the lag in the nematode cycle, it was possible to evaluate the experiment. In figure 7 below, reduction of nematodes was approximately 80% in the number of females per g of root. It could be verified that the plant that received the treatment with the biocompost with the strain *Paecilomyces lilacinus* Pl-1 developed excessively well in relation to the control, showing a bigger root system with a much smaller number of galls.

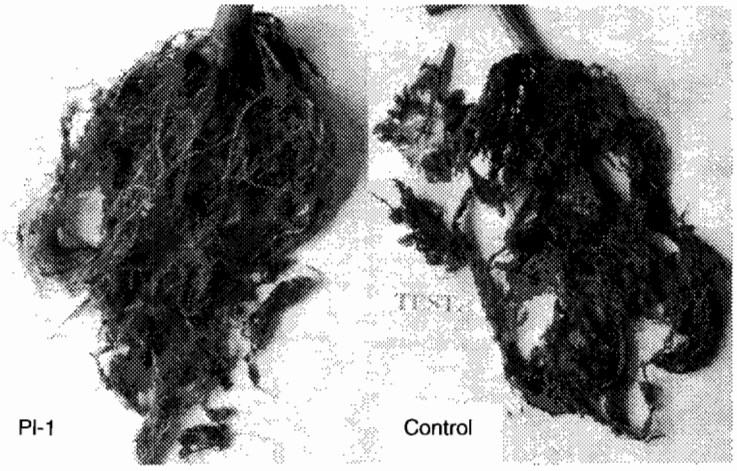

Figure 7. Comparison between control and *Coleus* inoculated with Pl-1.

The results in the reduction of females and root weight didn't have any influence on the sporulation index obtained during fermentation, as it was observed that the strain that produced more spores was Pl-2. The fungus Pl-1 that gave greater reduction is probably better adapted to the soil conditions as well as the nematode species and race employed in the experiment.

Conclusions and future approach

The utilization of a biocompost produced by solid state fermentation utilizing nematophagous fungi and coffee husk as substrate is possible and must be better studied. With the strain *Paecilomyces lilacinus* PL-1 80% of reduction in the coffee nematodes density (*Meloidogyne incognita* race 1) was reached, in a pot experiment. This is a significant result but further studies on the virulence of this strain should be carried out against other species and races of nematodes of the genre *Meloidogyne*. Screening of new strains should also be carried out. Studies on the fermentation conditions will be undertaken in order to enhance spore production and the virulence of the fungus.

Acknowledgements

CRS thanks CNPq for a scholarship under Scientific Productivity Scheme. DB thanks Lactec and IRD (DSF) for a doctorate scholarship.

References

1. Carneiro R.M.D.G., Carneiro R.G., Abrantes I.M.O., Santos M.S.N.A., Almeida M.R.A. (1996) *Journal of Nematology* **28** (2): 177-189.
2. Cayrol J.C. (1989) *P.H.M. - Revue Horticole*, **293**, 53 - 57.
3. Bourne J.M., Kerry B.R. (1999) *Soil Biology and Biochemistry* **31**,75 – 84
4. Ciancio A. (1995) *Appl. Nematol.* **18** (5), 451 - 454.
5. Dijksterhuis J., Veenhuis M., Harder W., Nordbring-Hertz B. (1994) *Advances in Microbial Physiology*, **36**, 111 - 143.
6. Siddiqui Z.A., Mahmood I. (1996) *Bioresource Technology* **58**, 229 - 239.
7. Jatala P. (1986) *Ann. Rev. Phytopathol.*, **24**, 453 - 489.
8. Kerry B.R. (1987) *In* Principles and Practice of nematode control in crops, Edited by Brown R.H., Kerry B.R., Academic Press, 233 - 263.
9. Nordbring-Hertz B. (1988) *Microbiological Sciences*, **5** (4), 108 - 116.
10. Roussos S., Bagnis C., Besnard C., Sigoillot C., Duponnois R., Augur C. (2000) *In* Coffee Biotechnology and Quality Proceedings of the 3rd International Seminar on Biotechnology in the Coffee Agro-Industry, Londrina, Brazil. Sera T., Soccol C.R., Pandey A., Roussos S. (eds). Dordrecht, p.277-286.
11. Soccol C.R, Pandey A. (2002) Coffee waste a new promising substrate to industrial production of microbial bio-products. Concise Encyclopedia of Bioresource Technology. The Haworth Press. Inc, New York, USA (In press).
12. Brand D., Pandey A., Roussos S., Soccol C.R. (2000) *Enzyme and Microbial Technology*, USA, **27**, 127-133.

Brand D., Roussos Sevastianos, Prochmann F.A., Pohl J., Soccol C.R. (2003).

Production of a biocompost by solid state fermentation against the coffee nematodes.

In : Roussos Sevastianos (ed.), Soccol C.R. (ed.), Pandey A. (ed.), Augur Christophe (ed.).

New horizons in biotechnology. Dordrecht : Kluwer, 403-412.

ISBN 1-4020-1718-9