0273-2289/04/118/0081/$25.00

Development of a Bionematicide With *Paecilomyces lilacinus* to Control *Meloidogyne incognita*

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Received April 9, 2003; Revised September 10, 2003; Accepted September 18, 2003

Abstract

Root-knot disease caused by *Meloidogyne incognita* is a matter of grave concern because it affects several economically important crop plants. The use of solid-state fermentation (SSF) may help to elaborate efficient formulations with fungi to be employed in the biologic control of nematodes. Attempts were made to select low-cost substrates for spore production of a strain of *Paecilomyces lilacinus* with known nematicide capacity. Coffee husks, cassava bagasse, and defatted soybean cake were utilized as substrates, and sugarcane bagasse was used as support. Fermentations were carried out in flasks covered with filter paper at 28°C for 10 d. The products obtained by SSF were evaluated for their nematicide activity in pot experiments containing one seedling of the plant *Coleus* inoculated with the nematode *M. incognita*. The plants were evaluated 2 mo after inoculation. Fermented products showed a reduction in the number of nematodes. The best results were obtained with defatted soybean cake, which showed almost 100% reduction in the number of nematodes; the reduction with coffee husk was 80% and with cassava bagasse was about 60%.

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Index Entries: Biocontrol; *Paecilomyces lilacinus*; *Meloidogyne incognita*; coffee husks; cassava bagasse; defatted soybean cake; sugarcane bagasse; solid-state fermentation.

Introduction

Root-knot nematodes of the genus *Meloidogyne* are serious plant pathogens affecting economically important crop production throughout the world, including cereals, fruits, and flowers *(1)*. Diseased plants show a progressive decline starting with leaf chlorosis, followed by flower and fruit fall, eventually leading to plant death in 2–4 yr. The root systems of the diseased plants show reduced growth and many galls leading to an extensive development of corky tissue on the main and secondary roots *(2)*.

The use of microbial agents for biocontrol of pests and plant diseases is becoming of paramount importance in many countries because of the problems caused by chemical pesticides *(2,3)*. Biocontrol agents of pests and plant diseases comprise a diverse group of microbes such as viruses, bacteria, fungi, and protozoa *(1–3)*, and among these, parasitic fungi directly penetrate targets. These are also resistant to adverse environmental conditions. Fungal parasites of nematode eggs have great potential as biocontrol agents against *Meloidogyne* spp. since sedentary females are infected and the eggs are destroyed *(4,5)*. The potentiality of *Paecilomyces lilacinus* as a biocontrol agent has been found equivalent to commonly used nematicides. *P. lilacinus* is a proven efficient biocontrol agent in its field application in controlling *Meloidogyne incognita (6,7)*. Infestations of soil with the fungus in field and greenhouse experiments have been reported to limit numbers of root-knot nematode galls and increase plant yields *(8–10)*.

Solid-state fermentation (SSF) has shown tremendous potential in effective utilization and value addition of agroindustrial byproducts *(11–13)*. Application of agroindustrial residues in bioprocesses, on one hand, provides an alternative for a sustainable equilibrium of natural organic material and, on the other, helps solve the environmental pollution problem, which their disposal otherwise would have caused. SSF could be an alternative for the production of efficient biocontrol agents for practical use, because mass production must be developed *(14)*.

Coffee husks, the main byproduct originated from the dry method of coffee processing, is rich in organics and nutrients. Recent attempts have focused on the application of coffee husks as substrate for the production of edible mushrooms *(15,16)* and plant hormones *(17)*. Cassava bagasse is made up of fibrous root material and contains starch (40–70%), which is generally not extractable by the processing units *(18)*. Application of cassava bagasse as substrate in SSF includes protein enrichment with strains of *Rhizopus (19)*, production of aromatic compounds *(20)*, and citric acid synthesis *(21)*. Defatted soybean cake, the residue obtained after oil extraction of soybean, could be used for the production of proteases owing to its high protein content *(22)*. Sugarcane bagasse is one of the largest cellulosic agroindustrial byproducts, containing approx 50% cellulose and 25% each

of hemicellulose and lignin. Extensive studies have been carried out on its utilization as substrate in SSF *(23)*.

The objective of the present work was to use agricultural byproducts as low-cost substrates for spore production of *P. lilacinus*. Coffee husks, cassava bagasse, and defatted soybean cake were utilized as substrate (carbon and energy source), and sugarcane bagasse was used as inert solid support. The fermentation products were tested for their nematicide activity in pot experiments containing the plant *Coleus* infected with eggs and larva of the nematode *M. incognita*.

Materials and Methods

Microorganism, Culture Medium, and Inoculum

The strain *P. lilacinus* (LPB-Pl-01) was maintained on potato dextrose agar in slants at 4°C. Culture was grown at 28°C for 10 d for the production of spores. The spores were harvested by homogenization with distilled water (50 mL with three drops of Tween-80) on a magnetic stirrer. The spores were counted using a Malassez cell.

Substrates and Support

Coffee husks, defatted soybean cake (Cocamar, Maringá-PR, Brazil), and cassava bagasse (Agroindustrial Paranaense de Polvilho, Paranavaí-PR, Brazil) were dried in an air oven at 55°C for 24 h, milled manually, and sieved to obtain fractions between 0.8- and 2.0-mm particle size. Sugarcane bagasse (obtained from the local market) was washed three times with distilled water to remove all the sugar and other soluble residues, dried, milled, and sieved as for the other substrates.

Solid-State Fermentation

In the first experiment, SSF was carried out in 250-mL conical flasks (mouth covered with filter paper). Different concentrations of coffee husks and cassava bagasse were tested, as shown in Table 1. Each flask was filled with 20 g of dry substrate and mixed with 20 mL of distilled water. The contents were well mixed and sterilized in an autoclave at 121°C for 20 min. After sterilization, the flasks were inoculated with 2.0×10^7 spores/g of dry substrate. The initial moisture content was made 65% by adding more sterile distilled water and also by considering the water coming from the inoculum. Flasks were incubated at 28°C for 10 d. Each assay was done with two replicates.

In the second experiment, the substrate constituted defatted soybean cake, coffee husk, and a mixture of sugarcane bagasse with the two substrates, as shown in Table 2. The content of substrate in each flask varied owing to the addition of sugarcane bagasse as support, which occupied a higher volume because of its light weight. The total content of the substrate was 20 g for assays 1, 2, and 5 and 12 g for assays 3 and 4. The initial moisture

Table 1
Proportions of Coffee Husks and Cassava Bagasse and Spore Concentration Obtained in Experiment 1

Assay	Coffee husk (%)	Cassava bagasse (%)	Number of spores (g dry substrate × 10^9)
1	100	0	2.60
2	75	25	6.80
3	60	40	7.27
4	15	85	7.40
5	0	100	7.60

Table 2
Proportions of Coffee Husks, Defatted Soybean Cake, and Sugarcane Bagasse and Spore Concentration Obtained in Experiment 2

Assay	Coffee husk (%)	Defatted soybean cake (%)	Sugarcane bagasse (%)	Number of spores (g dry substrate × 10^9)
1	100	0	0	3.54
2	50	50	0	5.82
3	83	0	17	5.26
4	0	83	17	15.3
5	0	100	0	42.7

content was 65% when no sugarcane bagasse was added and 75% with the addition of 2 g of support. The contents were mixed, autoclaved, inocu lated, and incubated as just described.

In another experiment, the effects of pH, moisture content, and spore concentration were evaluated.

The spores in fermented matter were harvested by adding 30 mL of distilled water and three drops of Tween-80 to 1 g of fermented product and mixing on a magnetic stirrer for 30 min. Proper dilutions were made and spore were counted using a Malassez cell.

Pot Experiments

Pot experiments were based on the results of the fermented products obtained by SSF against the nematode *M. incognita* race 1. The nematodes (isolated from coffee plantation) were reared in *Coleus*. The experiments were conducted in a greenhouse without thermal or illumination control and subject to environmental conditions. Two pots containing good-quality sterilized soil were prepared for tests of the selected fungi, and two more pots were utilized as control treatments (in which no fungus was inoculated). All the experiments were realized in two replicates. Each pot received one seedling of *Coleus*, which was chosen for being susceptible to the nematode action and for its resistance to other diseases.

A suspension of nematodes (*M. incognita* race 1) was prepared from the roots of a visually infected *Coleus*, with a high number of galls. Approximately 25 g of the matter obtained by SSF was homogenized with the soil and inoculated with 100 mL of nematode suspension. Each pot had the same disposition, with layers of soil, fermentation matter, and nematodes alternated around the root of each plant. The concentration of spores was 10^9 spores/g on wet basis (65% humidity), and the concentration of nematodes was 10,000 eggs and juveniles per pot.

Analysis was done by withdrawing a sample (portion) of the roots. After removing the plants from the pots, the roots were isolated, washed, and dried at room temperature (28–30°C) for 1 h. The roots were cut into 1-cm portions and two 1-g samples were weighed for female count under a stereomicroscope (magnification: ×45).

Results and Discussion

Solid-State Fermentation

In the first SSF experiment, cassava bagasse and coffee husks were used as substrates and compared for the production of spores. The addition of different proportions of cassava bagasse to coffee husks was done in order to achieve different C/N ratios. This also evaluated whether the virulence of the fungi was related to the nature of the substrate. Table 1 shows the final spore concentration of the fermented products obtained with different proportions of coffee husks and cassava bagasse. The concentration of spores in the final products increased proportionally as the concentration of cassava bagasse increased in the mixed substrate. When coffee husk was used alone, the final spore count was 2.6×10^9 spores/g of dry substrate, and for cassava bagasse was 7.6×10^9 spores/g of dry substrate. Thus, cassava bagasse alone as substrate was much superior than coffee husk or mixed substrates.

In the second experiment, defatted soybean cake and coffee husks were tested as substrates. Defatted soybean cake was employed owing to its high protein content (approx 40%). Leger et al. *(24)* reported that the virulence of nematophagous fungi against nematodes was related to the production of enzymes, probably proteases that destroyed the eggshell of the nematode and penetrated it in order to destroy the embryo inside the egg. Mixed substrate fermentation with sugarcane bagasse (inert material) was carried out in order to make the substrate less expensive and also to test whether this had any beneficial effect on spore production. The presence of sugarcane bagasse facilitated higher aeration possibilities in the substrate and retained higher quantities of water. Earlier studies on enzyme production by SSF with a mixture of sugarcane bagasse with different agricultural products in a proportion of 20:80 showed enhanced enzyme production *(25)*. Defatted soybean cake showed a spore production of 4.27×10^{10} spores/g of dry substrate, which when mixed with sugarcane bagasse (17%) reduced to 1.53×10^{10} spores/g of dry substrate (Table 2).

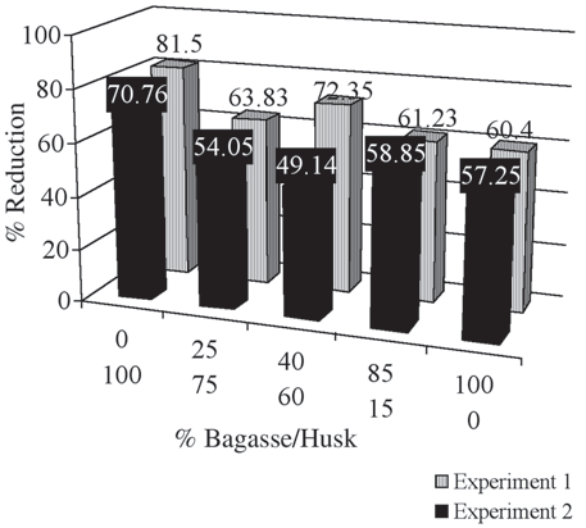

Fig. 1. Reduction in number of nematode females by employing fermented products obtained with mixtures of coffee husks and cassava bagasse.

The mixture of coffee husk and defatted soybean cake (1:1) resulted in the production of 5.82×10^9 spores/g of dry substrate, which did not differ significantly from the production for coffee husk alone and coffee husk with sugarcane bagasse (17%), 3.52×10^9 and 5.26×10^9 spores/g of dry substrate, respectively. All fermented products were assayed for their nematicide capacity as described next.

Pot Experiments

The first stage of experiments had an objective of evaluating the nematicide activity of the fermented products obtained with cassava bagasse, coffee husk, and their mixtures. The plant roots were dissected under a stereomicroscope for female counting. The results obtained for this experiment are presented in Fig. 1. When the quantity of cassava bagasse augmented the composition of the substrate, the number of nematode females fell, even when the quantity of spores (*see* Table 1) was higher. Probably the virulence of the fungi was related to the nature of the substrate employed in the fermentation. Most of the studies found in the literature combined the utilization of *P. lilacinus* developed in commercial media. When inoculated in pot experiments, its ability to control the nematodes increased with the degradation of organic matter. The decomposition of organic matter released nematicidal principles and the organic residual matter increased fungal activity and persistence *(26,27)*.

In the second stage of pot experiments, defatted soybean cake and coffee husks were the substrates, and sugarcane bagasse was added as inert material (support). The virulence of the fungi was much higher when defatted soybean cake was the substrate (*see* Fig. 2) and root galling was practically absent. The reduction in female nematodes was 99.6 and 99.3%,

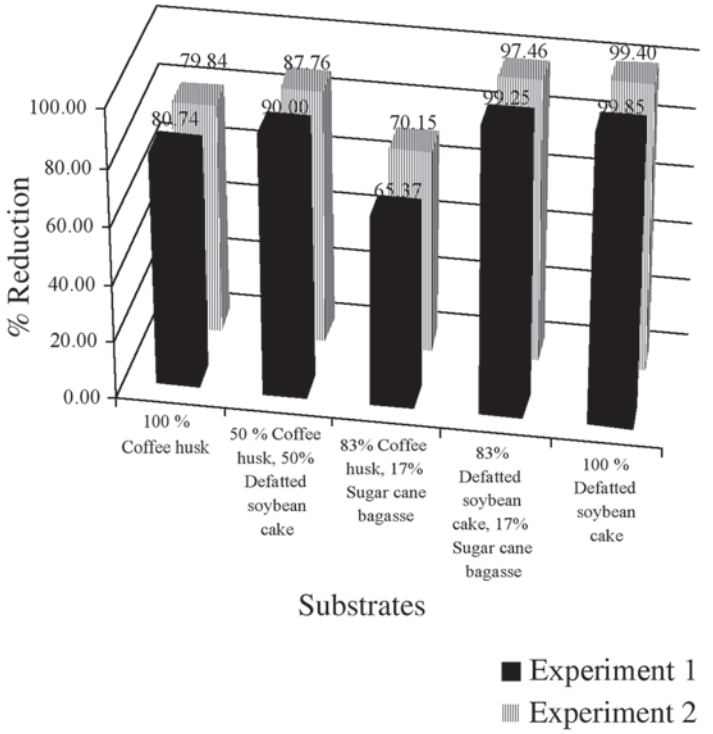

Fig. 2. Reduction in number of nematode females by employing fermented products obtained with coffee husks, defatted soybean cake, and sugarcane bagasse.

respectively, for the defatted soybean cake and defatted soybean cake mixed with sugarcane bagasse. In addition, the mixture of coffee husk and defatted soybean cake (1:1) showed a high reduction in the number of females (88%). The presence of *N* substances in the substrate perhaps enhanced the virulence of the fungi since the fungi possibly produced enzymes capable of degrading the nematodes' eggshell. When coffee husk was utilized alone, the reduction was on the order of 80%, confirming the results obtained in the first experiment. The addition of sugarcane bagasse to coffee husk was slightly less effective, reducing the number of female nematodes by about 70%.

Conclusion

The results proved the feasibility of using the strain *P. lilacinus* for spore production in SSF and its application as a biocontrol agent against the nematode *M. incognita* in pot experiments by utilizing the plant *Coleus*. The best spore production achieved was 4.27×10^{10} spores/g of dry substrate, which permitted a reduction in the number of female nematodes in pot experiments of almost 100%. Probably, the virulence of the fungi was related to the nature of the substrate employed in the fermentation, since the addition of 50% defatted soybean cake to coffee husk improved the

efficacy of nematode control by 10%. The fermented products should be studied for their application in different ratios of nematode population and fungi concentrations and also in other plants.

Acknowledgments

D.B. thanks LACTEC and IRD-France for a scholarship, the latter under continuous formation. C.R.S. thanks CNPq for a scholarship under the Scientific Productivity Scheme.

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