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FISIOLOGIA DE CRESCIMENTO E DE ESPORULAÇÃO DE FUNGOS NEMATÓFAGOS CULTIVADOS EM MEIO SÓLIDO

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Orientadores: Prof. Dr. Carlos Ricardo Soccol
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À Rafaela e à Lara com todo meu amor e para seus futuros...

Ao Rogério por me compreender, por seu apoio de sempre e por seu amor à nossas filhas.

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SUMÁRIO

LISTA DE ABREVIATURAS E SIGLAS

A_w – Atividade de água
BCA – Biological Control Agents
CEPPA – Centro de Pesquisa e Processamento de Alimentos
DNA – Desoxi-ribonucleic Acid
D - dias
EPA – Environmental Protection Agency
FES – Fermentação no Estado Sólido
HCl – Ácido clorídrico
HPLC – High performance Liquid Chromatography
IRD – Institut de Recherche pour le Développement
IAPAR – Institut Agronomique de l'état du Paraná
ITS - Internal Transcribed Spacer
J2 – Second Stage juveniles
LACTEC – Laboratoire de Recherche pour le Développement – Curitiba - Brasil
m – maintanance coefficient
MB – Methyl bromide
MEA – Malt Extract Agar
NaOH – hidróxido de sódio
NRRL- Northern Regional Research Laboratory – United States Department of Agriculture
LPB – Laboratório de Processos Biotecnológicos
ODS – Ozone deppleting Substance
OUR – Oxygen Uptake rate
PCR – Polymerase Chain Reaction
PDA – Potato Dextrose Agar
PVDF – Polyvinylidine difluoride
RAPD - Randomly amplified polymorphic DNA
RKN – Root-knot nematodes
RT – Room Temperature
SEAB – Secretaria de Abastecimento e Agriculura do Estado do Paraná
SCAR – Sequence Characterized Amplification Region
SmF – Submerged Liquid Fermentation
SSC – Solid State Cultures
SSF – Solid State Fermentation
UFPR – Universidade Federal do Paraná
UNEP - United Nations Environment Program
UV – Ultra-violet
YM – yeast malt
$Y_{x/o}$ – Biomass yield

RESUMO

O controle biológico representa uma alternativa importante aos pesticidas químicos que são perigosos para a saúde humana e para o meio ambiente. Vários estudos foram realizados com a utilização de fungos filamentosos que possuem ação contra os nematóides. Os nematóides do gênero *Meloidogyne* ou nematóide das galhas causam perdas econômicas importantes em muitas culturas agronômicas. O objetivo deste trabalho foi de estudar a utilização de resíduos agrícolas como substrato de baixo custo para a produção de esporos de fungos filamentosos nematófagos por fermentação no estado sólido (FES) e verificação da virulência dos produtos obtidos.

A fisiologia de crescimento e esporulação dos fungos filamentosos nematófagos dos gêneros *Paecilomyces* e *Verticillium* foi pesquisada. Três meios de cultura gelosados (Agar batata dextrosado, Agar extrato de malte e Agar extrato de casca de café) foram utilizados para determinar a velocidade de crescimento radial do micélio e a produção de biomassa. Foi realizado um acompanhamento das cinéticas de esporulação destas cepas por cultivo em meio sólido, em biorreator tipo esporulador de discos e em frascos Erlenmeyer. Experiências em vasos com plantas de *Coleus blumei* e os produtos obtidos por cultivo em casca de café demonstraram que a cepa *P. lilacinus* LPB-Pl-01 possui uma virulência mais significativa contra o nematóide *M. incognita* race 1. Esta cepa foi selecionada para a realização do trabalho.

Vários resíduos agro-industriais como o bagaço de mandioca e de cana-de-açúcar, o farelo de soja e igualmente os resíduos de camarão, foram utilizados sozinhos ou em combinação, como substrato para a produção de um composto contendo esporos de *P. lilacinus* LPB-Pl-01. Estes substratos foram selecionados de acordo com suas composições, afim de obter um produto final virulento e com uma quantidade importante de esporos do fungo nematófago. O composto escolhido é constituído de uma combinação de casca de café e farelo de soja (1:1). A produção de esporos neste substrato foi realizada em diferentes reatores; o melhor rendimento de esporos ($1,57 \times 10^{10}$ esporos/g de substrato seco) foi obtido em 8 dias de cultivo a 28º C, em um reator de bandejas. A influência da qualidade do composto inoculado em plantas de tomate contaminadas em *M.incognita* race 1 foi verificada: 0,175% do produto

demonstrou uma redução de 98% no numero de fêmeas deste nematóide sem prejudicar o sistema radicular.

O composto foi seco com circulação de ar em diferentes temperaturas: temperatura ambiente (20-26º C), 28, 32 e 35º C. O efeito do tratamento de secagem do composto na viabilidade de germinação de esporos em meio PDA foi determinado em 15, 30, 60, 90, 120 e 150 dias de armazenamento. O melhor tratamento de secagem foi à temperatura ambiente pois o composto não perdeu viabilidade apos 150 dias de armazenamento.

A influência da aeração e da umidade na cinética de produção de esporos da cepa de *P. lilacinus* LPB-Pl-01 em substrato constituído de 50 % casca de café e 50 % de farelo de soja foi acompanhada em biorreator tipo colunas de Raimbault. A respirometria (produção de CO_2 e consumo de O_2) permitiu de estabelecer importantes parâmetros biotecnológicos do processo como a velocidade de crescimento (0,08 h^{-1}), a produção de biomassa (0,545 g de biomassa/g de O_2 consumido) e o coeficiente de manutenção (0,1834 g de O_2 consumido/g de biomassa/h).

ABSTRACT

Biological control represents a great alternative to chemical pesticides which are hazardous for human health and the environment. Several studies were accomplished with filamentous fungi that have actions against nematodes. Nematodes of the *Meloidogyne* genre or root-knot nematodes are responsible for high agricultural losses in several cultures. The objective of the present work was to study the utilisation of agricultural by-products as low cost substrate for spore production of filamentous fungi in Solid State Fermentation (SSF) and verification of its nematicide capacity.

Growth and sporulation physiology of nematophagous fungi *Paecilomyces and Verticillium* were studied. Three culture media (Potato dextrose agar, Malt extract agar and Coffee Husk extract agar) were used to determine the apical growth velocity and biomass production. Sporulation kinetics of the strains were followed in SSF, disk fermenter and in Erlenmeyer flasks. Fermented products obtained in coffee husk were assayed for their nematicide potential in *Coleus blumei* plants. The strain *P. lilacinus* LPB-Pl-01 showed higher activity against the nematode *Meloidogyne incognita* Race 1 and was chosen for further studies.

Several agricultural by-products as cassava bagasse, sugar cane bagasse, defatted soybean cake and shrimp wastes were utilized alone or in combination as substrates for the production of a biocompost containing spores of the strain *P. lilacinus* LPB-Pl-01. The substrates were chosen in relation to their composition and the influences that they may have in the sporulation and in the virulence of the strain. The selected fermented product is composed of a mixture of defatted soybean cake and coffee husk (1:1). Sporulation of the strain in these substrate was followed in different reactors, best spore yield (1,57 E+10 spores/g of dry substrate) was achieved in 10 days of culture at 28°C, in a humid chamber containing perforated trays. The influence of the concentration of the fermented product in tomato plants contaminated with *M. Incognita* Race 1 was verified.

The fermented product was dried with air circulation in different temperatures. Effect of drying treatment was assayed through determination of spore viability after 15, 30, 60, 90, 120 and 150 days of storage. The influence of aeration and water content in the kinetics of spore production was followed in column type bioreactor. Respirometry analysis was accomplished and important biotechnological parameters were determined: growth velocity, biomass yield and maintenance coefficient.

1 INTRODUÇÃO

No decorrer do século 20, um aumento substancial na produção de alimentos foi grandemente apoiado no emprego de pesticidas químicos que possuem moléculas biocidas. Atualmente, a utilização inadequada de pesticidas, perigosos para a saúde humana e para o meio ambiente se tornou um problema generalizado. Ela conduziu a pesquisa de métodos menos poluentes para o controle de doenças de plantas, e ainda para responder ao objetivo do desenvolvimento sustentável da agricultura. Vários métodos de controle biológico, baseados em organismos supressores de pestes são as principais alternativas. Diversas tentativas e conceitos foram testados e preparações baseadas nestes agentes biológicos aparecem cada vez mais no mercado. Entretanto, mais pesquisas biotecnológicas são necessárias para que os mesmos se tornem substitutos práticos de pesticidas. Atualmente a utilização de biopesticidas ainda não possui uma eficácia comparável aos produtos químicos, mais prometem um futuro próspero (Gerhardson, 2002).

"O controle biológico utiliza populações de organismos vivos que podem ser parasitas, predadores, patogênicos, antagonistas ou competidores, para reduzir uma população de organismos nocivos, tornando-a menos abundante menos nociva do que era anteriormente"(De Bach, 1964, em Sidiqui, 1996). Os biopesticidas são por exemplo inseticidas preparados a partir de organismos vivos ou de substâncias que os mesmos produzem. Eles são fundamentados no principio do controle biológico: limitar a população ou a nocividade dos inimigos de culturas pela introdução no meio onde vivem de um seus inimigos naturais. Entretanto, a introdução de grandes populações de agentes de controle biológico no meio ambiente levanta a questão da biossegurança com relação às possíveis conseqüências ecológicas nas populações residentes e ao bom funcionamento do ecossistema. O impacto de um biopesticida sobre uma população fitopatogênica está diretamente ligado a especificidade do antagonismo. Embora seja difícil de estimar ou de avaliar o impacto geral sobre a microflora e a biofauna do solo, após a introdução massiva do mesmo (Siddiqui, et al., 2003).

As perdas mundiais causadas por nematóides ultrapassam 100 milhões de dólares por ano, sendo que a maioria destas perdas é atribuída aos nematóides das galhas. Os nematóides do gênero *Meloidogyne* (nematóide das galhas) causam prejuízos importantes a uma grande variedade de culturas. Estes parasitas vivem de substâncias nutritivas presentes no sistema

radicular, se multiplicam e invadem os tecidos das plantas privando-as assim de seu alimento. Com o objetivo de reduzir uma grande parte dos danos causados por nematóides, os fungos filamentosos nematófagos, seus inimigos naturais possuem estruturas de infecção adaptadas como armadilhas à laço ou esporos adesivos ou liberam toxinas no meio para imobilizar suas presas. Estes dispositivos afetam diretamente os nematóides ativos e reduzem o ataque às raízes por estes parasitas (Kerry, 2000). É necessário ter uma estratégia adaptada para enfrentar a invasão de nematóides e controlar sua população, em particular fazer diversos tratamentos com biopesticidas (Kerry, 1980).

A economia do Brasil como a da França é essencialmente baseada sobre a agricultura e é uma das mais importantes do mundo (café, cana-de-açúcar, soja, mandioca, frutas, etc.). No Brasil, quase todos estes produtos são exportados, e contribuem desta maneira ao desenvolvimento econômico do país. Entretanto, esta grande produção agroindustrial é responsável por uma produção muito elevada de resíduos sólidos e líquidos que ocasionam problemas ecológicos sérios. Nestes últimos anos houve uma tendência para aumentar a utilização eficaz destes resíduos como a polpa e a casca de café, o bagaço de mandioca e de cana-de-açúcar, o farelo de soja, etc... Estes sub-produtos podem constituir um excelente substrato para os processos de fermentação em meio sólido, pois os mesmos são ricos em fontes de carbono, nitrogênio e sais minerais. Igualmente, este sub-produtos podem ter importantes aplicações na biotecnologia, como substratos para o crescimento de fungos filamentosos para a obtenção de produtos com alto valor agregado: biopesticidas, enzimas, ácidos orgânicos, fungos comestíveis e metabólitos secundários (Soccol e Vandenberghe, 2003, Pandey, et al., 2000, Vandenberghe et al., 2000).

Os fungos filamentosos *Paecilomyces lilacinus* e *Verticillium chlamydosporium* são conhecidos por suas ações parasitas sobre os ovos e as fêmeas de *Meloidogyne*. Muitas pesquisas foram realizadas com estes fungos filamentosos para o controle de *Meloidogyne*, mais freqüentemente os métodos de produção de biomassa utilizados não estavam descritos ou eram realizados com meios de cultura onerosos (Stirling et al., 1998; Bourne et Kerry,1999; De Leij et al., 1992; Gaspard et. al., 1990 a et b; Khan et al., 1997; Mittal et al., 1995). A indústria alemã Prophyta comercializa um produto com *Paecilomyces lilacinus* em vários países. Três outras formulações deste fungo filamentoso são produzidas na África do Sul, na Austrália e na Colômbia. A utilização da Fermentação no Estado Sólido (FES) é uma ótima alternativa para a produção de agentes de controle biológico pois ela pode fornecer um produto estável e eficaz utilizando-se uma tecnologia de baixo custo. Neste últimos anos, um grande número de pesquisas foi realizado, em engenharia de fermentação no estado sólido

englobando os aspectos cinéticos, modelos matemáticos, design de biorreatores, controle e automação, que podem ser úteis para a produção comercial de produtos biotecnológicos (Pandey et al., 2003, Rahardjo, et al., 2006, Jones et al., 2004, Durand, 2003; Bellon-Maurel et al., 2003).

O objetivo principal desta tese foi de estudar a obtenção de um produto contra os nematóides das galhas à partir de fungos nematófagos cultivados por FES e utilizando-se preferencialmente os resíduos da agroindústria brasileira como substrato para a cultura de fungos filamentosos. Os trabalhos realizados são descritos em três partes.

A primeira parte compreende o estudo da fisiologia de crescimento e de esporulação de fungos nematófagos. O crescimento radial e a produção de biomassa de diferentes cepas de fungos filamentosos nematófagos, dos gêneros *Paecilomyces* e *Verticillium*, foram estudados empregando-se três meios de cultura. Estes meios são constituídos de diferentes fontes de carbono que são originários de extratos de batata (PDA), extrato de malte (MEA) e de extrato de casca de café (CHA). A esporulação das cepas foi estudada em frascos Erlenmeyer, em esporulador de discos e em colunas de Raimbault. Os parâmetros físicos de cultura como o pH, a quantidade de água e a temperatura foram estudados em FES com casca de café e com diversas cepas. A eficácia dos agentes de controle biológico obtidos foram testados através de experimentos em vasos utilizando-se a planta *Coleus blumei* contaminada com o nematóide *Meloidogyne incognita*. Estas experiências permitiram escolher uma cepa de fungo nematófago com a qual foram realizados os estudos de FES.

A segunda parte trata da escolha de um substrato sólido para a produção de esporos da cepa escolhida e da qualidade dos biopesticidas produzidos em cultura sólida. Diferentes resíduos da agroindústria brasileira foram testados sozinhos ou combinados como substratos de cultura em meios sólidos. Suas influências sobre a produção de esporos e sobre a atividade dos biopesticidas obtidos contra os nematóides foram verificadas. Após a escolha da cepa de fungo filamentoso mais eficaz e do substrato para a produção de conidiosporos, a cinética de produção de esporos foi seguida em cultura sólida. A produção de esporos foi realizada em diferentes tipos de fermentador. O estudo da secagem de esporos em diferentes temperaturas e o impacto sobre a viabilidade dos esporos produzidos foi verificado.

Enfim, na terceira parte, a influência da aeração e do stress hídrico dos meios de cultura sobre a esporulação de *Paecilomyces lilacinus* foi estudada, utilizando-se o fermentador de colunas de Raimbault. A respirometria deste fungo filamentoso foi seguida em linha e permitiu de estudar os principais parâmetros biotecnológicos do processos como a velocidade de crescimento, a produção de biomassa e o coeficiente de manutenção.

2 REVISÃO DE LITERATURA

Artigo 1

Utilização da Fermentação no Estado Sólido para a Produção de fungos filamentosos como Agentes de Controle Biológico: estudo sobre *Paecilomyces lilacinus* contra os nematóides das galhas

RESUMO

Os nematóides das galhas causam perdas consideráveis que ultrapassam U$ 100 bilhões por ano. Ainda, a resistência dos nematóides aos pesticidas e a retirada de produtos químicos do mercado conduziu pesquisadores de vários países a procurar novos métodos alternativos para o controle de nematóides. O controle biológico contra estes organismos por *Paecilomyces lilacinus* está bem estudado mais os processos de produção de esporos estão freqüentemente indisponíveis ou não atingiram a um nível industrial. A fermentação no Estado Sólido (FES) é um processo tecnológico apropriado para a produção massiva de biomassa dos agentes utilizados em controle biológico. Os conidiosporos produzidos por FES apresentam uma boa estabilidade e uma viabilidade importante e podem ser produzidos de maneira rentável. Os estudos de design de biorreatores são essenciais para o escalonamento de processos de FES compreendendo a produção massiva de esporos que podem ser utilizados no Controle Biológico. Este artigo descreve os nematóides das galhas e os prejuízos que eles causam na agricultura. Os principais métodos de controle de nematóides são apresentados compreendendo o controle biológico com *P. lilacinus* e as técnicas de biologia molecular são descritas para identificar e seguir o fungo no solo. Enfim, a FES pode ser o método de escolha para a produção de conidiosporos de fungos filamentosos de uma maneira competitiva, pois seu custo é baixo e apresenta algumas vantagens sobre a fermentação líquida. As características gerais da FES e um certo número de biorreatores, indispensáveis para o escalonamento da produção de agentes de controle biológico são descritos.

UTILIZATION OF SOLID STATE FERMENTATION FOR THE PRODUCTION OF FUNGAL BIOLOGICAL CONTROL AGENTS: CASE STUDY ON *Paecilomyces lilacinus* AGAINST ROOT-KNOT NEMATODES

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Key words: Solid State Fermentation, *Paecilomyces lilacinus*, *Meloidogyne incognita*, Biological Control.

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ABSTRACT

Root-knot nematodes (RKN) cause worldwide losses up to U$ 100 billion dollars annually. Nematodes resistance to pesticides and the withdrawal of chemicals from the market has led to the search of new methods for their control. Biological control of RKN with *Paecilomyces lilacinus* is well investigated but production systems are often unavailable or didn't attain much attention. Solid state fermentation (SSF) is a suitable technological process for the mass production of biological control agents (BCAs). Conidiospores produced by SSF are cost-effective and present good stability and viability. Studies of bioreactor design are essential for scaling up of a SSF process, including the mass production of fungal conidiospores.

2.1. Introduction

Nowadays, environmentally sound and economical feasible alternatives for pests control are subject of inumerous studies, due to the buildup of resistance to the pesticides in target pathogens populations and the withdrawal of pesticides from the market, because of environmental and public health concerns (Gehardson, 2002). Plant-parasitic nematodes specifically root-knot nematodes (RKN) are major pests of several economical important crop plants, causing serious yield losses. Methyl bromide (MB) was the most widely used soil fumigant, but it was already banished in developed countries and developing countries have till 2015 for its substitution, then other control measures are urgently required.

Biological control agents (BCAs) such as fungi can be a great alternative for their control, although its development is not a straightforward task. *Paecilomyces lilacinus* is a known soil hyphomycete and parasitizes RKN eggs and females and have been shown great nematicide capacity. Growth physiology of filamentous fungi is very important, when considering its production as BCA. A commercial patented strain is produced by the German manufacturer Prophyta, this strain is still object of inumerous researches (Khan, et al., 2003, 2004 and Kienwick and Sikora, 2006). The field applications of biofungicides is mainly accomplished by means of fungal conidiospores, which must be virulent and viable for long storage periods. Conidiospores can be advantageously produced by solid state fermentation (SSF) resulting in low cost and good quality product.

This paper describes the root-knot nematodes and the problems that they imply in agriculture. The main methods of nematodes control are presented and biological control with the fungus *P. lilacinus* is emphasized. Molecular biology techniques are demonstrated to identify and follow the fungus in the soil. Moreover, SSF can be the method of choice for producing filamentous fungi conidiospores in a competitive manner, as it presents low production costs and some advantages over submerged liquid fermentation (SmF). General characteristics of the method is presented as well as some aspects of SSF bioreactors that are useful for scale up strategies in the production of BCAs.

2.2. Plant-parasitic nematodes

The nematodes are roundworms that belongs to the phylum Nematoda, they occupy different ecological niches and may live as parasites of men, animals and plants. The nematodes are the most abundant creatures on earth, their majority are free-living, and consume bacteria and other microscopic organisms. Initially the nematodes should be considered as parasites and when the disease is developed into a host, in response to a great number of individuals then they are denominate as pathogens (Olsen, 2000).

Phytoparasitic nematodes can devastate several economic important cultures, causing significant losses. Phytoparasitic nematodes are obligate parasites that have evolved different parasitic strategies and relations with its host, to attain enough nutrients for their development and reproduction. The products of nematodes parasitic genes can be expressed as morphological structures (stylet), which allows to assess the parasitism of a particular host plant, where they can develop critical physiological functions in the interaction with the host (Davis et al., 2000).

The groups of phytoparasitic nematodes that have great economical importance are the sedentary endoparasites, which include the genera *Heterodera and Globodera* (cyst nematodes) and *Meloidogyne,* or root-knot nematodes and in less extension several migrant nematodes as *Pratylenchus* and *Radopholus*. Root-knot and cyst nematodes have complex interactions between their hosts and moreover present different characteristics of their parasitic cycle (Williamson and Gleason, 2003).

2.2.1. Phytoparasitic nematodes and agricultural losses

Phytoparasitic nematodes explore all vascular parts of the plants, but the species economically important infect the root, in consequence it's difficult to estimate the total loss attributed to nematodes due to the presence of other root pathogens. It is estimated that overall yield losses are more than 10% reaching 20% for some crops, in monetary terms world losses exceed certainly U$ 100 billions annually. The impact of plant parasitic nematodes in agriculture may also be measured by the strategies employed in their control. However in recent decades the utilization of chemical pesticides are being deregistered due to ground water contamination, avian and mammalian toxicity and food residues (Bird and Kaloshian, 2003).

2.2.2. The root-knot nematodes

The nematodes of the genre *Meloidogyne* are equally known as root-knot nematodes, in their parasitic cycle they develop knots in the plant roots. The root knots are giant cells of plants whose nematodes employ as nutrition source. The nematodes of the *Meloidogyne* genera have great economic importance and cause damage to several plants, the most described species include *M. arenaria*, *M. fallax*, *M. hapla*, *M. incognita*, *M. javanica* *M. naasi*, *M. trifoliophila, M. paranaensis* and others. *M. incognita* and *M. javanica* are the species more distributted in the world. The Meloidogynes attack several cultures: Solanaceae (tomato, potato), Curcubitaceae (water melon, cucumber), Leguminosae (beans), carrots, also highly pathogenic to some staple crops such as cereals, including rice, maize, soybean, banana, plantain, sweet potato and yam; or industrial crops such as tobacco, coffee, sugar cane, sugar beet, cotton and black pepper. Often this species also causes economic damage to fruit crops such as guava, pineapple, papaya and grapes (Lamberti, 1997). The infections by root-knot nematodes cause leaf chlorosis, galls on roots and the root system may be drastically reduced; plant stunting and wilting occur when severe infestations are present (Olsen, 2000).

2.2.2.1. Root-knot nematodes life-cycle

The nematodes are soil borne pathogens and feed themselves in the roots. Their life-cycle includes the stages of eggs, juveniles and adults. Nematodes hacth in the soil as second stage juveniles (J2). The vermiform juveniles are motile, they infect the roots preferentially in the zone of elongation or at the site of a lateral root emergence, they perforate the root walls with the stylet. After penetration, the nematode migrate intercellularly into the vascular cylinder, where it establishes a feeding site, that constitutes the giant cells which contains a granular cytoplasm and a great number of knots that are necessary for their development. This site becomes differentiated by cell division and sweeling, causing the formation of the galls or root-knots where the female is installed. The formed galls contains a gelatinous matrix, where the female lays hundreds of eggs (Davis, et al., 2000, 2004; Kaplan and Koevenig, 1989).

2.3. Methods for Nematodes Control

After 1950, the control of phytoparasitic nematodes is based in chemical pesticides although several nematicides are being withdrawal from the market due to issues such as environmental and public health concerns. Moreover the iminent interdiction of methyl bromide as soil fumigant, regarding its potential for ozone layer depletion and still the buildup resistance to pesticides of target population has led to the search of new alternatives for nematodes control (Fernandez, et al., 2001; UNEP, 2001). Possible control measures changes with climate conditions, socio-economical situation of the country, plant economy, chemical pesticides and resistant cultivars availability and the practicability of agricultural practices.

2.3.1. Resistant plants

Plants are resistant to nematodes when they have a reduced level of reproduction. The nematodes resistance genes are present in several crops and are an important component of various multiplication programs as for tomatoes, potatoes, cotton, soybeans and cereals. The resistance can be broad with action against several species of nematodes or narrow responsible only for specific biotypes (Williamson and Hussey, 1996).

Several resistance genes, dominant or semidominant were identified and cloned and are object of various studies (Zhang et al., 2002; Yamamoto and Hayashi, 2002; Lopez-Perez et al., 2006). The *Mi* gene of tomato is the more studied gene and confers resistance to *Meloidogyne incognita, M.javanica* and *M.arenaria* but not to *M.hapla*. The resistance is accompanied by a hypersensitive response in the host plant, with a region of necrotic cells of plants being visible around the head of the invading nematode within 12-24 h of inoculation on tomato roots. The coded protein contains a nucleotide binding site and a leucin rich repeat, protein motifs that are found in numerous plant resistance genes against a variety of pathogens (Williamson and Gleason, 2003; Bird and Kaloshian, 2003).

2.3.2. Crop rotation

Crop rotation is an important element for the maintenance or improvement of soil fertility and though for obtention of higher crop yields. Various cultures are followed on a certain order on

the same soil. The same succession of cultures reproducing in a regular time cycle, rotations can be biennial, triennial, four-year... Crop rotation can always be adopted against species with narrow ranges of plant-host, which is not the case of *Meloidogyne*. However, the order of plants and the time intervals between susceptible crops depend on nematodes species.
In the case of *Meloidogyne*, or at least with the species which lay eggs in a gelatinous matrix, at least three years between harvests must be passed. Harvests of high economic value crops are often intensively developed and can be difficult to find an order of suitable plants which will not change the economic equilibrium (Milligan et al., 1998).

2.3.3. Chemical control

Plant-parasitic nematodes are more vulnerable as juveniles (J2) in soil when searching for the roots of host plants. Once endoparasitic species have penetrated a root, chemical control is more difficult as compounds have to be non-phytotoxic. There are several nematicides that can be used effectively for nematode pests of annual crops but there appears to be little prospect for management of nematodes in many susceptible perennial crops without repeated application of nematicides (Gowen, 1997).

There are two kinds of chemical products that can be utilized against plant parasitic nematodes: soil fumigants and nematicides or pesticides. Their incorporation in soil depends on the form of the formulation, can be by injection, spray, by mechanical means and irrigation pipes. Products to nematodes control are normally applied before planting for fumigants or at the time of planting in the case of pesticides. Fumigants are highly effective against nematodes, their efficacy is related to their high volatility at ambient temperatures. All fumigants have low molecular weights and occur as gases or liquids. As they volatilize, the gas diffuses through the spaces between soil particles where the nematodes are killed. The most known fumigant is methyl bromide, it is mainly used in high valued crops such as strawberries and tomatoes and in lesser amount to grains and traded commodities, however it was characterized as ozone depleting substance (ODS) though it was added to the substances controlled by the Montreal protocol whose use must be eliminating by 2015 in developing countries meanwhile its use was already banished in developed countries in 2005. Fumigants such as Chloropicrin, Dazomet and Metam Sodium present good activities against nematodes. Nematicides are available as liquids, granules or solids, they may kill by contact or by systemic action, they can control a wide range or be species specific, consisting mainly of

organophosphates and organocarbamates and differ from one country to another (UNEP, 2001).

2.3.4. Biological control

Biological control is a pest management strategy that utilizes deliberate introduction of living natural enemies to lower the population level of a target pest (Delfosse, 2005). This enemies are commonly named biological control agents (BCAs), which must demonstrate some characteristics for success: rapid colonization of the soil, persistence, virulence, predictable control below economic threshold, easy production and application, good storage, low cost, compatible with agrochemicals and safe (Kerry, 1987).

In nature it is observed that plant parasitic nematodes can be attacked by many enemies such as viruses, bacteria, rickettsias, fungi, and others, although much more attention was given to fungi and bacteria in the research of biological control. Biological control can be natural, when a natural population of organisms have increased to reduce or suppress nematodes multiplication, or induced when biological control agents have been introduced by man. This introduction have two approaches: inundation or microbial pesticide application for rapid control of a pest and introduction or mass release to provide long lasting control. The suppression can be non specific or specific when only one or two organisms are involved (Jatala, 1986; Davies et al., 1991; Kerry, 1987; Akhtar and Malik, 2000).

Several attempts were accomplished with the utilization of bacteria in the control of nematodes, the bacteria can be divided in two groups: nematode parasitic and non parasitic rhizobacteria. The most studied bacteria is *Pasteuria penetrans,* an obligate endoparasite of *Meloidogyne*, followed by strains of *Pseudomonas* (Siddiqui, 2004; Siddiqui and Shaukat, 2003; Siddiqui and Mahmood, 1999; Tzortzakakis et al., 1999; Ali, et al.,2002; Dabiré et al., 2001; Mateille et al., 2002; Payne, 1992).

Nematophagous fungi – are organisms that may control the development plant-parasitic nematodes, they attack living nematodes or their eggs and utilize them as source of nutrients. They are classified or named by the different action mechanisms that they may develop against the nematodes, in a determined phase of its life cycle. The nematode-trapping fungi develops special mycelium structures in forms of traps in response to the presence of nematodes in the soil, the structures may adhere or not to the nematodes cuticle (Jansson and Nordbring Hertz, 1980). The endoparasitic fungi were so denominated because their spores must be ingested or adhered by nematodes. Finally, there are fungal species that are parasites

of root-knot and cyst nematodes, which are parasites of eggs or females of these nematodes (Mankau, 1980; Dijksterhuis et al., 1994, Nordbring-Hertz,B, 1988).

Several steps should be followed when developing commercially viable BCAs:

1. Isolation and selection of BCAs from the environment; Attention should be given in agent maintenance;
2. Studies of ecology, physiology and taxonomy of potential BCAs;
3. Laboratory and/or field tests to identify the most virulent and fitted strains;
4. Economical mass production of the chosen strains;
5. Studies on formulation strategies and compatibility with application techniques;
6. Risk assessment trials, BCA should be safe to humans and non target species.

Ecological and economical consequences should be carefully considered when releasing BCAs in the environment. Programs must be only considered when the benefits are greater than the costs (Jetter, 2005). BCAs are generally highly species specific, but can present indirect impact on non- target species, causing environmental disequilibrium. However, if the agent introduced demonstrates high efficacy, this problem is overcome once they will reduce their own population through feedback (Pearson, 2005).

In this article, the nematode infection process will be described for the egg parasites, and particular emphasis will be given to process developed for the fungus *Paecilomyces lilacinus.*

2.3.4.1. Infection process

The infection process of nematophagous fungi have been elucidated through the application of biochemistry and molecular biology techniques, involving enzyme characterization whose roles are in the penetration of the eggshell or the nematode body wall and the identification of nematicidal toxins. Nematodes are provided of two distinct barriers to infection: the eggshell and the cuticle. Eggshells of root-knot nematodes are composed of three layers: the outer vitelline layer, composed mainly of proteins, chitin and inner lipoprotein layers. The thickness of these layers varies considerably among nematode genera. The cuticle plays an important role in motility, maintenance of morphology integrity and also providing protection from the environment and pathogens (Morton, 2004). Fungi are applied in soil mainly as spores, which must be active and virulent, they colonise the rhizosphere of plants where sedentary females

and eggs are found. Spores will germinate and form appressoria, which is a hyphal structure that secrete extracellular enzymes depending on the recognition of the host surface hydrophobicity (Lopez-Llorca, et al., 2002). The fungus may also be able to adjust pH to regulate its optimum enzyme activity, as described for *Metarhizium anisopliae* (St Leger, et al., 1999), as enzymes secreted by nematophagous fungi are serologically and functionally related (Segers, et al., 1995, 1997). Serine proteases and chitinases from *Paecilomyces lilacinus* strains were purified and characterized from culture media containing egg yolk and chitin (Bonants,et al., 1995; Khan et al., 2003; Huang et al., 2004). The enzymes produced by the commercial strain 251 were applied to *Meloidogyne javanica* eggs promoting significant differences in the eggshell and reduction in egg hacthing (Khan et al., 2004). Equally, eggs of the nematode *Globodera palida* were treated with an endochitinase and a protease from *V. suchlasporium* showing surface damage when compared to untreated control (Tikhonov, et al., 2002). Park, et al., (2004) researched the influence of leucinostatins, secondary metabolite produced by *P.lilacinus* in the colonization of *M.javanica* eggs, showing positive results but on the other hand showed that chitinase activity may be related to parasitism and not in the degradation of pathogens wall.

2.3.4.2. *Paecilomyces lilacinus*

This fungus is found in the majority of agricultural soils, it can be frequently isolated from *Meloidogyne* eggs and females. *Paecilomyces* belongs to the division *Eumycota*, class *Deuteromycetes*, order *Moniliales* and family *Moniliaceae*. The conidiophores of the genus *Paecilomyces* ramify in grouped branches or irregularly. The conidia are separated from the phialides in form of chains. *P. lilacinus* shows fast hyphal growth. The conidiophores are up to 600 µm high and develop groups of lateral branches, from which each 2-4 bottled-shaped phialides grow. The ellipsoid, 2.5-3.0 µm long and 2.0-2.2 µm broad conidia are of lilac colour. The facultative egg parasite *P.lilacinus* is sometimes able to infect mobile nematode stages or sedentary females, but it is most aggressive against eggs (Onions, et al., 1981, Jacobs, 2002).

The development of *P.lilacinus* as biological control agent (BCA) depends on several factors considering its age, virulence, viability, inoculum concentration, methods of application among others, the environmental conditions as soil type, fertility, organic matter, amendments, temperature and pH and also host susceptibility (Kerry, 2000). Several works

were accomplished with the utilization of this fungus in the control of plant parasitic nematodes, where important information are given and can be useful when developing BCA, although little information is given about the production system utilized. Fermentation parameters such as water content, aeration, utilized fermenter, inoculation, pH, were not described by the authors.

Temperature had a significant effect in the cultivation of *P. lilacinus*, best biomass was obtained in potato dextrose broth (PDB) in the temperatures between 24-30°C. Also the temperature of the soil demonstrated a great influence in the control of *M. incognita* with *P.lilacinus* (Cabanillas, et al., 1989a). Cabanillas et al (1989b) studied the survival of *P. lilacinus* spores produced in PDA, in carriers such as alginate pellets, diatomaceous earth, wheat grain, soil and soil plus chitin and also its effects against *M.inconita* in microplot experiments.

The fungus grown in wheat, was also tested against *M. javanica* on tobacco with or without the addition of phenamiphos and ethoprop in microplot experiments during 2 years having Vetch as winter culture. The fungal survived in the soil although it wasn't able to control nematodes development, showing that the type of root system may have an important role in control by *P.lilacinus* (Hewlett, et al., 1988).

The fungus isolate was produced in rice grains and tested in pot experiments with the addition of chitin (0-1% w/w) to tomato plants infected with *M. arenaria*. Results indicated that the combination of *P.lilacinus* and chitin were effective for the control of the nematode tested (Culbreath et al., 1986).

Tomato cultures infested with *M. incognita* were protected at different levels by the fungus *P. lilacinus*. The protection grade against this nematode with *P. lilacinus* was positively related with the quantity of applied fungus and application time. The best protection against the nematode in tomato was reached with 10 and 20 g of fungus infested wheat, which resulted in a raise of 3 and 4 times in tomato yield, respectively when comparing with plants treated only with the nematode. The best protection achieved against this pathogen was when the fungus was applied in soil 10 days before plantation and during plantation (Cabanillas and Barker, 1989).

Spores of *P. lilacinus* produced in potato dextrose agar (PDA) were tested in microplot experiments alone and in combination with chitin in controlling *M. incognita* in egg plant, tomato and chickpea. Results showed that the best treatment employed consisted of the addition of fungus spore suspension and chitin, on the development of the plants inoculated with *M incognita*, showing lower gall formation. When utilizing only fungi, treatment was

less effective when comparing to the addition of fungus and chitin, however when chitin was employed alone, root galling was higher in all plants tested. It seems that chitin serve as the substrate or food base for selective development of the biocontrol agent in the soil. There are two theories which may explain the action of chitin against nematodes: chitin decomposition releases ammonia, which acts as a nematicide on J2 of root-knot nematodes; and chitin may increase population of chitinolyitic microflora, that may parasitize nematodes eggs and egg sacs (Mittal, 1995).

P. lilacinus was also utilized alone and in combination with bone meal, horn meal and several oil cakes to control *M. javanica* in tomato plants. Methodology for spore production of the fungus wasn't supplied by the authors. Results indicated that *P. lilacinus* was effective as females, egg masses and eggs were parasitized. The addition of the organic ammendments showed increased activity and persistence of the fungus in the soil (Khan and Saxena, 1997).

The effect of culture growth, on *P. lilacinus* spores size, ultrastructure, UV tolerance, were determined with aerial spores produced in PDA and submerged spores produced in media containing glucose and mineral solution. Aerial spores where more constant in size, but were smaller than submerged spores, although rodlet layer was found only in aerial spores. Aerial spores were more tolerant to UV radiation, showing also better viability after drying and storage, however nematophagous activity were similar (Holland, et al., 2002).

A commercial patented strain PL 251 is registered for sale in several countries, is produced by the German manufacturer Prophyta and consist of a water dispersible granule that can be used for a variety of crop plants (EPA, 2005). This strain was assayed for the production of paecilotoxin and other toxins with anti-microbial activities showing no detectable levels (Khan et al., 2003). This product was evaluated for its potential to control *M. incognita* and *M. hapla* Chitwood in tomato, demonstrating that a single pre-planting application is sufficient for nematodes control (Kiewnick and Sikora, 2006a and b).

Different molecular approaches were tested to identify and monitor the fungus in the soil. Species specific primers were developed for the identification of *P. lilacinus* based on sequence information from the ITS gene. The primers generated a single fragment of 130 base pairs, specific to *P. lilacinus*, which permitted to detect the fungus from soil, roots and nematodes eggs. Also, through Real-time PCR primers and a Taq Man probe, it was possible to quantify the population of the fungus (Atkins et al., 2005). RAPD (randomly amplified polymorphic DNA) techniques were also attempted to monitor and to differentiate a strain of *P. lilacinus* from a strain of *Pochonia chlamydosporia*. Two specific fragments from each strain were chosen and were further cloned, sequenced, and used to design sequence-

characterized amplification region (SCAR) primers specific for the two strains, which were used in classical PCR reaction to determinate the detection limits (Zhu et al., 2006). Philogenetic analysis from 5.8S rDNA and internal transcribed spacer (ITS1 and ITS2) sequences were realized for identification and taxonomy of different *Paecilomyces* species (Inglis and Tigano, 2006).

2.4. Growth Physiology of Filamentous Fungi

The spore production of filamentous fungi is an important stage in the reproduction of fungi, it consists on the formation and liberation of conidiospores. Life-cycle of filamentous fungi comprehends five steps, that are the dormancy of the spore, germination of the spore, apical mycelium growth, conidiogenesis and conidiospore production. The normal development of the mycelium and good conidiogenesis are the main conditions for an important spore production. This conidiospore production is directly related to the quantity and the nature of the carbon and nitrogen sources available in a culture media and depends on several factors: inoculation mode, salinity, and carbon/nitrogen ratio, aeration and water content (Roussos, 1985). The conidiospores are characterized by a low water activity, absence of citoplasm movements, and reduced metabolic activity. In favourable conditions germination takes place forming a vegetative tube, which is the base of a future mycelium. A spore is considered germinated when the length of the longest germ tube was greater than the greatest dimension of the swollen spore. Different techniques, other than microscopic examinations, can be used to asses mould germination, Gompertz equation and logistic function can be used for fitting germination data (Dantigny et al., 2006). Determination of the optimal cultivation conditions for large numbers of conidiospore production of filamentous fungi used as BCA is very important for commercial practice, especially for the decrease of mass production costs. Several studies using fungal BCAs where carried out with the objective to enhance conidiospore production. Chen et al., 2005 studied the addition of different carbon and nitrogen sources in the sporulation of *Coniothyrium minitans* by SSF with wheat. *P. fumosoroseus*, an entomopathogenic fungus, cultivated in SSF and SmF using the same fermentation broth and culture conditions demonstrated that in SSF the carbon source was mainly utilized for the production of biomass, while in SmF the fungus produced more insecticidal metabolites (Assaf et al., 2006). When more biomass is synthesized by a fungus,

consequently more conidiospores are formed resulting in a higher spore yield in SSF than SmF.

2.5. Solid State Fermentation (SSF)

SSF may be defined as the growth of microorganism in the absence of free water, however, the substrate must contain enough water to support growth and metabolism of microorganisms (Cannel and Moo-Young, 1980; Pandey, 2003). The fermentation can be carried out in two types of matrices, that comprehends a solid substrate which functions as source of nutrients and also serves as support and an inert support which can be impregnated with a liquid nutritive media (Saucedo-Castaneda, 1991). The most utilized substrates have amilaceous or lignocellulosic origin . Several materials are utilized as inert supports: sugar-cane bagasse, amberlite, vermiculite, polyurethane foam, etc.

SSF presents several advantages over SmF but the choice of the method should be investigated regarding physiology of the microorganism and end product. Comparison of SSF and SmF was frequently realized (Holker and Lenz, 2005; Lonsane, et al., 1985). Some advantages of SSF processes:

- Simplicity of culture media;
- Absence of liquid residues;
- Reduction of contamination due to low water content;
- Culture conditions mimics the natural environment;
- Ease of aeration (humid or dry) regarding the porosity of the material;
- Direct utilization of the fermented material;
- Ease of products extraction because it is present in high concentrations;
- Ease to apply an hydric stress and dry the fermented product *in situ*.

The problems or disadvantages that may be develop during a SSF process are: excess of heat generation (difficulties in heat and mass transfers), fermentation parameters control (pH, water content), biomass estimation, substrates pre-treatment among others.

SSF processes simulate the living conditions of many higher filamentous fungi. For this reason, SSF is the cultivation method of choice for instance in cases in which the

biotechnological process is required to include morphological and metabolic differentiation into substrate-penetrating and aerial hyphae, such as in the production of conidiospores (Holker and Lenz, 2005).

There is lack of information about the influence of nutritional and physical parameters on the physiology and kinetics of growth and sporulation of *P.lilacinus* in SSF, being the biomass estimation an important parameter for their characterization however its rather difficult to measure biomass in SSF. As filamentous fungi grows their hyphes penetrate in the solid matrix and its impossible to separate substrate from mycelium then a direct measurement of biomass is out of question. Indirect measures of biomass are required and comprehends analysis of biomass components as glucosamine, ergosterol (De Carvalho et al., 2006, Lareo et al., 2006; Sobal Cruz, 2002), nucleic acids and proteins (Vandenberghe, 2000). Certain considerations must be done when choosing a biomass component: it should be well adapted to the microorganism, present little or no influence with substrate and must be constant through development. The most adequate method to measure biomass consists in measure the production of CO_2 and the O_2 consumption by the microorganisms during fermentation, which allows the calculation of biomass and specific growth of the microorganism inside the reactor through stoichiometric relations between biomass synthesis and oxygen consumption (Saucedo-Castaneda, 1994. Pintado et al., 1998).

2.5.1. Important parameters in SSF

First of all, based on the type of the microorganism that can belong to the natural microflora of the substrate and pure culture. Ensiling and composting are the two methods that utilize the natural microflora. Pure culture are main utilized for the production of fungal conidiospores, secondary metabolites, antibiotics and others high valued products (Pandey, 1992). Several groups of microorganisms may grow in solid substrates, although filamentous fungi have higher capacity to grow in the absence of free water due to their physiological, enzymological and biochemical properties. Filamentous fungi are more appropriate for cultivation in SSF because its growth is a combination of the apical extension of the hyphes associated to the generation of new hyphes by mycelium ramification, which permit the access of the fungi between the solid matrix to form a solid structure. The penetration in the substrate enhances

the access to the available nutrients between the particles and allows the fungi to realize its metabolic activity (Raimbault, 1998).

In SSF, the quantity of water present in the media is function of the substrate water retention capacity, this quantity should be sufficient for the growth of microorganisms without destructing the solid structure or reduce the porosity of substrate or support (Gervais et al., 1988, Gervais and Molin 2003). The water present in the substrate may play different roles: influences in the microorganisms constitution, serve as vehicle for enzymes, nutrients and metabolites and also in oxygen solubilization (Oriol et al., 1988). High moisture content of the substrate can lead to a reduce porosity of the solid matrix, weak oxygen diffusion and raise the risks for bacterial contamination, however, low water content leads to a limited growth and reduce the microorganism accessibility to substrate (Lonsane, et al., 1985).

The control of the gaseous environment in aerobic SSF is a fundamental factor for microorganisms growth, which depends on the air flux rate through the substrate and the velocity of O_2 consumption by microorganisms (Raimbault and Alazard, 1980). Aeration provides O_2 for aerobic growth and metabolism, helps to control moisture and temperature and also eliminates CO_2 and some volatile metabolites. The gaseous environment may significantly affect biomass and enzymes production levels.

The initial pH of the substrate in SSF is adjusted to optimal for microorganisms growth. The pH value varies in accordance to microorganisms metabolism, by acid production during fermentation or by the formation of urea which tend to elevate the pH. With the objective of avoid high pH variations Raimbault and Alazard (1980), utilized a solution of mineral salts with buffer capacity.

2.5.2. SSF and the production of Biological Control Agents (BCA)

An important parameter that must be considered when selecting a BCA for mass commercial development is the availability of a cost-effective production and stabilization technology that gives an appropriate effective formulation (Spadaro and Gulino, 2005). BCA inoculum are mainly applied for control of pests as spores and SSF offer several advantages for its production regarding the quality of the product obtained, as aerial spores are more tolerant to UV radiation, show higher stability, are more resistant to drying and exhibit higher germination rates for longer storage periods, this can be explained by the presence of a

hydrophobic rodlet layer formed during the process (Agosin, et al., 1997, Roussos et al., 2000). Another advantage that SSF may give for BCAs production is the possible utilization of agricultural by-products as substrate for fermentation. The generation of very high amounts of residues cause serious environmental problems and SSF may provide their efficient utilization and value-addition and moreover leads to a less expensive process (Soccol and Vandenbreghe, 2003). Table 1 shows applications of SSF for the production of BCA spores in different substrates or supports.

SSF processes allow to reduce labor and technical difficulties and often formulation is no further necessary. The fermented product can be air dried or by utilizing rotavapor and directly used or spores can be extracted for further formulation (Jenkins et al., 1998). Roussos et al., 1989 studied different methods for the conservation of filamentous fungi spores produced by SSF and demonstrated that the temperature has a significant effect on spore viability.

Table1. BCAs produced in SSF in different substrates or supports.

Microorganism	Substrate/support	Application	Reference
Coniothyrium minitans	Oats	Fungal antagonist	Oostra et al., 2000
Epicoccum nigrum	Peat/vermiculite	Brown rot of fruits	Larena et al., 2004
Verticillium chamydosporium	Sand /barley bran mixture	Nematophagous	Bourne and Kerry, 1999
Hirsutella rhossiliensis	Corn grits	Nematophagous	Chen and Liu, 2005
Trichoderma harzianum	Sugar cane bagasse	Fungal antagonist	Roussos et al., 1991
Paecilomyces lilacinus	Coffee husk	Nematophagous	Brand et al., 2003
Metarhizium anisopliae	Rice/sugarcane bagasse	Entomopathogenic	Arzumanov et al., 2005
Bacillus thuringiensis	Wheat	Insecticidal	Vimala Devi et al., 2005
Beauveria bassiana	Potato waste	Entomopathogenic	Soccol, et al., 1997

Mass production of spores must be achieved for BCAs commercialization purposes, though scale up is the one of the most important steps in its development. Different bioreactors have been designed to overcome the problems that a SSF problems may pose and they may be employed for the production of fungal biopesticides.

2.5.3. SSF Bioreactors

The bioreactors employed in SSF processes give the environment for the growth and activity of microorganisms (Pandey., 1991). SSF bioreactors are subject of several studies for the production of biopesticides, metabolites and mainly oriental food, which envisage the industrial production of such products (Jones, E.E. et al., 2004; Pandey et al., 2001; Larena et al., 2004; Saucedo-Castaneda et al., 1992). The main problems in the scale up of SSF bioprocesses are: variations of the formed biomass, the development of great quantities of inoculum, the sterilization of the substrate, heat generation from microorganisms metabolism and in consequence the agitation, aeration, pH control and others (Lonsane at al., 1992). The factors that affect the design of SSF bioreactors are: the substrates or supports used considering their size, mechanic resistance; the oxygen transfer which has a direct influence in the temperature and the water retention capacity of the solid media; the gaseous phase that is between the particles of substrate or support; the morphology of the micoorganisms and the need of a sterilization process (Durand, 2003, Raghavarao, et al., 2003).

SSF bioreactors may be classified in accordance to the quantity of susbtrate utilized in the process and they are divided in two categories: laboratory scale (g-kg) and pilot and industrial scale(kg-ton). Another type of classification is based on the design of SSF fermenters which may present or not agitation and or aeration devices. Laboratory scale bioreactor comprises simple devices such as Petri dishes, Erlenmeyer flasks, jars and Roux bottles, that are mainly utilized for screening of microorganisms and substrates, altough in this type of bioreactors, aeration and agitation controls are not possible. Autoclavable plastic bags are also useful and commonly used for the production of fungal inoculum, it was utilized for the production of *Pochonia chlamidosporia*, a nematode parasitic fungus, in substrates consisted of rice and corn grains (Wang et al., 2005).

Column type bioreactors, are well studied and may provide on line information of the microorganisms respiration, they are mainly utilized for optimization studies (Raimbault and Alazard, 1980). This bioreactor served as model for the construction of several others as aeration can be controled and cool the substrate by evaporation, and also heat generation can be minimized by convection by the shape of the columns and heat exchange through glass walls with thermostatized water bath. Barranco-Florido et al., (2002) utilized this type of bioreactor for the selection of strains of *Verticillium lecanii*, an entomopathogenic fungus, in sugarcane pith bagasse impregnated with mineral media and cuticle of *Sphenarium*

purpurascens for induction of proteases and chitinases. Conidia production of *Beauveria* sp for the control of mate caterpillars were also investigated in column bioreactors (Santa et al., 2004), also the influence of aeration and moisture content in the sporulation of *Metarhizium anisopliae* var. *acridum* were assayed (Arzumanov et al., 2005).

Zymotis, a large scale fermenter, is composed of heat exchanger plates, has capacity for until 12 kg of dry substrate can control temperature, moisture and aeration during fermentation, was utilized for the production of cellulases by *Trichoderma harzianum* (Roussos, et al., 1993) and for the production of fungal biopesticide (Roussos, et al., 2000).

When passing to pilot and industrial scale reactors, several process characteristics must be considered: difficulties in heat removal, compactation of solid media, microorganisms properties as resistance to agitation and oxygen demand, necessity of substrate pretreatment and handling. Based in this characteristics fermenter can fall in the following categories: unmixed,intermitently mixed and continously mixed reactors with or without air cirulation. Tray fermenters are extensively used in industries and show less inconvenients to scale up. They can be built in wood, metal, plastic and may show or not perforations. They are ussualy placed in thermostated rooms and though require large incubation area, being difficult to maintain a sterile process. Reactors based in continuous agitation comprehends rotating drum, perforated drum and horizontal paddle mixer. They were designed with the aim to increase contact between reactor wall and solid media and also to provide oxygen. However they present several disadvantages as agglomeration of substrate, difficulties in temperature regulation, low oxygen transfer and agitation may lead to substrate structure modification Presenting a similar device used in Zymotis reactor, to reduce heat and need of high air flux a bioreactor was patented by the German industry Prophyta, which indeed produces the strain PL-251, it presents a tower format containing perforated plates where heat exchangers are located at the bottom and substrate in the top and also through this plates occurs the passage of sterile air (Durand, 2003). One reactor patented by Durand et al (1997) was used for the production of fungal conidiospores for biological control, it has a planetary agitation device and capacity for 50l and controls of temperature and relative humidity as well as sterilization (De Vrije et al., 2001).

Scale up is still a bottleneck for the broad use of SSF in industrial processes, but the development of rational and computer controlled process in the last decades brought advances in SSF, including progress in the modelling of microbial growth on solid substrates and energy and mass transfer in different types of bioreactors. New methods for measuring SSF parameters as water content and biomass, the use of statistical tools for process optimization

were also developed (Oostra, J. et al., 2000; Holker and Lenz, 2005; Couto, and Sanroman, 2006; Chen et al., 2005).

2.6. References

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3. MATERIAL E MÉTODOS

3.1. Microorganismos

3.1.1. Cepas

Dois gêneros de fungos filamentosos, parasitas de ovos e fêmeas de nematóides das galhas, foram estudados para colocar em evidência suas capacidades de esporulação em resíduos da agroindústria brasileira e também sua atividade nematicida. As cepas utilizadas neste trabalho possuem origens distintas (Tabela 3.1).

TABELA 3.1. Nome e origem das cepas de fungos filamentosos nematófagos.

Nome dos fungos	Coleção de origem	Código de referência
Paecilomyces lilacinus	NRRL	LPB-Pl-01
Paecilomyces lilacinus	NRRL	LPB-Pl-02
Paecilomyces lilacinus	Iapar, Brasil	LPB-Pl-03
Verticillium chlamydosporium	Iapar, Brasil	LPB-Vc-01

NRRL – Northern Regional Research Laboratory - USDA, Iapar – Instituto Agronômico do Paraná, Brasil. LPB – Laboratório de Processos Biotecnológicos, UFPR, Brasil.

3.1.2. Conservação das cepas

As cepas de fungos nematófagos são cultivadas e conservadas em meio "Potato Dextrose Agar" (PDA), 39g/l (Sigma). 15 ml de meio são distribuídos em tubos de ensaio, fechados com algodão e esterilizados à 121°C durante 20 minutes. O meio esterilizado é resfriado em posição inclinada. As cepas são inoculadas com auxílio de uma alça de platina e incubadas durante 7 dias à 28°C. Elas são conservadas à + 4°C durante três meses.

3.2. Fisiologia de crescimento e esporulação de fungos nematófagos

3.2.1. Meios de cultura

3.2.1.1. Meio à base de extratos de batata

O meio PDA, 39 g/l, (Sigma) é utilizado para estudar a fisiologia de crescimento e de esporulação das cepas (determinação do modo de inoculação, crescimento radial e produção de biomassa e de esporos).

O meio PDB (Potato Dextrose Broth) 24 g/l (Sigma) é utilizado para a produção de esporos em FES utilizando-se o bagaço de cana-de-açúcar como suporte sólido.

Os meios PDA e PDB são compostos de: 20g/l de glucose e 4 g/l de extrato de batata e 17 g/l de ágar para o PDA.

3.2.1.2. Meio à base de Extrato de Malte

O meio MEA (Malt Extrait Agar), 33,6 g/l (Sigma) é utilizado para os estudos de crescimento radial e produção de biomassa.

Ele é composto de: 12,75 g/l de maltose, 2,75 g/l de dextrina, 2,35 g/l de gliceroll, 0,78 g/l de peptona e 15 g/l de ágar.

3.2.1.3. Meio à base de Extrato de casca de café (CHA)

O meio CHA é preparado da seguinte maneira: 100 g de casca de café com uma granulometria inferior à 0,8 mm são pesados e transferidos para frasco Erlenmeyer de 2 l, adiciona-se 1000 l de água destilada. Esta mistura é colocada em autoclave à 100ºC, durante uma hora. Esta suspensão é filtrada e o volume é completado à 1 l. 20 g/l de ágar bacteriológico (Biobras) é adicionado. O pH do meio é ajustado à 4,5. O meio CHA é esterilizado à 121ºC durante 20 minutos.

3.2.2. Preparação da suspensão de esporos

A suspensão de esporos é preparada adicionando-se 5 mL de água destilada contendo Tween 80 (0,01 g/l) em tubos de ensaio contendo as cepas de fungos filamentosos nematófagos, raspando-se com auxílio de uma alça de platina para remover os esporos.

3.2.3. Determinação do modo de inoculação

As três cepas de *P. lilacinus* foram escolhidas para esta experiência que tem como objetivo determinar o modo de inoculação mais eficaz em termos de rendimento de esporos para o preparo de inóculos. Dois modos de inoculação foram testados: a inoculação em superfície e em profundidade dos meios gelosados.

Após preparar o meio PDA, 50 ml são introduzidos em frascos Erlenmeyer de 250 ml e esterilizados à 121°C durante 20 minutes. A inoculação em profundidade é feita com 1 ml da suspensão de esporos (§3.2.2.) com auxílio de uma pipeta. Os frascos Erlenmeyer são agitados e deixados à temperatura ambiente para que o ágar se solidifique.

A inoculação em superfície (após a solidificação do ágar) é realizada com 0,2 ml da suspensão de esporos e com auxílio de uma alça de Drigalski a suspensão de esporos é distribuída sobre a superfície do ágar estéril. A incubação das culturas é mantida a 28°C durante 11 dias.

3.2.4. Produção de esporos de fungos nematófagos

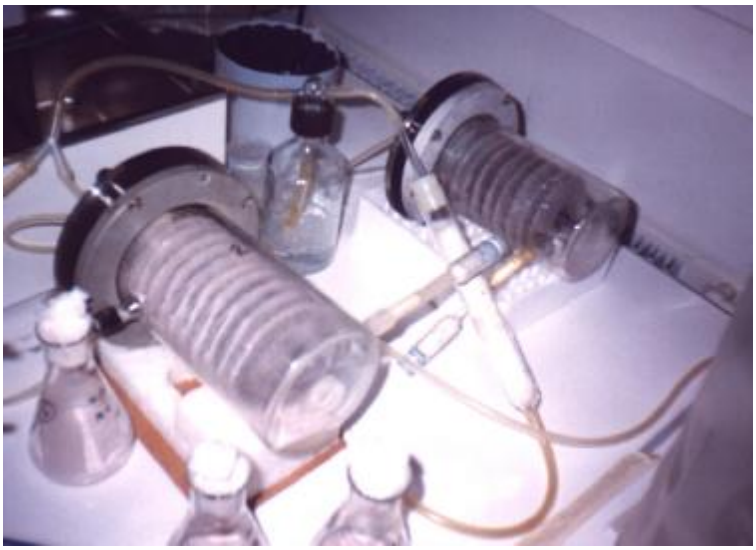
Dois dispositivos foram comparados para a produção de esporos de fungos nematófagos:

- Erlenmeyers de 250 ml;
- Fermentador à discos.

3.2.4.1. Descrição do Fermentador de discos

O Fermentador de discos é constituído de uma cuba de vidro de 25 cm de comprimento e um diâmetro interno de 9,2 cm com uma capacidade total de 1,5 l. Na cuba, existe uma entrada na parte alta e uma saída na parte baixa. Ele é equipado com 10 discos de 5 cm de diâmetro dispostos sobre um eixo, desenvolvendo uma superfície útil de 127 cm^2. Os discos são constituídos de duas grelhas de aço inoxidável de 2 mm de espessura e malha de 4 mm, o espaçamento entre os discos é de 10 mm. Eles são separados por anéis colocados no eixo central entre cada disco. O último anel do eixo central é utilizado para fixar cada disco. A cuba de vidro é fechada na parte superior com uma placa de aço inoxidável que possui três entradas e saídas fixas (Roussos, 1985). (Figura 3.1)

FIGURA 3.1. Dois fermentadores de discos utilizados para a produção de esporos de *P. lilacinus* Pl-1.



3.2.4.2. Funcionamento do Fermentador a discos

O meio PDA (600 ml) é esterilizado à 121°C durante 20 minutos no fermentador e resfriado à 40° C com auxílio da passagem de ar comprimido por uma entrada da placa e rotação dos discos. O meio é inoculado de maneira estéril com uma suspensão de esporos (2,0 x 10^7 esporos/g de substrato carbonado). Coloca-se uma aeração importante e uma rotação lenta dos discos para se permitir uma distribuição uniforme do meio inoculado sobre os discos. Quando o meio inoculado ainda está líquido, 8 amostras de aproximadamente 25 g de meio cada uma são retiradas e introduzidas de maneira estéril em Erlenmeyers de 250 ml previamente esterilizados para comparar a produção de esporos nos dois dispositivos. O excedente de meio é retirado pela parte baixa da cuba. A aeração e a agitação são mantidas até a completa solidificação do meio. O fermentador de discos e os frascos Erlenmeyer são incubados a temperatura ambiente (18-24°C).

3.2.4.3. Coleta dos esporos e determinação da concentração do inóculo

Após o período de incubação, 30 ml de água destilada estéril contendo Tween 80 e uma barra magnética são introduzidas nos Erlenmeyers, e 250 ml no fermentador a discos. Os Erlenmeyers e os discos do fermentador são agitados durante 10 minutos. As suspensões de esporos são recuparadas em Erlenmeyers estéreis. 1 ml da suspensão de esporos é depositada sobre uma célula de Malassez para o cálculo do número de esporos produzidos pelas cepas testadas. Em alguns casos, uma diluição adequada da suspensão de esporos é realizada para se

poder contar entre 30 e 100 esporos por unidade de contagem sob microscópio (Leica DMLS). Todas as medidas foram realizadas em triplicata a partir da mesma solução mãe de esporos. A concentração de esporos do inóculo (esporos/ml) é determinada pela seguinte fórmula.

Concentração de esporos do inóculo = média do número de esporos * 10^5 /(diluição)

3.2.5. Crescimento radial e biomassa de fungos nematófagos

Para estudar a fisiologia de crescimento de fungos nematófagos três meios de cultura foram utilizados (PDA, MEA e CHA). Os meios PDA e MEA são preparados, esterilizados e distribuídos em placas de Petri (52 cm de diâmetro) à razão de 5 ml/placa. Para medir a biomassa, um filme de celofane estéril (condição úmida) é colocado sobre o meio aproximadamente 16 horas antes da inoculação para permitir uma absorção do ágar pelo filme. As placas de Petri devem ser mantidas em posição inclinada para evitar a condensação de água e a possibilidade de contaminação. A inoculação é realizada com auxílio de uma alça, os esporos são depositados no centro de cada placa de Petri, contendo uma folha de papel celofane. As placas são incubadas a 28°C durante 7 dias. O diâmetro das colônias é medido com auxílio de uma régua graduada. O crescimento radial representa a distância percorrida sobre a superfície do meio de cultura pelo micélio, expressa em mm/h. (Sobal, 2002).

A biomassa é estimada depositando-se a folha de papel celofane contendo o micélio num recipiente previamente tarado. Após a secagem à 105°C durante 24 horas o peso seco da biomassa é medido e expresso em μg/h.

Para estudar a velocidade de crescimento de fungos em meio CHA uma outra estratégia é utilizada. O meio CHA é distribuído em placas de Petri estéreis de 75 mm de diâmetro contendo 20 ml/placa. Após a solidificação do meio uma gota de suspensão de esporos de cada cepa de *Paecilomyces* e de *Verticillium*, é colocada no centro da placa. As placas são incubadas à 28ºC. O diâmetro de cada colônia é medido com auxílio de uma régua graduada, até que um dos raios do micélio atinja as bordas da placa. A biomassa produzida na superfície do meio gelosado é medida pela dissolução do ágar em banho maria e separação da biomassa por uma filtração em papel de filtro previamente tarado. A biomassa é seca à 105°C durante 24 horas, resfriada em dessecador, pesada e expressa em μg/h. As experiências são realizadas

em duplicatas. Em todos os casos as cinéticas foram realizadas com uma amostragem de 2 placas por meio de cultura.

3.3. Substratos

3.3.1. Origem

As cascas de café provêm da indústria torrefadora Café Damasco, situada em Curitiba, Brasil. Café Damasco utiliza a variedade *Coffea arabica* cultivada na região noroeste de estado do Paraná, e utiliza a via seca para a obtenção dos grãos verdes.

O bagaço de mandioca provém da Agroindustrial Paranaense De Polvilho Ltda- Paranavaí-PR, Brasil.

O farelo de soja foi fornecido pelo CEPPA (Centre de Pesquisa e Processamento de Alimentos) em Curitiba, Brasil.

O Bagaço de cana-de-açúcar foi adquirido num centro de produção de caldo de cana (Rei da Cana, Curitiba, Brasil), ele é lavado várias vezes para retirar o açúcar remanescente.

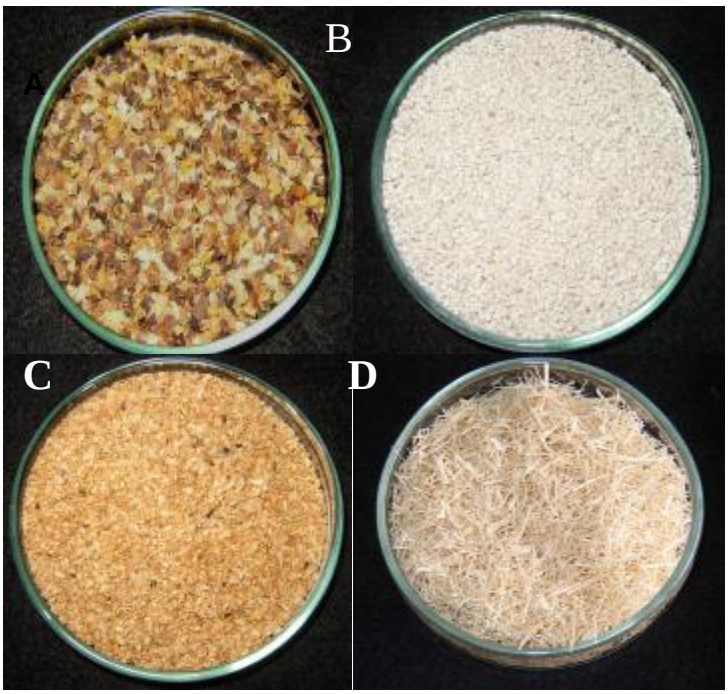
Os resíduos de camarão foram recuperados num centro de distribuição de camarões “limpos” em Curitiba, Brasil.

3.3.2. Pré-tratamento dos substratos

Primeiramente, os substratos são secos em estufa de bandejas com circulação de ar à 55ºC, durante 48 horas, até atingir uma umidade de aproximadamente 8% à 10%.

Posteriormente, os substratos são moídos e selecionados. Três frações são obtidas. A primeira fração compreende os tamanhos superiores a 2 mm, o segundo entre 0,8 e 2,0 mm e o terceiro inferiores a 0,8mm. A fração entre 0,8 e 2,0 mm é utilizada para os estudos de FES (Figura 3.2).

FIGURA 3.2. Substratos tratados 0,8 – 2,0 mm: A) Casca de Café; B) Bagaço de mandioca; C) Farelo de soja, e D) Bagaço de cana-de-açúcar.



3.4. Fermentação no Estado Sólido (FES)

3.4.1. Produção de inóculo

O modo de inoculação em profundidade de meios gelosados foi escolhido para a produção de inóculo das quatro cepas de fungos nematófagos. A produção de inóculo é realizada em frasco Erlenmeyer de 250 ml, a partir de um tubo de ensaio contendo a cepa desejada. 50 ml de meio PDA esterilizado e resfriado a aproximadamente 40°C, são inoculados com 1 ml de uma suspensão de esporos. Os frascos Erlenmeyer são incubados a 28°C durante 7 dias.

3.4.2. Reatores utilizados para as FES

Três tipos de reatores foram utilizados para os estudos de fermentação no estado sólido: frascos de vidro, um dispositivo úmido e as colunas de Raimbault.

3.4.2.1. Frascos de vidro com aeração natural

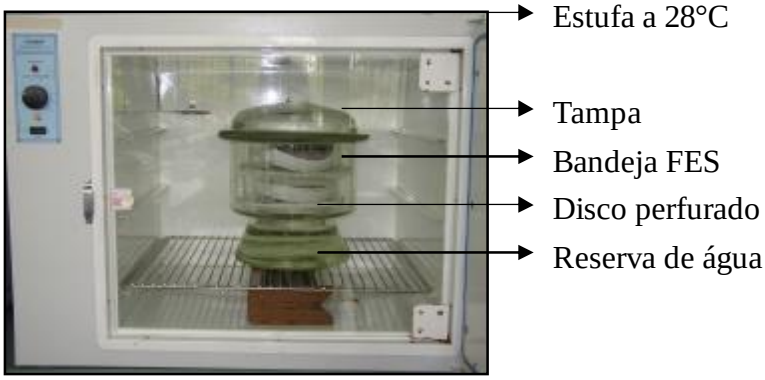
Os frascos de vidro (Figura 3.3), possuem uma capacidade de 500 g, a troca de ar é feita através de uma cobertura de papel de filtro. Os frascos são preenchidos com 20 g de substrato peso seco. Eles são utilizados para os estudos de seleção da cepa (§3.4.4), do substrato (§ 3.4.5), para a cinética de produção de esporos (§3.4.6) e para a capacidade nematicida das cepas (§3.8).

FIGURA 3.3. Frascos de vidro com substrato fermentado.

3.4.2.2. Dispositivo úmido com aeração natural

O dispositivo úmido (Figura 3.4) consiste em uma grande cuba de vidro fechada na parte superior onde se encontra uma abertura que permite as trocas de gás através de algodão. Possui uma capacidade de 8l, diâmetro interno e altura de 25 cm e de 22 cm respectivamente. No fundo do reator adiciona-se 1l de água para saturar o ar em umidade. Três bandejas, de um diâmetro de 15 cm e altura de 3 cm, com uma capacidade de 100 g de substrato peso seco cada são colocadas no interior do reator. Elas são separadas por discos perfurados situados a 7 cm um do outro. Este dispositivo é utilizado para verificar a possibilidade de extrapolação da produção de esporos de ***P. lilacinus*** Pl-01 em um substrato constituído de 50% casca de café e 50% de farelo de soja (§3.4.7)

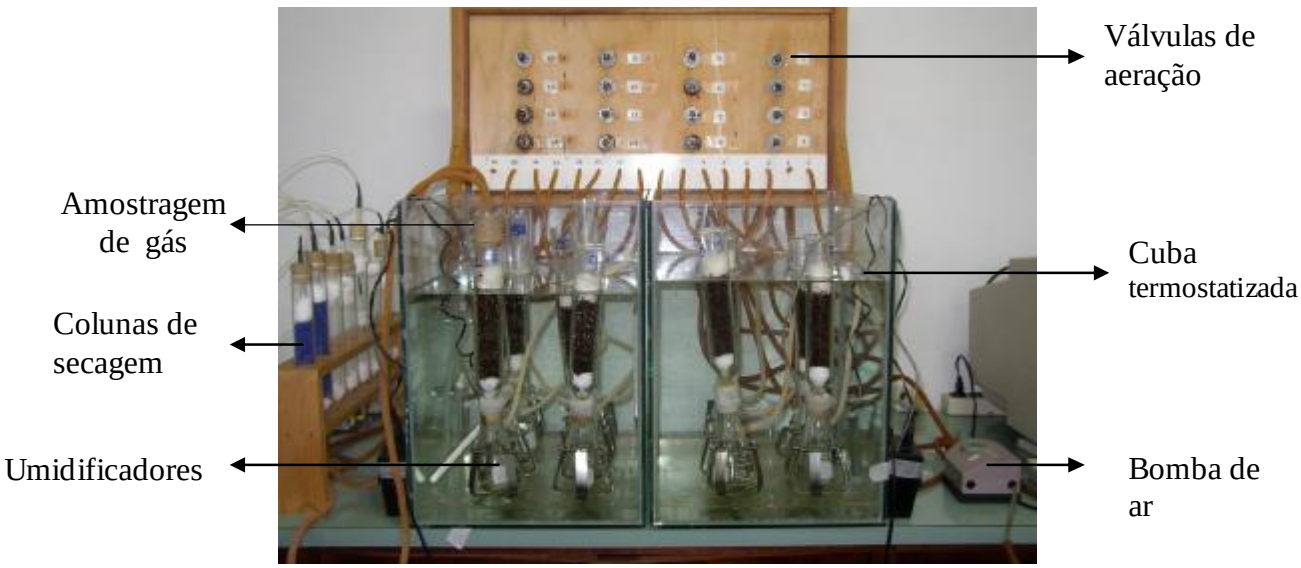
FIGURA 3.4. Dispositivo utilizado para a produção de esporos de fungos nematófagos cultivados em FES.



3.4.2.3. Colunas de Raimbault com aeração forçada

Foi empregado um equipamento específico para a FES em colunas de baixa capacidade, acoplado a um sistema de aeração e temperatura, semelhante ao desenvolvido por Raimbault e Alazard (1980). Os biorreatores são constituídos por colunas de vidro de 4cm de diâmetro e 20 cm de comprimento, com volume útil é de aproximadamente 250 ml. Uma vez preenchidas com o substrato estéril, as colunas são montadas sobre um umidificador e introduzidas em um banho-maria termostatizado com temperatura controlada. O controle de fluxo de ar saturado em água é feito por intermédio de microválvulas. O equipamento é composto de duas cubas de vidro, que permitem a instalação de 8 colunas em cada cuba, que podem ser retiradas individualmente, sem provocar perturbações às outras colunas, conforme demonstrado na Figura 3.5.

FIGURA 3.5. Dispositivo FES de laboratório com duas cubas termostatizadas que podem reunir 8 colunas de Raimbault.



Este tipo de reator é utilizado para estudar a fisiologia de esporulação da cepa de *P. lilacinus* Pl-01 com aeração controlada (§3.4.8). Ele permite também de estudar continuamente a respirometria, consumo de O_2 e produção de CO_2.

3.4.3. Esterilização dos substratos

Para as FES realizadas em frascos de vidro, 20 g de substrato peso seco são colocados no reator e adicionados de 20 ml de água destilada. Eles são fechados com papel de filtro e esterilizados durante 20 min em autoclave à 121°C.

Para as FES conduzidas em dispositivo úmido e em colunas de Raimbault, o substrato é pesado em um recipiente de vidro e a mesma quantidade de água em peso é adicionada para a esterilização (121°C, 20 minutes) em autoclave.

3.4.4. Seleção da cepa de fungo nematófago em reator tipo frasco de vidro

Num primeiro momento, as fermentações são realizadas com as quatro cepas de fungos nematófagos, *P. lilacinus* Pl-1, Pl-2 e Pl-3 e *V. chlamidosporium* Vc-1 utilizando-se a casca de café como substrato. Após esterilização (§3.4.3), os frascos são inoculados com 2,0 x 10^7 esporos/g de substrato seco, adicionados da quantidade de água necessária para atingir a umidade desejada. Os parâmetros físicos (pH, temperatura de incubação e umidade inicial do substrato) são testados com objetivo de otimizar a produção de esporos por FES pelos fungos nematófagos. A Tabela 3.2. mostra os fatores e os níveis testados através de uma planificação experimental 2^{3-1} tratada pelo software STATISTICA 5.0. Os ensaios são realizados em duplicata.

TABELA 3.2. Fatores físicos de fermentação e níveis testados.

Nível Fatores	- 1	0	+ 1
Umidade inicial (%)	60	65	70
Temperatura (º C)	26	28	30
pH	3,5	4,5	5,5

Para selecionar a cepa com a melhor atividade nematicida contra o nematóide *Meloidogyne incognita* Raça 1 os ensaios em vasos são realizados (§3.8) com adição dos produtos obtidos por FES em casca de café em condições otimizadas (umidade de 65%, pH 4,5 e temperatura 28°C). O índice de esporulação (§3.5.1), umidade (§3.5.2) e pH (§3.5.3) final dos produtos obtidos por FES são determinados.

3.4.5. Seleção do substrato para a produção de esporos de *P.lilacinus* LPB-Pl-01

Vários substratos são testados para a produção de um biocomposto contendo esporos viáveis e virulentos da cepa de *P. lilacinus* LPB-Pl-01, contra o nematóide *M. incognita* raça 1. Estes substratos são escolhido de acordo com suas composições (presença de proteínas, quitina) ou para melhorar a composição de substratos previamente testados. O bagaço de mandioca, o farelo de soja, as cascas de café e os resíduos de camarão são utilizados como substrato e o bagaço de cana-de-açúcar é utilizado como suporte.

3.4.5.1. Bagaço de mandioca e Resíduos de camarão

Os resíduos de camarão, ricos em quitina, misturados com o bagaço de mandioca (1:2 p/p) são utilizados como substrato com o objetivo de induzir a virulência dos esporos da cepa de fungo filamentoso nematófago Pl-01. Duas planificações experimentais são realizadas para otimizar a produção de esporos da cepa Pl-01 neste substrato. Os fatores testados na primeira planificação são demonstrados na tabela 3.3.

TABELA 3.3. Modelo 2^{3-1} para otimizar a produção de esporos da cepa Pl-01 em resíduos de camarão e bagaço de mandioca (1:2 p/p).

Fatores	pH	Umidade inicial (%)	Taxa de inoculação (ml 10^7/g ss)
1	5.5	75	3.0
2	5.5	65	1.0
3	4.5	75	1.0
4	4.5	65	3.0
5	5.0	70	2.0

ss = substrato seco

Através de uma segunda planificação 3^2, somente foram testados os fatores que apresentaram uma influência significativa na primeira. Os fatores testados são pH (4.0, 4.5 e 5.0) e a umidade inicial (64, 67 e 70%). Estas experiências são realizadas emduplicata.

Com as condições otimizadas (pH 4,0, umidade de 67%, temperatura de 28°C e taxa de inoculação 2,0 x 10^7 esporos/g de substrato seco), uma cinética de produção de esporos foi realizada durante 15 dias. As amostras (dois frascos por dia) são retiradas todos os dias para a determinação do índice de esporulação (§3.5.1), da umidade (§3.5.2) e do pH (§3.5.3). O produto obtido por FES com as condições otimizadas de cultura é testado para verificar a atividade em material vegetal (§3.8).

3.4.5.2. Bagaço de mandioca e casca de café

A mistura de bagaço de mandioca mais casca de café em diferentes concentrações (Tabela 3.4) é testada com objetivo de aumentar a relação C/N e verificar a influência desta relação na produção de esporos de *P. lilacinus* Pl-01. As FES são realizadas a 28°C, umidade de 65% e taxa de inoculação de 2,0 x 10^7 esporos/g de substrato seco durante 10 dias de incubação. O índice de esporulação (§3.5.1), a umidade (§3.5.2) e o pH (§3.5.3) final dos produtos obtidos por FES são determinados. Cada produto é testado para sua atividade nematicida em material vegetal (§3.8).

TABELA 3.4. Diferentes concentrações de casca de café e bagaço de mandioca testados para a produção de esporos de *P. lilacinus* Pl-01.

Ensaios	% Cascas de café	% Bagaçode mandioca
1	100	0
2	75	25
3	60	40
4	15	85
5	0	100

3.4.5.3. Farelo de soja, cascas de café e bagaço de cana-de-açúcar

Em uma outra experiência, os substratos são constituídos de farelo de soja, casca de café e de mistura de bagaço de cana-de-açúcar (Tabela 3.5). As FES são realizadas a 28°C, umidade de 65% e taxa de inoculação de 2,0 x 10^7 esporos/g de substrato seco durante 10 dias de incubação. O índice de esporulação (§3.5.1), a umidade (§3.5.2) e o pH (§3.5.3) final dos produtos obtidos por FES são determinados. Cada produto é testado para sua atividade nematicida em material vegetal (§3.8).

TABELA 3.5. Diferentes concentrações de casca de café, farelo de soja e de bagaço de cana-de-açúcar testados para a produção de esporos de *P. lilacinus* Pl-01.

Ensaios	% Cascas de café	% Farelo de soja	% Bagaço de cana-de-açúcar
1	100	0	0
2	50	50	0
3	83	0	17
4	0	83	17
5	0	100	0

3.4.5.4. Farelo de soja, cascas de café

A mistura de farelo de soja com cascas de café é testada em diferentes concentrações (Tabela 3.6) para a produção de esporos de *P. lilacinus* Pl-01. As FESsão realizadas à 28°C, umidade de 67% e taxa de inoculação de 2,0 x 10^7 esporos/g de substrato seco durante 10 dias de incubação. O índice de esporulação (§3.5.1), a umidade (§3.5.2) e o pH (§3.5.3) final dos produtos obtidos por FES são determinados. Cada produto é testado para sua atividade nematicida em material vegetal (§3.8).

TABELA 3.6. Diferentes concentrações de cascas de café e farelo de soja testados para a produção de esporos de *P. lilacinus* Pl-01.

Ensaios	% Cascas de café	% Farelo de soja
1	100	0
2	0	100
3	50	50
4	75	25
5	25	75

3.4.6. Cinética de esporulação da cepa *P. lilacinus* Pl-01

A cinética é realizada em um substrato constituído por 50% de cascas de café e 50% de farelo de soja. A produção de esporos da cepa de *P. lilacinus* Pl-01 foi acompanhada durante 12 dias de incubação à 28°C. A umidade inicial é de 67%, o pH de 4,5 e a taxa de inoculação de 2,0 x 10^7 esporos/g de substrato seco. Dois frascos são retirados cada dia para verificar a produção de esporos (§3.5.1), o pH (§3.5.2) e a umidade (§3.5.3) do produto obtido por FES.

3.4.7. Extrapolação em dispositivo úmido

A mistura de dois substratos (cascas de café 50% e farelo de soja 50%) é pesada em um recipiente, a adição de água e a esterilização são realizadas como descrito anteriormente (§3.4.3.). Após inoculação do substrato, a quantidade de cada recipiente contendo respectivamente 50, 75 e 100g de substrato seco é transferida para as bandejas, que são colocadas no interior do dispositivo, com 1l de água estéril no fundo para a saturação do ar e fechada na parte superior com algodão para permitir as trocas de gás. A espessura do substrato nas bandejas para os pesos de 50, 75 e 100 g (matéria seca) é respectivamente de 1,4; 2,1 e 2,8 cm.

Após 8 dias de incubação a 28°C, as bandejas são retiradas do dispositivo e colocadas em fluxo laminar em condições assépticas. Após homogeneização apropriada, a contagem de esporos (§3.5.1) foi realizada utilizando-se uma câmara de Malassez. Após, os produtos são secos por passagem de ar seco (§3.6) e a viabilidade dos esporos é determinada (§3.7).

3.4.8. Fisiologia da esporulação em reator FES tipo coluna

3.4.8.1. Preparo das colunas de fermentação

As colunas de Raimbault, e os umidificadores são esterilizados a 121ºC durante 20 minutos. A inoculação do substrato estéril é realizada em recipientes de vidro e após uma intensa homogeneização, as colunas são preenchidas (40g/coluna), assepticamente em câmara de fluxo laminar. Cada coluna é pesada antes e após preenchimento com o substrato e colocadas sobre os umidificadores, contendo aproximadamente 150 ml de água destilada, para umidificar o ar antes dele passar pelo reator. As colunas são introduzidas nas cubas de FES equipadas com regulação de temperatura e fluxo de ar. A água das cubas à 28ºC é ajustada com auxílio de um termostato. Cada coluna é ligada a uma válvula de ar que permite de ajustar o fluxo de ar ao valor desejado (0-120 ml/min), controlado através de um aerômetro (OMEL) localizado na saída de cada coluna.

3.4.8.2. Otimização da umidade inicial e da aeração

Um modelo 3^2 (Tabela 3.7) gerado pelo programa STATISTICA 5.0, é utilizado para otimizar os parâmetros umidade inicial e fluxo de ar com o objetivo de aumentar a produção de esporos da cepa de *P. lilacinus* Pl-01 em biorreator tipo colunas de Raimbault. A taxa de inoculação é de 2,0 x 10^7 esporos/g de substrato seco. As experiências são realizadas em duplicata, resultando num total de 20 colunas. A produção de esporos (§3.5.1), a umidade final (§3.5.2) e o pH (§3.5.3) são determinados após 10 dias de incubação à 28°C.

TABELA 3.7. Modelo 3^2 para a otimização dos fatores umidade inicial e aeração para a produção de esporos da cepa Pl-1 em colunas de Raimbault.

Ensaio	Umidade inicial (%)	Fluxo de ar (ml/min/coluna)
1	63	30
2	63	60
3	63	90
4	65	30
5	65	60
6	65	90
7	67	30
8	67	60
9	67	90
10 (C)	65	60

3.4.8.3.Cinética de produção de esporos

A FES é realizada com as condições otimizadas de cultura: pH 4,5, umidade inicial de 65% e fluxo de ar de 60 ml/min/coluna. A cinética de produção de esporos e a respirometria (§3.5.5), são acompanhadas durante 10 dias de incubação a 28°C. Cada dia duas colunas são retiradas para a determinação da produção de esporos (§3.5.1), da umidade (§3.5.2), do pH (§3.5.3), dos açúcares totais (§3.5.4), e da biomassa (§3.5.6).

3.5. Técnicas de Análise

3.5.1. Contagem de esporos e Índice de esporulação

A concentração de esporos nos produtos obtidos por FES foi analisada em 1 g de substrato úmido adicionado de 30 ml de água destilada e três gotas de Tween 80. Esta suspensão é agitada com uma barra magnética durante 30 minutos para separar os esporos do meio de cultura. Após diluições sucessivas os esporos foram contados em câmara de Malassez sob o microscópio (Leica DMLS). 1 ml da diluição conveniente foi colocada na câmara de Malassez para o cálculo de número de esporos da cepa testada. O índice de esporulação (IS) representa o número de esporos produzidos por grama de matéria seca (Roussos, 1985). Este índice é calculado pela seguinte fórmula:

Índice de esporulação = média do número de esporos * 10^5 * 30/(diluição * g matéria seca)

3.5.2. Umidade

Entre 5 e 10 g de amostra seca são pesadas em placas de Petri previamente taradas em balança de precisão. A amostra é seca durante 24 horas à 105ºC, resfriada em dessecador e pesada novamente. A perda em água da amostra permite de expressar a matéria seca (quantidade de sólidos por 100 g de amostra fresca) ou a quantidade de água (massa de água contida em 100 g de amostra fresca). O peso seco é calculado pela seguinte equação e é expresso em porcentagem de matéria seca ou em porcentagem de umidade:

Umidade, g % MS =(A x 100) / P

Ou:

A = diferença entre o peso da amostra úmida e peso da amostra seca,

P = peso da amostra úmida,

MS = matéria seca

3.5.3. Medida do pH

A determinação do pH é efetuada diretamente em uma suspensão obtida com o produto obtido por FES em água destilada (2 g/20 ml). A medida é realizada por meio de um pHmetro digital munido de um eletrodo combinado. A calibração do aparelho é realizada com soluções tampões de pH 4,0 e pH 7,0.

3.5.4. Dosagem de açúcares totais

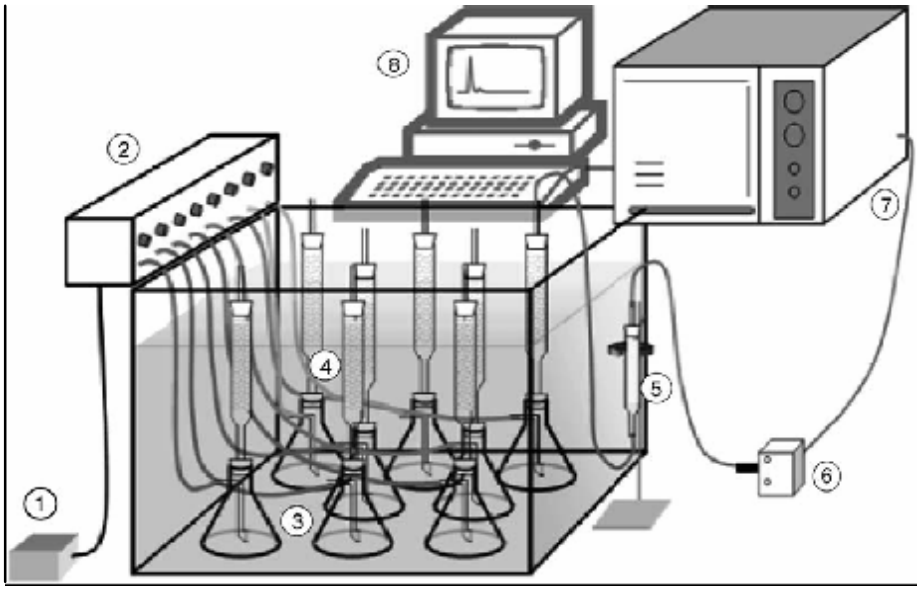
Os açúcares totais são dosados pelo método de Somogyi-Nelson (Nelson, 1944; Somogyi, 1945) após hidrólise ácida. 1 g de substrato ou de produto é pesado em frasco Erlenmeyer de 250 ml, 50 ml de água destilada e 1 ml de HCl concentrado são adicionados. Os frascos são colocados em banho-maria a 100°C durante 20 minutos. Depois o pH é neutralizado com uma solução de NaOH (3M e 1M). O volume é completado à 100 ml em balão volumétrico e a suspensão é homogeneizada e filtrada. A amostra a ser dosada é convenientemente diluída de modo a obter uma concentração em açúcares de 100 mg/l. A 1 ml desta diluição, adiciona-se 1 ml do reativo de Somogyi. A mistura é incubada em banho-maria à 100°C durante 10 minutos e depois resfriada. Adiciona-se 1 ml do reativo de Nelson. A mistura ainda é homogeneizada e finalmente se adiciona 7 ml de água destilada. A densidade ótica é lida à 535 nm. A curva padrão é feita com glicose (20-200mg/l)

3.5.5. Respirometria

Para estudar o metabolismo respiratório da cepa de *Paecilomyces lilacinus* LPB-PL-01, o consumo de oxigênio e a produção de CO_2, são analisados em linha (Figura 3.6). Esta técnica de análise dos efluentes gasosos na saída de cada biorreator é utilizada para estimar indiretamente a biomassa do microorganismo e permite também monitorar as diferentes fases de crescimento sem sacrificar as colunas.

Os efluentes gasosos que saem do reator tipo coluna são desumidificados pela passagem em colunas de sílica gel e analisados por cromatografia gasosa. A taxa de consumo de oxigênio (OUR) e o CO_2 produzido são determinados através de um Cromatógrafo gasoso (CG).

FIGURA 3.6. Esquema de análise automatizada de CO_2 e de O_2 durante a FES: 1. Bomba de ar; 2. Sistema de distribuição de ar; 3. Umidificadores; 4. Colunas de Raimbault em banho-maria; 5. Coluna de sílica gel; 6.Válvula automática de amostragem; 7. Cromatógrafo gasoso; 8. Computador com software para aquisição de dados.



O fermentador foi acoplado a um cromatógrafo (SHIMADZU –GC-8A), ligado a um computador (COMPAQ-XT486). Os dados foram armazenados pelo programa CHROMA, que é um integrador de cromatogramas. Ele recebe os sinais analógicos do detector do cromatógrafo e os converte em sinais numéricos. O cromatógrafo operou com um detector de condutividade térmica, um injetor automático e uma coluna Alltech CTR1, que consiste em uma coluna concêntrica dupla, com a parte externa formada de um filtro molecular de 5Aº e a parte interna de uma mistura de polímeros porosos (Poropack). Esta coluna permite a separação de oxigênio, nitrogênio, ar, metano, dióxido e monóxido de carbono em diferentes tempos de retenção. O cromatógrafo gasoso operou nas seguintes condições:

Temperatura do detector:	60ºC
Temperatura da coluna	60ºC
Fase gasosa:	Hélio
Fluxo de Hélio:	30 mL/min
Pressão de Hélio:	1 bar
Corrente do catarômetro:	120 mA
Volume de injeção:	300 µL
Gás para calibração:	Ar: CO_2(0,0) / O_2 (21,0) / N_2(79,0)
	Mistura 1: CO_2(5,0) / O_2(5,0) / N_2(90,0)
	Mistura 2:CO_2(10,0) / O_2(15,0) / N_2(75,0)

Nestas condições, o tempo de retenção de cada componente pode ser expresso como mostrado na Tabela 3.8.

TABELA 3.8. Tempos de retenção de alguns gases, monitorados em CPG.

Componente	Tempo de retenção (min)
Ar	0,62
CO_2	0,95
O_2	5,72
N_2	8,02

3.5.6. Determinação de biomassa através da dosagem de ergosterol

A medida da biomassa de fungos filamentosos, produzidas ao longo de FES em diferentes substratos, é realizada indiretamente, utilizando-se a análise de ergosterol, presente na parede celular de fungos por comparação com a concentração em ergosterol do microorganismo estudado, determinado em meio padrão (YM).

3.5.6.1. Preparo das amostras

3.5.6.1.1. Biomassa de P. lilacinus em meio YM

A composição do meio YM é a seguinte (glucose, 10 g; extrato de levedura, 3 g; extrato de malte, 3 g; peptona, 5 g; água q.s.p. 1l). O meio YM é inoculado com 2,0 x 10^7 esporos/ml e incubado à 28°C. Após 5, 7 e 10 dias de cultura, a biomassa é recuperada por filtração. Após lavagem da biomassa com água destilada, ela é seca à 50°C durante 16 horas. 0,3 g da biomassa seca é utilizada para a determinação do ergosterol.

3.5.6.1.2. Produtos obtidos por FES

Os produtos obtidos por FES utilizados para determinação de biomassa foram provenientes dos estudos cinéticos realizados em biorreator do tipo colunas, sendo também realizadas as determinações de produção de CO_2 e consumo de oxigênio pelo fungo *Paecilomyces lilacinus* PL-1. Foi utilizado 1 g de produto úmido em tempos determinados no decorrer do estudo cinético.

3.5.6.2. Saponificação das amostras para extração do ergosterol

As amostras foram pesadas em frascos de vidro com tampa. Adicionou-se 4 mL de álcool etílico absoluto e 2 mL de NaOH 2M, os frascos foram semi-fechados e colocados em banho-maria à 70º C durante 1 hora sob agitação manual periódica. Após este período de incubação adicionou-se 4 mL de HCl 1M à cada frasco e após 2 mL de solução 1 M de $KHCO_3$, posterior à adição de cada reagente as amostras foram agitadas

3.5.6.3. Extração do ergosterol

A extração foi realizada com n-hexano. Inicialmente foi adicionados aos frascos 4 mL de n-hexano, os quais foram então submetidos a uma agitação manual de 30 s. O conteúdo dos frasco foi passado para um tubo de centrífuga e então centrifugados a 4000 rpm durante 3 minutos. Reservou-se a fração de n-hexano em tubo de ensaio, ao restante foram adicionados novamente 4mL e posteriormente 2 mL de n-hexano para nova etapa de extração, centrifugação e recuperação de material extraído. Evaporou-se o solvente das amostras em dessecador à vácuo em estufa a 50º C. A cada tubo foi então adicionado 500 µL de n-hexano para determinação do conteúdo de ergosterol das amostras. As amostras foram filtradas em membranas de PVDF.

3.5.6.4. Dosagem de ergosterol por HPLC

A determinação de ergosterol dos extratos foi realizada por cromatografia líquida de alta eficiência. Utilizou-se um cromatógrafo Varian ProStar, com coluna C_{18} e detetor PDA (matriz de fotodiodos) regulado a 282 nm. As condições de eluição do analito foram desenvolvidas por Carvalho et al. 2005.

Utilizou-se 10 µL de amostra. A fase móvel utilizada foi metanol puro (de 0 a 3 min), acetonitrila pura de 3 a 10 minutos, e metanol puro de 10 a 15 minutos com vazão de 2mL/min, para eluir outros componentes presentes na amostra. O tempo de retenção obtido foi de aproximadamente 12 minutos. Como amostra padrão foi utilizada uma solução de ergosterol PA a 10000µg/mL , com diluições a 5000, 2000 e 1000 µg/mL. A curva padrão obtida foi em (µg/mL): y = 55585x + 9E+06, sendo que y corresponde à área e x à concentração de ergosterol, com regressão linear igual a 0,999.

3.5.6.5. Determinação da recuperação de ergosterol nas amostras

A uma amostra de fermentado com conteúdo de ergosterol conhecido, adicionou-se 0,3 g de ergosterol P.A.. Esta amostra dopada foi submetida às mesmas condições de saponificação,

extração e determinação de ergosterol utilizada para as amostras em análise. Determinou-se então que a recuperação de ergosterol foi de 49,3%.

3.6. Secagem dos produtos obtidos por FES

Os estudos de secagem foram realizados com circulação de ar a temperatura ambiente (entre 20 e 26ºC), e à temperatura controlada (28°C, 32 e 35°C). Em condições assépticas, o produto obtido por FES (10 g base úmida) é colocado em colunas de vidro, entre dois pedaços de algodão, que serviu como agente de esterilização para a passagem do ar. A bomba de ar estava conectada a uma coluna de sílica gel que por sua vez estava conectada a cada coluna contendo os produtos a secar durante o tempo necessário para se obter uma umidade entre 5 e 10%.

3.7. Viabilidade dos produtos secos

A determinação da viabilidade dos esporos de fungos nematófagos nos produtos obtidos por FES secos é realizada em meio PDA. Primeiramente, pesou-se 1g de fermentado em frasco Erlenmeyer de 250 mL contendo 100 mL de água destilada estéril, 3 gotas de Tween 80 e fragmentos de porcelana para facilitar a dissolução do material fermentado e conseqüente liberação dos esporos. O frasco Erlenmeyer foi deixado por 30 minutos a 150 rpm. Após 30 minutos foram feitas diluições sucessivas até 10^{-9} para verificação da viabilidade. Plaqueou-se 1 mL das diluições 10^{-7} até 10^{-9} em triplicata e adicionou-se 20 mL de meio PDA (pour plate) à aproximadamente 40ºC. As placas foram incubadas à 28º C por 4 dias quando se verificou o número de colônias do fungo nas respectivas diluições. Os resultados foram comparados com as amostras de produtos obtidos por FES não submetidos a secagem.

3.8. Teste de atividade em material vegetal

3.8.1. Nematóides e material vegetal

Os nematóides (isolados de plantações de café) foram colocados a nossa disposição pelo Instituto Agronômico do Paraná (IAPAR) situado em Londrina, Brasil. Os nematóides são mantidos e multiplicados em plantas de *Coleus blumei* e em plantas de tomate (*Lycopersicum esculentum* Mill cv. Rutgers) cultivados em solo Latossolo vermelho-amarelo (Oxisol).

Os grãos de tomate são semeados em pequenos vasos (250 g de solo) e mantidos à 25°C durante 10 dias para a germinação. Estacas da planta de *Coleus blumei* são cuidadosamente cortadas e selecionadas e posteriormente plantadas para promover o crescimento das raízes e mantidos em casa de vegetação. Após 3 semanas, as plantas são selecionadas para a realização das experiências em vasos e para a conservação e propagação dos inóculos de nematóide.

Os nematóides são repicados a cada 6 meses por renovação de 2/3 do solo e uma nova planta de *Coleus* ou de tomate. As raízes da planta infestada são utilizadas para preparar uma suspensão de nematóides que é utilizada nos experimentos em vasos e também para a multiplicação da população de nematóides.

3.8.2. Experimentos em vasos

Os experimentos foram realizados no Centro de Diagnóstico Marcos Enrietti da Secretaria do Estado e Abastecimento do Paraná localizado na Faculdade de Ciências Agrárias da Universidade Federal do Paraná. Vasos com capacidade de 3 kg de solo são utilizados, onde o solo a suspensão de nematóides e os produtos obtidos por FES são distribuídos de acordo com cada tratamento. Sempre se tomou cuidado de se utilizar as plantas que possuíam as mesmas características de dimensão, idade e número de folhas para tornar os experimentos mais homogêneos. Os vasos são distribuídos de maneira aleatória na mesa que é previamente desinfetada com solução de hipoclorito de sódio 5%. As experiências foram realizadas em casa de vegetação (Figura 3.7) sem controle de temperatura e iluminação particulares e submetidas a condições ambientais.

FIGURA 3.7. Casa de vegetação com vasos em começo de experimento.

3.8.3. Preparo da suspensão de nematóides

As suspensões de nematóides são preparadas a partir de raízes de tomates ou *Coleus* contaminadas (Figura 3.8.) com o nematóide das galhas *Meloidogyne incognita* Raça 1 que apresentava várias galhas e conseqüente massa de ovos, de acordo com a planta utilizada para cada experimento. As raízes são cortadas em pedaços de aproximadamente 2 cm e colocadas e um liquidificador (Walita modelo Roma) com água destilada. A suspensão é misturada 3 vezes durante 15 segundos. Os juvenis e os ovos de nematóides eram contados com auxílio de uma câmara de contagem (1 ml da suspensão) sob o microscópio (Zeiss, Standar 25), com o objetivo de e estabelecer a taxa de inoculação para cada tratamento.

FIGURA 3.8. Raiz de *Coleus* contaminadas com *M. incognita* raça 1.

3.8.4. Medida de efeito nematicida dos produtos obtidos por FES

Estes experimentos são baseados na atividade dos produtos obtidos por FES contra o nematóide *Meloidogyne incognita* raça 1. Após 3 meses de cultura, os experimentos são interrompidos. As plantas são cuidadosamente retiradas dos vasos e as raízes são lavadas para retirar o solo. As raízes são colocadas sob papel filtro para secar durante 1 hora a temperatura ambiente. Para cada planta duas amostras de 1 g de raiz são pesadas para a contagem de fêmeas em lupa binocular por dissecação direta da raiz (x 45) (Wild, M8). As amostras destinadas a contagem de fêmeas são armazenadas em frascos contendo solução de formol à 5%.

O cálculo para estimar a redução do número de fêmeas é realizado por comparação com o número de fêmeas presentes nas raízes de plantas infestadas que não receberam nenhum tratamento. O remanescente das raízes é colocado a 60°C durante 24 horas para a determinação do peso seco.

4. RESULTADOS E DISCUSSÃO

Parte 1: Fisiologia de Crescimento e de Esporulação de fungos nematófagos

4.1. Capítulo I: Crescimento Radial de Fungos Nematófagos e Produção de Biomassa

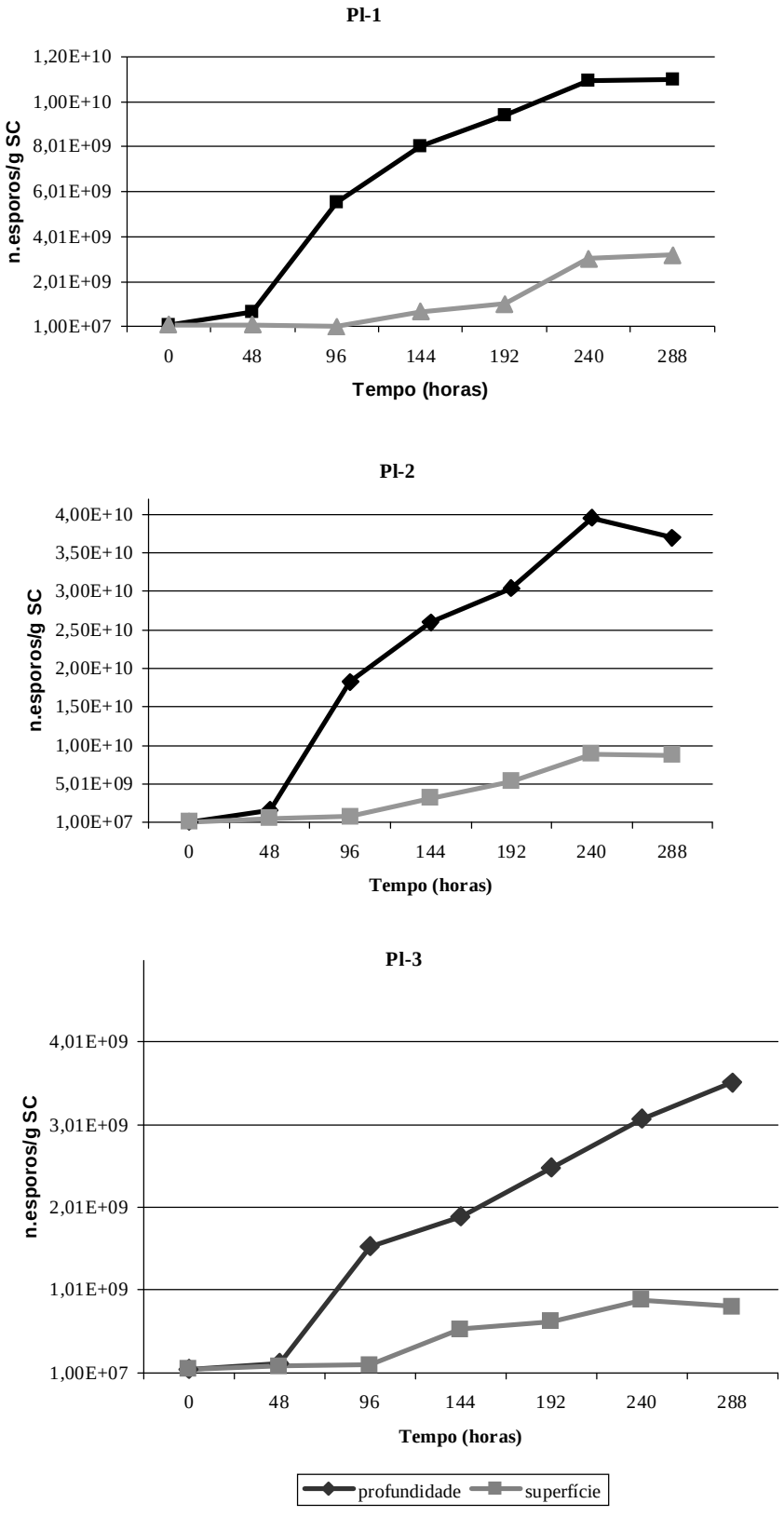
Neste capítulo, o modo de inoculação dos meios de cultura, a fisiologia de crescimento e a esporulação de fungos nematófagos serão abordados. Duas estratégias diferentes de inoculação, em profundidade e em superfície, foram testadas para verificar suas influências nas cinéticas de esporulação de três cepas de fungos filamentosos. A fisiologia de crescimento de fungos filamentosos nematófagos estuda a germinação de esporos, o crescimento radial do micélio com a extensão de suas hifas para a formação de biomassa, e a conidiogênese com a formação de um conidióforo e liberação de conidiosporos. Estas etapas fisiológicas do fungo são estudadas utilizando-se dois meios de cultura : o meio batata dextrosado (PDA) e o meio extrato de malte (MEA). A última parte deste capítulo compreende as cinéticas de esporulação de *Paecilomyces lilacinus* cultivado em FES utilizando-se o bagaço de cana-de-açúcar como suporte impregnado com o caldo de batata dextrosado (PDB) para a produção de biomassa.

4.1.1. Efeito do modo de inoculação dos meios gelosados sobre a germinação e produção de esporos

A germinação de um esporo de fungo filamentoso é a continuação da longa etapa de dormência e permite o início de um estado de atividade metabólica. Três etapas sucessivas caracterizam a germinação de um esporo: 1) A ativação do esporos em dormência em resposta à condições ambientais apropriadas; 2) A absorção de água e a restauração do metabolismo do fungo e 3) A emergência do tubo germinativo (Dantigny et al., 2006). Existem várias vantagens para a utilização de um esporo como "starter" com relação ao micélio. Inicialmente o esporo apresenta um estado de dormência e pode germinar, o que não acontece para o micélio. Uma outra diferença notável se manifesta no momento da inoculação. A distribuição dos esporos nos meios de cultura líquidos ou em FES é muito homogênea levando-se em consideração que o esporo é a forma unicelular, contrariamente ao micélio que deve ser moído para se obter uma inoculação mais homogênea. Ao contrário, o micélio é ativo no momento da inoculação e deste modo a fase de adaptação é mais rápida. Para os fungos filamentosos os esporos são as formas mais apropriadas para a inoculação dos meios de cultura e dois modos de inoculação foram testados para verificar suas influências na produção de esporos de fungos nematófagos.

As cepas de *P. lilacinus* Pl-1, Pl-2 e Pl-3 foram testadas quanto a sua capacidade de produção de esporos utilizando-se dois diferentes tipos de inoculação do meio PDA. Para a inoculação em profundidade ou em superfície do meio gelosado (PDA) as cinéticas de esporulação das cepas foram acompanhadas durante 288 horas de incubação a 28°C (Fig 4.1.1.).Para as três cepas testadas, a quantidade de esporos produzidos foi sempre superior com a inoculação em profundidade do meio PDA em relação aos obtidos com os meios de cultura inoculadas na superfície. A inoculação com esporos em profundidade de um meio de cultura é o modo de inoculação mais apropriado para a produção de esporos de fungos filamentosos em meios gelosados. Entretanto para a inoculação em profundidade o micélio se desenvolve de maneira sincronizada, não existe um efeito de inibição entre as colônias e a colonização da superfície dos meios de cultura é homogênea. Deste modo, a germinação de esporos, o crescimento do micélio são sincronizados levando a uma produção elevada de biomassa e esporos.

FIGURA 4.1.1. Efeito do modo de inoculação em profundidade e em superfície do meio PDA sobre as cinéticas de *P. lilacinus* Pl-1, Pl-2 e Pl-3. (SC= Substrato carbonado)



A cepa *P. lilacinus* Pl-2 produziu 3,9 x 10^{10} esporos/g substrato carbonado quando ela foi inoculada em profundidade e 8,02 x 10^{9} quando ela foi inoculada em superfície. Para a cepa *P. lilacinus* Pl-1 1,09 x 10^{10} em profundidade e 8,05 x 10^{9} em superfície e para a cepa *P. lilacinus* Pl-3, 3,1 x 10^{9} e 8,9 x 10^{8} esporos/g de substrato carbonado, respectivamente inoculados em profundidade e em superfície. Pode-se observar uma cinética precoce quando se utiliza a inoculação em profundidade foi utilizada para as três cepas testadas (figura 4.1.1.). Ao contrário, para a inoculação em superfície dos meios de cultura, verifica-se uma fase de latência acentuada até 96 horas de cultura. A produção de esporos começou a partir de 48h de cultura e atinge seu máximo em 240 h para as cepas *P. lilacinus* Pl-1 e Pl-2. A cepa *P. lilacinus* Pl-1 demonstrou uma cinética regular e a cepa *P. lilacinus* Pl-2 produziu mais esporos devido provavelmente ela ter produzido mais biomassa. Este experimento permitiu escolher o modo de inoculação em profundidade dos meios de cultura tendo em vista que todas as cepas produziram uma quantidade maior de esporos quando inoculadas desta maneira.

4.1.2. Comparacão do crescimento radial e da produção de biomassa

Quando as condições de cultura são favoráveis os esporos dos fungos filamentosos vai germinar e ocorre a formação de um tubo germinativo que se desenvolve de maneira apical e formará o micélio. A propagação do fungo é feita pela fabricação de hifas que vão se alongar por ramificação para formar na superfície de um meio gelosado as colônias com uma grande rede de micélio que caracteriza o crescimento radial dos fungos filamentosos. Este modo de desenvolvimento das colônias de fungos filamentosos em função do tempo denomina-se velocidade de crescimento radial. Quando as condições de cultura são favoráveis, o desenvolvimento dos fungos filamentosos é diretamente ligado a quantidade e a qualidade da fonte de carbono e nitrogênio presentes em um meio de cultura. Os principais parâmetros que influenciam a fisiologia de crescimento são: 1) a quantidade de água disponível; 2) a temperatura e 3) a presença de oxigênio. Os fungos podem se desenvolver em substratos carbonados naturais, levando-se em consideração que seus sistemas enzimáticos para a degradação destes substratos são bem adaptados. Dois meios de cultura (PDA e MEA) foram escolhidos para verificar a influência da fonte de carbono sobre a velocidade de crescimento radial das cepas de fungos filamentosos nematófagos estudados. Estes meios favorecem a

germinação de esporos e o desenvolvimento do micélio. Os resultados obtidos para este experimento estão agrupados nas Tabelas 4.1.1 e 4.1.2.

TABELA 4.1.1. Velocidade comparada de crescimento radial (mm/h) para 4 cepas de fungos nematófagos cultivados em MEA e PDA a 28°C durante 166 horas.

Tempo	Cepas							
	Vc-1		Pl-1		Pl-2		Pl-3	
(Horas)	MEA	PDA	MEA	PDA	MEA	PDA	MEA	PDA
0	0	0	0	0	0	0	0	0
42	0,17	0,18	0,21	0,15	0,20	0,24	ND	ND
65	0,16	0,16	0,22	0,21	0,22	0,24	0,21	0,22
94	0,18	0,14	0,25	0,25	0,24	0,25	0,23	0,22
137	0,18	0,15	0,25	0,27	0,24	0,25	0,23	0,22
166	0,15	0,22	0,26	0,31	0,27	0,24	0,24	0,27
Média	0,17	0,17	0,24	0,24	0,24	0,24	0,23	0,23

TABELA 4.1.2. Evolução comparativa da produção de biomassa (µg/h) para 4 cepas de fungos nematófagos cultivados em MEA e PDA a 28°C durante 166 horas.

Tempo	Cepas							
	VC-1		Pl-1		Pl-2		Pl-3	
(Horas)	MEA	PDA	MEA	PDA	MEA	PDA	MEA	PDA
0	0	0	0	0	0	**0**	0	0
47	28,7	30,9	34,1	21,3	14,9	**42,6**	43,6	34,0
94	87,8	86,2	114,4	109,6	126,6	**196,8**	101,1	98,4
137	97,3	118,9	181,02	175,6	170,4	**313,9**	156,2	156,6
166	104,8	141,3	237,05	315,9	297,0	**402,7**	194,0	286,4

A velocidade de crescimento radial de uma cepa é constante e caracteriza cada fungo filamentoso. Ela exprime a capacidade de um fungo em invadir uma superfície sólida em condições experimentais bem precisas. Os valores médios obtidos para as velocidades de crescimento radial são idênticos nos meios PDA e MEA para as cepas de *P. lilacinus* e para *V. chlamydosporium* (Tabela 4.1.1.). As cepas de *P. lilacinus* Pl-1 e Pl-2 possuem uma velocidade de crescimento mais elevada: 0,24 mm/h seguida de 0,23 mm/h para a cepa de *P. lilacinus* Pl-03. A cepa de *V. chlamydosporium* Vc-01 apresentou uma velocidade de crescimento radial inferior de 0,17 mm/h. Embora tenham uma velocidade de crescimento radial similar, a produção de biomassa (micélio e esporos) foi ainda mais importante em meio PDA levando-se em consideração que a dextrose e o amido são mais facilmente assimiláveis para a formação de biomassa que a fonte de carbono presente no extrato de malte Bernabeu e Lopez-Llorca (2002) isolaram várias cepas de fungos nematófagos e caracterizaram seu desenvolvimento. As cepas de *V. chlamydosporium* isoladas apresentaram uma velocidade de crescimento radial menos importante quando comparadas com cepas de *P. lilacinus* e também *V. lecanii*, confirmando os resultados obtidos neste experimento.

Na Tabela 4.1.2. pode-se observar que a produção de biomassa é mais importante (1,4 vezes superior) para o meio PDA que para o meio MEA, para todas as cepas utilizadas em 166 horas de cultura a 28°C. A produção de biomassa para a cepa de *V. chlamydosporium* Vc-1 é praticamente igual nos dois meios de cultura estudados até 94 horas de cultura e após este tempo a produção de biomassa é mais importante no meio PDA até o fim da cultura. Para as cepas de *P. lilacinus* Pl-1 e Pl-3 no início da cultura (47 h) a biomassa foi mais elevada em meio MEA do que em PDA, entretanto após 137 h encontrou-se quantidades de biomassa superiores em meio PDA. Provavelmente o meio PDA induz uma melhor esporulação das cepas VC-1, Pl-1 e Pl-3 no fim da cultura, assim os valores de biomassa apresentados para 166 horas representam principalmente os esporos produzidos pelas cepas. A cepa Pl-2 é particular, ela foi a mais performante para a produção de biomassa e desde o início do cultivo ela apresentou mais biomassa no meio PDA. Esta cepa também produziu mais esporos em meio PDA, quando foram realizadas a cinéticas de esporulação e provavelmente é por esta razão que ela produziu mais biomassa, que é constituída de micélio e também de esporos.

4.1.3. Produção de esporos em frascos Erlenmeyer e em Fermentador de discos

A conidiação é o processo de formação e liberação de esporos pelos fungos filamentosos, quando os parâmetros ambientais e nutricionais se tornam críticos para seus ciclos de vida. Os esporos são unidades de reprodução e de conservação dos fungos filamentosos, capazes de suportar condições extremas e resistem aos ataques químicos e físicos. Eles começam seu crescimento quando as condições se tornam favoráveis para seu desenvolvimento (Bowen et al., 2000). A produção de esporos é freqüentemente expressa em índice de esporulação, que indica o número de esporos produzidos por grama de matéria seca ou por grama de substrato carbonado inicialmente presente nos meios de cultura (Roussos, 1985). O meio PDA foi escolhido para estudas a esporulação de três cepas de fungos nematófagos cultivados em frascos Erlenmeyer e num fermentador de discos. Durante a incubação do fermentador em temperatura ambiente (18°C – 24°C) acompanhou-se em paralelo uma cinética de esporulação em frascos Erlenmeyer (Figura 4.1.2). A cinética de esporulação nos frascos permitiu de estabelecer o tempo ótimo para a produção de esporos. As três cepas e seus rendimentos respectivos em esporos por grama de substrato carbonado inicialmente presente no meio PDA, em 14 dias de incubação estão demonstrados na Tabela 4.1.3.

TABELA 4.1.3. Índice de esporulação e quantidade de esporos produzidos pelas cepas de fungos nematófagos após 14 dias de cultura em temperatura ambiente.

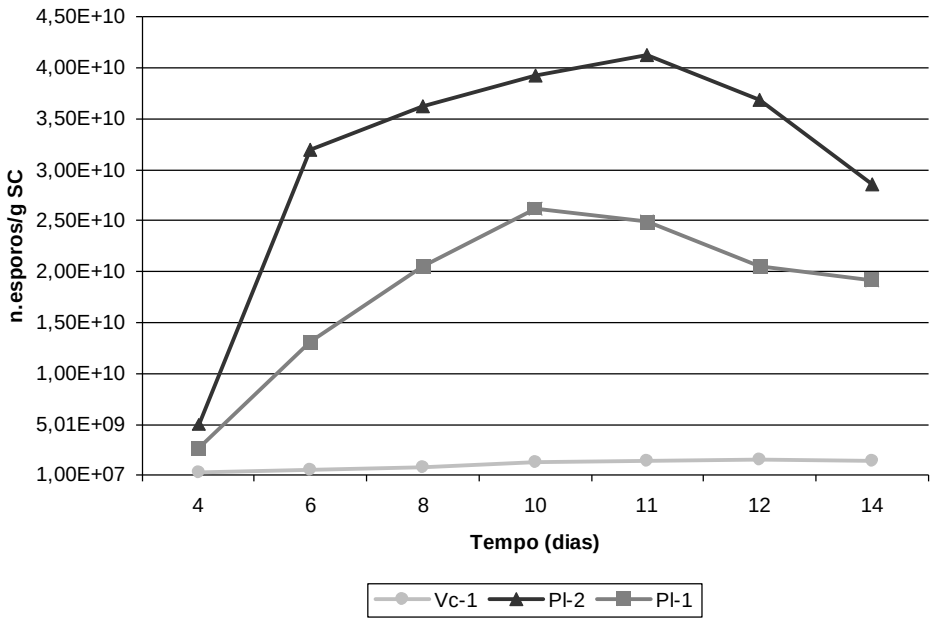
Reator	Fermentador de discos		Erlenmeyer	
Cepas	(n. esporos/g SC)	(n. esporos/fermentador)	(n. esporos/g SC)	(n.esporos/Erlenmeyer)
Vc-1	$7,67 \times 10^8$	$5,54 \times 10^9$	$1,48 \times 10^9$	$8,8 \times 10^8$
Pl-1	$7,49 \times 10^9$	$5,39 \times 10^{10}$	$1,92 \times 10^{10}$	$1,15 \times 10^{10}$
Pl-2	$1,13 \times 10^{10}$	$8,14 \times 10^{10}$	$2,86 \times 10^{10}$	$1,71 \times 10^{10}$

SC = substrato carbonado inicialmente presente no substrato

O fermentador de discos utilizado permitiu a obtenção de um inóculo concentrado mas com um índice de esporulação um pouco mais baixo do que em frascos Erlenmeyer. Entretanto, os esporos produzidos no fermentador de discos podem ser utilizados como inóculo para iniciar uma FES em grande escala. É necessário de se dispor de uma quantidade importante de inóculo composto de esporos limpos e viáveis na ordem de 2×10^7 esporos/g substrato peso seco.

Na figura 4.1.2 pode-se observar que a cepa de *P. lilacinus* Pl-2 produziu 4,13 x 10^{10} esporos/g substrato carbonado inicialmente presente no meio após 11 dias de cultura. A cepa de *P. lilacinus* Pl-1 produziu 2,62 x 10^{10} esporos/g SC e a cepa de *V. chlamydosporium* Vc-1 1,58 x 10^{9} esporos/g SC após 10 dias de cultura em temperatura ambiente em frascos Erlenmeyer. A cepa de *Verticillium* não produziu muitos esporos embora observou-se a presença de muitos clamidiosporos na câmara de contagem que consiste de uma grande célula com vários esporos dentro, isto é muito interessante, uma vez que este tipo de esporo pode germinar no solo em condições desfavoráveis (seca, desnutrição). Provavelmente a temperatura utilizada para a incubação das culturas não seja a condição ótima para o crescimento e conseqüente esporulação destes fungos.

FIGURA 4.1.2. Cinética comparativa de produção de esporos em frascos Erlenmeyer em meio PDA a temperatura ambiente (18 – 24° C).



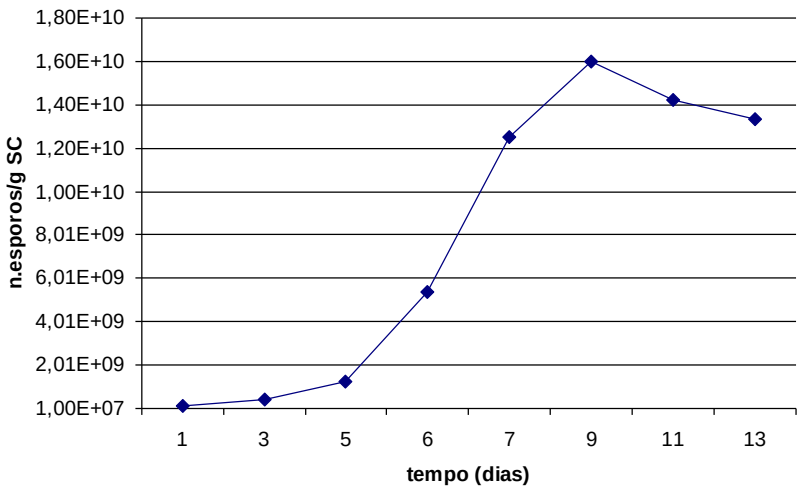
4.1.4. Produção de esporos por FES

A FES consiste em uma técnica para a cultura de microorganismos em um suporte ou substrato sólido para a produção de biomassa ou para a produção de metabólitos destes microorganismos. O bagaço de cana-de-açúcar, é um sub-produto agro-alimentar abundante no Brasil e já foi enormemente repertoriado na literatura por sua utilização como suporte para

FES (Roussos, 1985). Este suporte natural possui uma enorme capacidade de absorção (aproximadamente 90% de seu peso em água) e neste experimento ele foi utilizado para a absorção do meio PDB (Potato dextrose broth). A cepa *P. lilacinus* Pl-1 foi escolhida para o estudo da produção de esporos em colunas de fermentação (Raimbault & Alazard, 1981). Com este fermentador de FES, é possível de aportar o oxigênio e a umidade necessários para o metabolismo do microorganismo através de uma aeração forçada. Em um primeiro experimento, utilizou-se o bagaço de cana-de-açúcar como suporte, impregnado com o meio PDB, para comparar a produção de esporos obtida em frascos Erlenmeyer. Para as FES utilizou-se uma aeração de 30 ml/min. Com 10 dias de incubação obteve-se um rendimento de 9,87 x 10^8 esporos/g de suporte seco.

Num segundo experimento utilizou-se uma quantidade de PDB três vezes mais concentrado (72 g/l) que anteriormente e uma aeração de 30 ml/min/coluna utilizando-se sempre uma quantidade idêntica de meio inoculado nas colunas. Esta cepa produziu 1,62 x 10^{10} esporos/g de substrato carbonado em 15 dias de cultivo.

FIGURA 4.1.3. Produção de esporos de *P. lilacinus* Pl-1 em bagaço de cana-de-açúcar impregnado com PDB três vezes concentrado



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A fisiologia de esporulação das cepas de fungos nematófagos disponíveis no laboratório foi acompanhada em diferentes reatores (frascos Erlenmeyer, colunas de Raimbault e esporulador de discos) em maiôs de cultura comerciais contendo a dextrose ou o amido como fonte de carbono. O resumo para as duas cepas de *Paecilomyces lilacinus* (Pl-1 e

Pl-2) que apresentaram os índices de esporulação mais importantes está demonstrado na Tabela 4.1.4.

TABELA 4.1.4. Índices de esporulação (n.esporos/g SC) de *P. lilacinus* Pl-1 e Pl-2 cultivados em diferentes dispositivos de esporulação segundo o tempo da incubação.

Cepa	*P. lilacinus* Pl-1		*P. lilacinus* Pl-2	
Tempo de incubação (dias)	9	14	11	14
Erlenmeyer	$2,62 \times 10^{10}$	$1,92 \times 10^{10}$	$4,13 \times 10^{10}$	$2,78 \times 10^{10}$
Fermentador de dsicos	ND	$7,49 \times 10^{9}$	ND	$1,13 \times 10^{10}$
Colunas de Raimbault	$1,60 \times 10^{10}$	$1,35 \times 10^{10}$	ND	ND

ND = Não determinado; SC = Substrato carbonado

A cepa *P. lilacinus* Pl-2 produziu uma quantidade mais expressiva de esporos em frascos Erlenmeyer e em fermentador de discos em 14 dias de cultura que a cepa *P. lilacinus* Pl-1, entretanto a quantidade de esporos produzidos em frascos Erlenmeyer foi superior do que em fermentador de discos. A produção máxima de esporos desta cepa foi obtida em 11 dias de cultura, contrariamente para a cepa de *P. lilacinus* Pl-1 foi em 9 dias de cultura em frascos Erlenmeyer. A cepa *P. lilacinus* Pl-1 apresentou um comportamento idêntico à cepa *P. lilacinus* Pl-2, pois sua produção de esporos também foi mais importante em frascos e no fim do cultivo o número de esporos diminui. A cinética de produção de esporos pela cepa *P. lilacinus* Pl-1 é comparável em frascos Erlenmeyer e em colunas de Raimbault, onde a produção mais importante para os dois dispositivos foi obtida em 9 dias de crescimento, mas ainda a produção em frascos foi mais significativa. O fato de que a temperatura de cultivo utilizada nas experiências em fermentador de discos e em frascos Erlenmeyer não era a mais apropriada para o desenvolvimento dos fungos nematófagos, pode ter uma influência sobre a produção de esporos nestes reatores. Também, em colunas de Raimbault, a quantidade de substrato carbonado, utilizada com o meio PDB três vezes concentrado foi ainda inferior quando se compara com o meio PDA quando o ágar é considerado um suporte. A relação de substrato carbonado é de 1,4 por grama de ágar e de 0,288 por grama de bagaço com o PDB três vezes concentrado. Ainda, a aeração bem como a temperatura devem ser otimizados para a produção de esporos dos fungos nematófagos neste dispositivo.

Anteriormente, vários trabalhos sobre a produção de esporos de fungos filamentosos cultivados em FES foram estudados em meios de cultura contendo o amido como fonte de carbono (Roussos, 1985; Soccol et al., 1997). Um grande número de resíduos da agro-

indústria (Bagaço de mandioca, resíduos de batata, casca de café, etc.) podem ser utilizados para a produção de esporos de fungos nematófagos. Estes resíduos podem ser utilizados como fonte de carbono e nitrogênio ou como suporte sólido para a produção de esporos. Enfim, os fatores físicos de crescimento : aeração, temperatura de incubação e o pH dos meios de cultura devem ser igualmente otimizados para cada cepa.

4.2. Capítulo II: Seleção da cepa de fungo nematófago

ARTIGO 2 – Produção de um biocomposto por fermentação no estado sólido contra os nematóides do café

Resumo

Os fungos nematófagos *Paecilomyces lilacinus* e *Verticillium chlamydosporium* são conhecidos por serem parasitas de ovos de nematóides. Quatro cepas de fungos filamentosos foram testadas quanto a sua capacidade de crescimento em ágar à base de extrato de casca de café (100 g/l). As cepas de *P. lilacinus* Pl-1, Pl-2 e Pl-3 apresentaram uma velocidade de crescimento radial de 0,20, 0,18 e 0,19 mmh^{-1}, respectivamente. As cepas Pl-1 e Pl-2 produziram uma quantidade de biomassa mais significativa (128 e 132 mg, respectivamente em 12 dias). A única cepa de *V. chlamydosporium*, apresentou uma velocidade de crescimento radial de 0,12 mmh^{-1} e uma produção de biomassa de 69 mg em 12 dias. As cascas de café foram testadas como substrato para a produção de esporos destas cepas. Duas planificações experimentais foram realizadas para a otimização dos parâmetros de fermentação: umidade inicial, pH inicial e temperatura de incubação. Os produtos obtidos por FES com as condições otimizadas de cultura foram testadas quanto à sua atividade contra o nematóide *Meloidogyne incognita* raça 1 através de experimentos em vasos com a planta *Coleus*. A cepa Pl-1 demonstrou uma atividade mais eficaz, pois a mesma reduziu em 80% o número de fêmeas de nematóides.

Brand, D., Roussos, S., Prochmann, F.A., Pohl, J., Soccol, C.R. Production of a biocompost by solid state fermentation against the coffee nematodes. In: New Horizons in Biotechnology. Editors: Sevastianos Roussos, Carlos Ricardo Soccol, Ashok Pandey, Cristopher Augur. Kluwer Academic Publishers, 2003, V. 1, p. 449-458

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

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Introduction

Phytosanitary problems caused by nematodes presents an important economic incidence all over the world in different agricultural cultures, mainly in coffee plantations. In reason of its extreme resistance, its great physiologic variability and its underground life the nematode combat is very difficult. The most common species that cause largest damages in the agriculture belong to the *Meloidogyne* genera, also called root-knot nematodes (Carneiro *et al.*, 1996).

The more common practices used in the nematode combat have been the use of varieties of resistant cultures, rotation of cultures and application of chemicals mainly phosphates and carbamates. Even so this last method, although very efficient and frequently used it is extremely dangerous to men and animals, due to its wide action spectrum, they disturb the ecological balance of places in which they are used, they propitiate the 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, underground water and the own culture that is being treated, they affect the health of animals equally of men. As function of such facts, concerning researches about biological fight comes enlarging its space as a concrete alternative in the substitutes search to the chemical nematicides. Predatory mushrooms, that capture nematodes or destroy its eggs, mycorrhizal mushrooms, toxins produced by microorganisms and plants, can be used shortly with success in the combat to nematodes of different cultures.(Cayrol, 1989; Bourne *et al.*, 1999; Ciancio, 1995; Dijksterhuis, *et al.*, 1994)

The term Biological control can be defined as the utilization of living organisms populations, parasites, predatory, pathogens, antagonistic or competitors in maintaining another organism's population density at a lower average than would occur in their absence. (De Bach, 1964. In Sidiqui, 1996). 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 cause 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 microorganisms 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 on various developmental stages of nematodes. (Jatala, 1996; Kerry, 1986, Nordbring-Hertz, 1988)

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 accomplished with a natural solid substrat or support for the development of the fungi. The use of agricultural residues or by-products such as cassava bagasse, coffee husk and pulp, sugar cane bagasse makes SSF even more interesting as it can supply good efficiency and stability of the final product (Roussos, *et al.,* 2000; Soccol, *et al,* 2002)

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.*

Experimental

Micro-organisms and culture media

The strains of nematophagous fungi utilized in this work were parasites of nematodes eggs *Paecilomyces lilacinus* designated as Pl-1, Pl-2 and Pl-3 and one strain of *Verticillium chlamydosporium*, Vc-1 nematodes eggs and cysts parasites. They were maintained in Potato dextrose agar (PDA) and cultivated in coffee husk extract media for the production of inocullum. (Brand, *et al.,*2000)

Growth Physiology of nematophagous fungi

In an agar medium containing 100 g/L of coffee husk extract (Brand, 2000) 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 during 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 28º C. The diameter of each colony was measured every 24 hours and the biomass was weighted

after 12 days by the dissolution of the agar and separation of mycelia on filter paper and dried at 100º C for 24 hours.

The strains belonging to the *Paecilomyces* genera showed similar radial growth velocity, 0,20 mmh^{-1}, 0,18 $mmh^{-1}$and 0,19 mmh^{-1}respectively for Pl-1, Pl-2 and Pl-3. The fungi parasite of nematodes cysts and eggs grows slower than the fungi parasites of eggs and presents a radial growth velocity of 0,13 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 less efficient in producing biomass but although were capable to assimilate and metabolize the components present in the coffee husk.

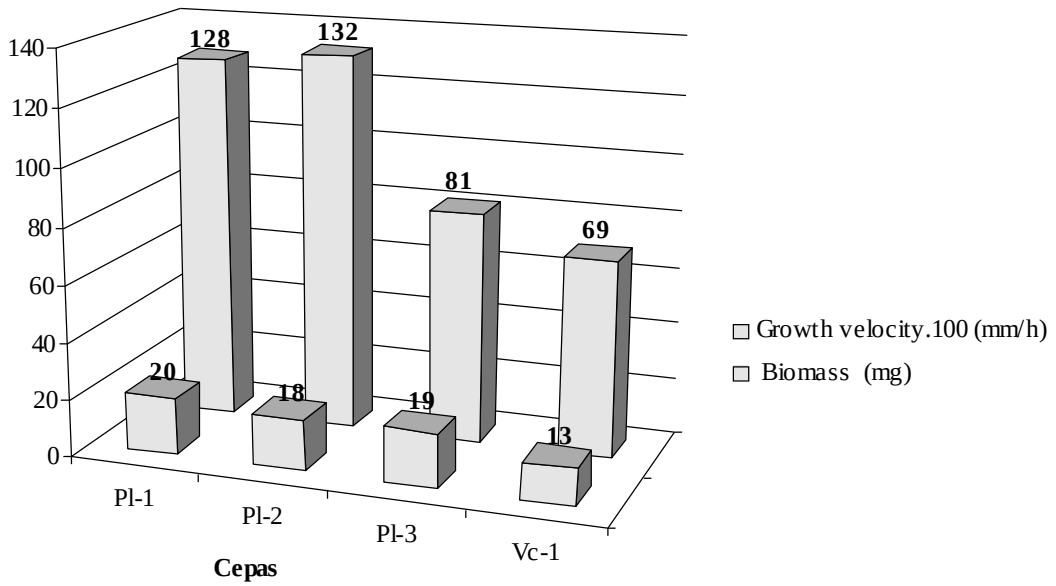

Figure 1. Radial growth velocity and biomass production of nematophagous fungi in 12 days.

Solid state fermentation

Experiments were conducted by utilizing coffee husk as substrate, 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 of 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 were 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 28º C and spores were counted in Malassez cell. Studies of the fermentation physical conditions, such as pH, temperature and initial moisture content were done initially by using an experimental design 2^{3-1}. The optimization of this culture conditions was accomplished in order to obtain better concentration of spores (the response variable). Table 1 shows the real and coded values for all strains tested.

Table 1. Real and coded values of experimental design 1

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

The inoculation rate employed was always 2 E+07 spores/g of substrate in dry weight basis and the incubation period was 7 days at 28º C for each strain studied. After incubation the spores were counted in Malassez cell. A total of 5 g 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 for counting.

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 showed by the 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 what means that the range employed its above of the ideal condition for spore production. The variables temperature and initial moisture content were not significant at level of 5%. For the fungi *Verticillium chlamydosporium* the most important factor in spore production was the temperature, the range employed was above ideal.

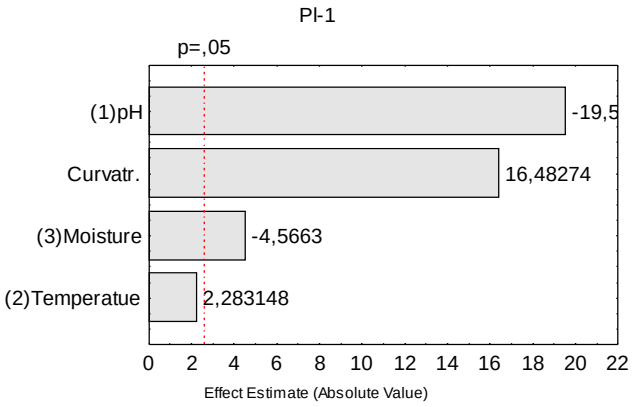

Figure 2. Pareto chart of effects for strain Pl-1

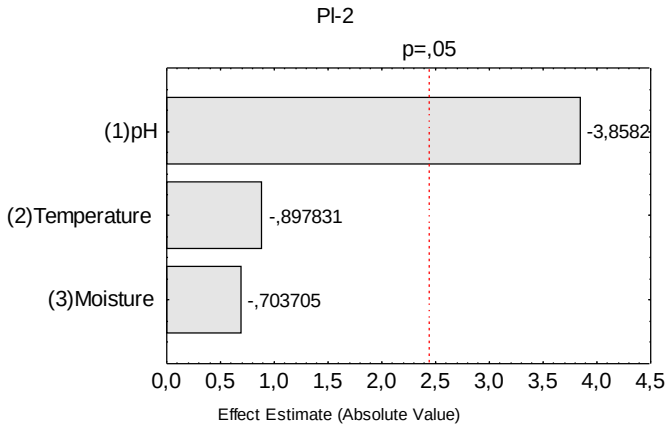

Figure 3. Pareto chart of effects for train Pl-2

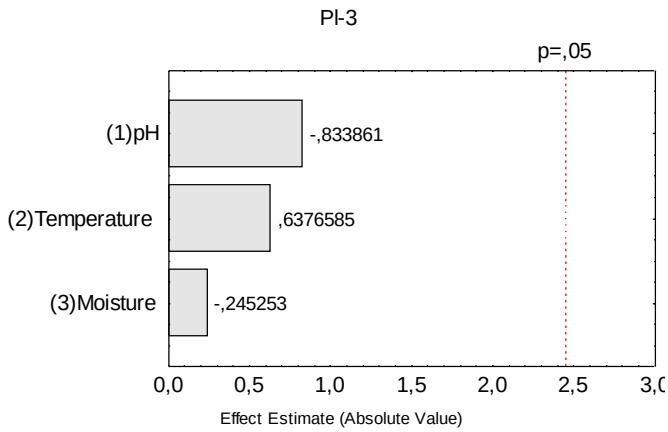

Figure 4. Pareto chart of effects for strain Pl-3

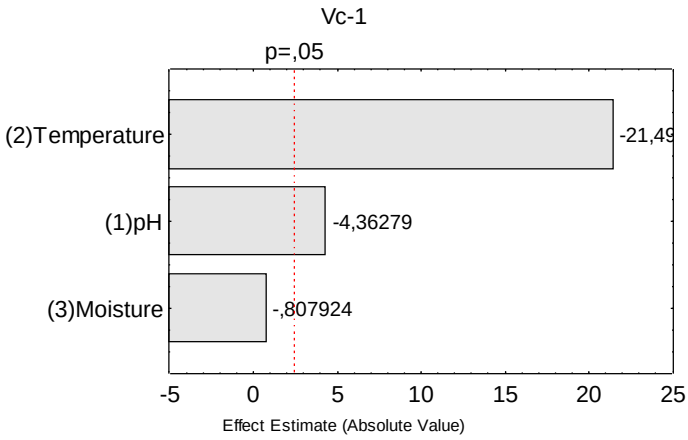

Figure 5. Pareto chart of effects for strain Vc-1

All strains of *Paecilomyces lilacinus* produced more than 3.5 E+09 spores/g coffee husk (dry weight), being the strain Pl-2 the one that showed better spore production reaching 6.4 E+09 spores/g.

The best conditions of spore production were achieved with the plan 3^2, but the number of spores didn't raise a decimal order, due to such fact the conditions employed for each strain was established as initial moisture of 65%, natural pH of the substrate and incubation

temperature of 28º C. With this conditions, fermentation were carried out in order to utilize the biocompost obtained in pot experiments as described below.

Pot experiments

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

The nematodes (isolated from coffee plantation) were reared on tomato as well as in *Coleus*. The experiments were realized in glasshouse without thermal, neither illumination controls, being subjected to environmental conditions. Two vases containing good quality sterilized soil were prepared for the tests of the selected fungi and more two vases were utilized as control treatments (in whose no fungi was inoculated). All experiment were realized with two replicates. Each pot received one seedling of the plant *Coleus*, which was chosen for being susceptible to the 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 2ºC) retarded the development of the nematodes as well as its parasitic action. The period of two months is referred in literature as ideal for temperatures around 25º C. Temperatures below 18º C reduce the activity of the nematodes and equally the action of nematophagous fungi, amplifying the period of plant infection. Medium temperatures inferior to 5º C may paralyze the nematode action. A suspension of nematodes (*Meloydogyne incognita* race 1) was prepared from roots visually infected of *Coleus*, 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 10^9 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, the roots were isolated, washed and dried at room temperature. The roots were weighed and 5% were

evaluated. The results obtained indicated a significant reduction of nematodes 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	Fresh 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 characterized a reduction in the number of females in the pots treated with the biocompost containing the strain of nematophagous fungi *Paecilomyces lilacinus* Pl-1. In this case 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 propitiated a reduction in the number of nematode females in order of 15 – 25 % inferior to the values observed for the control pots (figure 6).

Figure 6. Comparison of roots evaluated in the experiment

In spite of the retard in the nematodes cycle, it was possible to evaluate the experiment. In figure 7 below the best result is standing out, the medium reduction of the nematodes was approximately 80% in the number of females per g of root. It can 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 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 influence with the sporulation index obtained during fermentation, as it was observed that the strain that produced more spores was Pl-2. The fungi Pl-1 that gave the greater reduction is probably better adapted to the soil conditions as well as the nematode species and race employed in the experiment.

Conclusions and futuristic 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 it was reached 80% of reduction in the coffee nematodes density (*Meloidogyne incognita* race 1) in a pot experiment, this is a significant result but further studies of the virulence of this strain should be studied against other species and races of nematodes of the genre *Meloidogyne*, as well as the others strains employed. Studies of the fermentation conditions will be studied in order to enhance the virulence of the fungi .

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Parte 2: SELEÇÃO DO SUBSTRATO E QUALIDADE DOS BIOPESTICIDAS

4.3. Capítulo III:

Utilização de resíduos de camarão misturados com bagaço de mandioca como substrato para a produção de um biocomposto nematicida

ARTIGO 3 - Utilização de resíduos de camarão misturados com bagaço de mandioca como substrato para a produção de um biocomposto nematicida

Resumo

O objetivo deste estudo foi de desenvolver um bionematicida contendo o fungo filamentoso *Paecilomyces lilacinus* utilizando-se a Fermentação no Estado Sólido (FES). O substrato foi constituído de uma mistura de 33,3% de resíduos de camarão e de 66,6% de bagaço de mandioca. Duas planificações experimentais foram realizadas para se encontrar as melhores condições para a produção de esporos da cepa de fungo filamentoso. A primeira planificação 2^{3-1} foi utilizada, com três fatores: 1) taxa de inoculação, 2) pH inicial e 3) umidade inicial do substrato. Na segunda planificação, somente os fatores significativos da experiência anterior foram testados. A cinética de produção de esporos foi acompanhada durante 15 dias com as condições otimizadas: 67% de umidade inicial, pH 4,5 e taxa de inoculação de 2.0 E+07 esporos.g^{-1} de substrato seco. A produção máxima de esporos foi muito significativa na ordem de 1,02E+10 esporos.g^{-1} substrato seco. O biocomposto obtido foi testado através de experimentos em vasos com plantas de tomate. Três grupos foram testados : um grupo controle, um grupo com os biocompostos obtidos por FES em presença de nematóides e outro inoculado somente com nematóides. Para o tratamento com o biocomposto em presença de nematóides a redução do número de fêmeas foi elevado (60%) quando comparado com o tratamento em vasos inoculado somente com nematóides.

USE OF SHRIMP WASTES MIXED WITH CASSAVA BAGASSE AS SUBSTRATES FOR THE PRODUCTION OF A NEMATOCIDAL BIOCOMPOST

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Key words: *Paecilomyces lilacinus*, solid state fermentation, cassava bagasse, shrimp wastes, biological control, myconematicide, nematodes.

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Abstract

The aim of the present work was to develop a bionematicide by solid state fermentation of *Paecilomyces lilacinus* LPB-Pl-01. The substrate tested was constituted of 33.3% shrimp wastes and 66.6% cassava bagasse. Two experimental designs were conducted to achieve best spore concentration of the fungal strain. The first design was 2^{3-1} and the variables tested were inoculation rate (2.2 x 10^7, 4.5 x 10^7 and 6.7 x 10^7 spores.g^{-1} dry substrate), initial moisture content (65%, 70% and 75%) and initial pH (4.5, 5.0 and 5.5). In the second design 3^2, pH and initial moisture content were tested again, although pH was the only significant factor in the first design. Kinetics of spore production was followed for 15 days with optimized conditions: 67% initial moisture content, 2.2 x 10^7 spores g^{-1} dry substrate, and initial pH 4.5. The biocompost was utilized in pot experiments for evaluation of its capacity against root-knot nematodes in tomato plants. The pot experiment consisted of 3 groups: control, treatment with biocompost (fungus) + nematodes, and only nematodes with 4 replicates. Number of females and root weight were evaluated after 2 months. Maximal spore production was achieved after 11 days of fermentation reaching 1.02 x 10^{10} spores g^{-1}dry substrate. Reduction in the number of nematodes females was around 60% for the myconematicide in relation to the group inoculated with only nematodes.

Introduction

Plant-parasitic nematodes exploit diverse parts of crop plants being the root the most preferred feeding site. Economically important groups of nematodes are sedentary endoparasites which include the genera *Heterodera* and *Globodera* (cyst nematodes), as well as *Meloidogyne* (root-knot nematodes), being the latter the most widespread *(14)*. It has been estimated that the overall yield losses are over 10% approaching 20% for some crops. Worldwide losses certainly exceed US $ 100 billion dollars annually *(1)*.

The host range of root-knot nematodes is very extensive and it is difficult to find plants that are not hosts. Many vegetables, bedding plants, shrubs and trees are susceptible. Root-knot nematode infections cause plant decline and at extreme concentration plant death. Plants may show leaf chlorosis, and are rather unproductive. However, the extent of damage may vary with plant, time of infection and cultural conditions. The roots present knots (swellings) which are the most typical symptom of the infection, these knots may vary upon the host. Root-knot nematodes feed on roots and their life cycle involves egg, juvenile and adult stages. Eggs hatch into juveniles and those that enter the root and develop into females are sedentary which grow inside the root and lay hundreds of eggs on the root surface *(11)*.

The most common practices used in root-knot nematodes control comprise crop rotation, use of resistant plants, and mainly the utilization of expensive chemical nematicides. Although many chemical nematicides are being prohibited due to issues such as ground water contamination, mammalian and avian toxicity, and food residues, they are still used. Resistant plants remain as the most important control measure against nematodes, unfortunately resistance was not found for many crop plants and nematodes were able to overcome this in some cases *(10)*.

Nowadays strains of the filamentous fungus *Paecilomyces lilacinus* are being developed as biological control agents against root-knot, cyst and other plant-parasitic species of nematodes. The inoculum applied at field conditions consists mainly of spores, which must be able to survive and to infect the plant-parasitic nematodes (*4)*. In the initial step, this nematophagous fungus in contact with a nematode must penetrate its cuticle and eggshell, probably by the combination of mechanical activity and hydrolytic enzymes, such as protease and chitinase due to the composition of the nematode structures. These enzymes are called the virulence factors of the strains of nematophagous fungi and they have already been identified, cloned and expressed *(5, 6, 7)*.

Solid state fermentation (SSF) may be employed for mass production of biological control agents (BCA) (*12*), although submerged fermentation (SmF) is still the main technique utilized for commercial purpose. Studies for the development of processes of *Paecilomyces lilacinus* spores mass production under SSF were mostly using agricultural raw material, such as wheat. The use of agricultural by-products is attempted in this work. Cassava bagasse consists mainly of fibrous material and starch (40-70%), the principal carbon source; however, it has poor nitrogen content. Many SSF bioprocesses have been developed with this by-product, such as protein enrichment, production of aroma compounds, citric acid, and pigments *(13).* The other constituent of the substrate is shrimp wastes, whose proximate composition is 50% of chitin, 25% of protein and 25% of calcium carbonate. In this way cassava bagasse is supplemented with a nitrogen source, and the substrate contains a significant amount of chitin for the induction of chitinase synthesis. The addition of chitin to substrates has been studied to investigate development and sporulation of arbuscular mycorrhizal fungi, showing positive effects (*3).* Matsumoto *et al.* studied the utilization of shrimp waste silage for the production of N-acetylhexosaminidase of *Verticillium lecanii* in SmF and SSF systems, proving that the substrate was an efficient inducer of the enzyme (*9).*

The objective of the present work was to produce a virulent biocompost by SSF, with the strain of *Paecilomyces lilacinus* LPB-Pl-01 in a low cost substrate. The substrate was constituted of cassava bagasse and shrimp wastes in order to have a great amount of active spores. The final fermentation product was tested against the nematode *Meloidogyne incognita* race 1 in tomato plants in order to verify the fungal strain activity.

Materials and Methods

Microorganism and inoculum production. The strain of nematophagous fungi utilized in this work was *Paecilomyces lilacinus* (Thom) Samson LPB-Pl-01. This strain was maintained in potato-dextrose-agar (PDA) at 4 C and periodically transferred to agar slants. A spore suspension was prepared from one agar slant, 5 mL of sterile water was added to the tube and spores were scraped with the aid of o platin loop. For inoculum preparation 50 mL of PDA were sterilized in 250 mL Erlenmeyer flasks; before solidification of the medium, 1 mL of the spore suspension containing approximately $2x10^8$ spores/mL was added to each flask. The flasks were incubated at 28 C for 7 days. Spores were harvested by magnetic stirring and glass beads with 30 mL of distilled water and drops of tween 80. After serial dilutions spores were

counted in a Malassez cell under microscope. Final inoculum concentration was of approximately 2 x10^8 spores/mL.

Substrate pre-treatment. Cassava bagasse was dried in an air oven for 48 h at 55 C. This material was then milled and classified to obtain a particle size of 0.8-2.0 mm. Shrimp wastes (not edible parts) were washed three times and allowed to dry in air oven in the same manner as for cassava bagasse. Shrimp wastes were then milled, and fractions smaller than 2.0 mm were used for experimental work. The substrate consisted of 66.6% of cassava bagasse and 33.3% of shrimp wastes.

Solid state fermentations. SSFs were carried out with a total of 9 g of substrate in glass flasks covered with filter paper for allowing gas exchanges. To each flask containing 9 g of dry substrate was added half of the volume of the water necessary to reach the desired initial moisture content. The substrate was homogenized and sterilized in autoclave for 20 min at 121 C. The substrate was subsequently inoculated with the desired spore concentration. The spore suspension was added to sterile water in enough quantity to reach the desired moisture content. The pH was adjusted with HCL 1N. Incubation was at 28 C for 10 days.

Two experimental designs were realized in order to optimize the physical parameters of fermentation by using the software STATISTICA version 5.1 (StatSoft, Inc. Tulsa, OK, USA, 1996). The first experiment consisted of a 2^{3-1} design, and the parameters tested were pH, initial moisture content of the substrate and inoculation rate as shown in **Table 1**. The experiment was carried out with two replicates.

The variable pH was the only significant factor at the level of 5% in the first experiment, a new experimental design 3^2 with the variables pH and initial moisture content was performed in order to attain the best fermentation conditions for spore production of *Paecilomyces lilacinus* LPB-Pl-01 in the mixture of cassava bagasse and shrimp wastes. The variables pH (4.0, 4.5 and 5.0) and initial moisture content (64%, 67% and 70%) were tested in three levels and they were totally randomized among each other, resulting in a total of 10 assays that were accomplished with two replicates.

With the optimized fermentation conditions of the substrate, a kinetic study was carried out to verify the spore production during 15 days, being the samples withdrawal daily for spore count, determination of pH and moisture content.

Spore production. The spore concentration in fermented products were assayed by taking 1 g of wet fermented substrate, which was magnetic stirred for 30 min with 30 mL of distilled water. After serial dilutions the spores were counted in Malassez cell under

microscope. The number of spores is expressed by grams of dry substrate, as it was taken into account the final moisture content of the fermented product.

Nematicide activity of optimized fermented product. The activity of the fermented products against nematodes of the species *Meloidogyne incognita* (Kofoid and White) Chitwood Race 1 was evaluated by pot experiments. Nematodes were reared in tomato plants (L*ycopersicum esculentum* Mill. cv Rutgers). A nematode suspension was made by taking roots visually infected by nematodes. Roots were cut in 2 cm pieces, adding water and shaking for 15 s in a blender. Juveniles and eggs were counted under microscope in order to establish the inoculation rate for each treatment. Experiments were conducted with 4 replicates, and consisted of 1 control (no fungus or nematodes), 1 treatment [fermented product (fungus) + nematodes], and 1 treatment (only nematodes).

Each pot received good quality sterile soil, 1 seedling of tomato with same age, and had the same disposition, with layers of soil, fermented product and nematode suspension alternated around the root of each plant, depending on the case. The fermented product (15 g) was inoculated in wet basis (around 67% moisture content) with spore concentration of 10^9 spores g^{-1} of substrate. The concentration of nematodes was of 1,500 eggs and juveniles per pot. The experiment was conducted in a greenhouse without thermal neither illumination controls. After 2 months the plants were removed from pots, the roots were isolated, washed and dried at room temperature for 1 h. The roots were cut in 1 cm portions and 2 subsamples of 1 g were weighed for female count under stereomicroscope (magnification of 45x). The root remains were used for determination of dry weight, and were dried at 60° C for 24 h. Data for female count and root dry weight were subjected to analysis of variance (one-way ANOVA) and treatment differences were determined by Tukey's test. Differences among means were considered significant at a probability level of 5% ($p< 0.05$).

Results and Discussion

Solid state fermentations. The mixture of shrimp wastes and cassava bagasse was a good substrate for spore production of the fungal strain LPB-Pl-01 of *Paecilomyces lilacinus*. The first optimization experiment (2^{3-1}) showed that the only significant parameter at the level of 5% was the pH, as demonstrated by the Pareto Chart of effects (**Fig. 1**). The spore production results are shown in **Table 2**. The best spore production was achieved with initial pH of 5.0, moisture content of 70%, and inoculation rate of 2.2×10^7 spores g^{-1} dry substrate, being of

2.2 x10^{10} spores g^{-1}dry substrate. The pH was the significant parameter and it was observed that at pH values of 5.5 the production of spores was less effective. In the contour graph (**Fig. 2**) tendencies for better spore production of the fungal strain on the substrate consisting of the mixture of 2:1 cassava bagasse and shrimp wastes are demonstrated. In this first optimization step, a 2^{3-1} factorial design was used and the interpretation of the contour graph shows the tendencies for a better spore production, even though the best spore production achieved in this is not among the values plotted in the graph but it should be taken in consideration that the only significant factor in this optimization step is the pH of the substrate.

In the second step of the optimization study, through a 3^2 complete experimental design, the fermentation parameters pH and initial moisture content were assayed in three levels. Even though initial moisture content was not significant in the first experiment it was tested again in this step. Spore production in this optimization step was more informative as can be seen in **Table 3**. Best results aiming maximal spore production were achieved with the optimized condition of pH 4.0, initial moisture content of 67%, and inoculation rate of 2.2 x 10^7 spores g^{-1} of dry substrate (**Fig. 3**). This fermentation condition yielded a production of 3.45 and 3.18 x 10^{10} spores g^{-1} for each replicate (**Table 3**). In this experiment, tests 10A and 10B are the central points that were used to reinforce and reassure where the best conditions of the experiments could be found, although tests 2A and 2B presented also high spore production. The contour graph (**Fig. 3**) shows that the best results were achieved with pH 4.0 and 67% initial moisture content. However, the only significant factor in this step of optimization is the quadratic effect of moisture content, and the pH was not a significant factor in this design (**Fig. 4**).

Kinetics of the fermentation was accomplished with pH 4.5, and moisture content of 67%, in order to verify when the maximal spore production would occur aiming to reduce time of fermentation. Spore production along 15 days of fermentation is shown in **Fig. 5**. Maximal spore production (1.02 x10^{10} spores.g^{-1} dry substrate was achieved at the 11th day of fermentation. The parameters of pH and initial moisture content were also followed along fermentation. The pH values and initial moisture content increased along fermentation reaching 8.6 and 75%, respectively, at the end of the process.

Activity of fermented products against nematodes. To verify if the spores produced from *Paecilomyces lilacinus* LPB-Pl-01 were effective against root-knot nematodes of *Meloidogyne incognita* Race 1, three types of treatments were accomplished. Assessments of nematode female count and root weight are shown in Table 4. Root weights didn't show any stastitical differences at level of 5% by one way analysis of variance (p = 0.2253, f = 1,1767),

but means of female count were significant different (p = 0,0090, f = 8,314). Tukey's multiple comparison test was used to determine which means of female counts were different among the treatments. Statistical differences were found only when comparing nematodes with control treatment ($p < 0.01$), but they were not significant for the fermented product when compared to control and nematodes treatment. The presence of nematodes in fungus + nematodes and nematodes treatments led to the formation of giant cells, though the root tissue was much healthier in the control and with the utilization of the fermented product when compared to only nematodes.

The percentage reduction in female count was 60.5%, but it is important to mention that the average of root weights for the fungus + nematodes treatment was a little higher than the control. Liang *et al.* (*8*) investigated the utilization of a shrimp and crab shell powder medium for the production of protease of *Monascus purpureus*, whose culture supernatants exhibited enhanced vegetable growth when applied to rapes. In other studies conducted by Brand *et al. (2)*, the utilization of fermented products containing coffee husk and defatted soybean cake and their mixtures, the reduction in nematode females reached 90%. The mixture of 2:1 cassava bagasse and shrimp wastes, even being a good substrate for spore production of *Paecilomyces lilacinus* LPB-Pl-01 and having great amounts of chitin and protein in its composition for the possible induction of enzymes and better efficacy on nematodes control was not as great as the substrates tested in earlier studies *(2)*.

In conclusion, the utilization of residues of cassava bagasse and shrimp wastes as substrates for spore production of the fungal strain *Paecilomyces lilacinus* LPB-PL-01 was outstanding reaching 3.49 x 10^{10} spores g-1 dry substrate. The best conditions achieved for spore production were a pH ranging from 4.0-4.5 and a moisture content of 67%. Although its utilization as a myconematicide against *Meloidogyne incognita* Race 1 in tomato plants requires further studies, a reduction of 60.5% in the number of nematode females was recorded. Lower and higher concentrations of the fermented product may be tested to enhance the activity against nematodes.

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Table 1. Design (2^{3-1}) for SSF optimization with cassava bagasse and shrimp wastes as growing substrate for *Paecilomyces lilacinus.*

Assays	pH	Initial moisture content (%)	Inoculation rate (No. spores.10^7/g dry substrate)	Spore concentration (No. of spores x10^8 / mL)
1	5.5	75	6.7	6.0
2	5.5	65	2.2	2.0
3	4.5	75	2.2	2.0
4	4.5	65	6.7	6.0
5	5.0	70	4.5	4.0

Table 2. Spore production by the fungus *Paecilomyces lilacinus* LPB-Pl-01 in the experimental design (2^{3-1}) with shrimp wastes and cassava bagasse as substrate.

Replicate	pH	Initial moisture (%)	Inoculation rate (No. Spores.10^7/g dry substrate)	Number of spores (No. spores 10^{10}/g dry substrate)
1	5.5	75	6.7	0.72
1	5.5	65	2.2	0.44
1	4.5	75	2.2	1.2
1	4.5	65	6.7	1.36
1	5.0	70	4.5	2.22
2	5.5	75	6.7	0.71
2	5.5	65	2.2	0.42
2	4.5	75	2.2	1.71
2	4.5	65	6.7	1.07
2	5.0	70	4.5	1.94

Table 3. Spore production by *Paecilomyces lilacinus* LPB-Pl-01 in the experimental design (3^2) with substrate consisting of 2:1 cassava bagasse and shrimp wastes.

Replicates	Initial pH	Initial moisture content (%)	Final pH	Final moisture content	Number of spores (No. spores 10^{10}/g dry substrate)
1A	4.0	64	7.5	62.93	2.27
1B	4.0	64	7.3	63.28	2.17
2A	4.0	67	7.6	70.86	3.45
2B	4.0	67	7.7	70.33	3.18
3A	4.0	70	7.7	73.82	1.72
3B	4.0	70	7.8	75.39	1.95
4A	4.5	64	7.8	61.75	2.10
4B	4.5	64	7.6	58.38	1.08
5A	4.5	67	7.6	70.26	1.72
5B	4.5	67	7.7	70.20	2.47
6A	4.5	70	7.8	74.86	2.80
6B	4.5	70	7.8	75.40	1.65
7A	5.0	64	7.7	64.19	2.18
7B	5.0	64	7.6	65.63	2.49
8A	5.0	67	7.6	65.75	2.27
8B	5.0	67	7.6	67.09	2.01
9A	5.0	70	7.7	73.18	1.45
9B	5.0	70	7.8	74.56	1.67
10A	4.5	67	7.7	72.09	3.49
10B	4.5	67	7.7	68.60	3.25

Table 4. Tomato plants root weight and female count for testing the nematicide activity of the strain of *Paecilomyces lilacinus* LPB-Pl-01 produced on 2:1 cassava bagasse and shrimp wastes (average of two samplings).

Experiment	Replicate	Root dry weight (g)	Average of female count
Control	1	0.62	0.0
Control	2	1.11	0.0
Control	3	0.60	0.0
Control	4	1.00	0.0
Average		0.83	0.0
Standard deviation		0.26	0.0
Fungus + nematodes	1	1.21	44.5
Fungus + nematodes	2	1.17	47.0
Fungus + nematodes	3	0.83	51.0
Fungus + nematodes	4	1.72	50.0
Average		1.23	48.13
Standard deviation		0.36	2.95
Nematodes	1	0.94	139.5
Nematodes	2	0.68	119.0
Nematodes	3	1.10	111.0
Nematodes	4	1.31	117.5
Average		1.01	121.75
Standard deviation		0.23	10.68
% Reduction in number of nematodes			60.5

Control = No fungus, no nematodes. Fungus + nematodes = Fermented products and nematodes. Nematodes= Only nematodes.

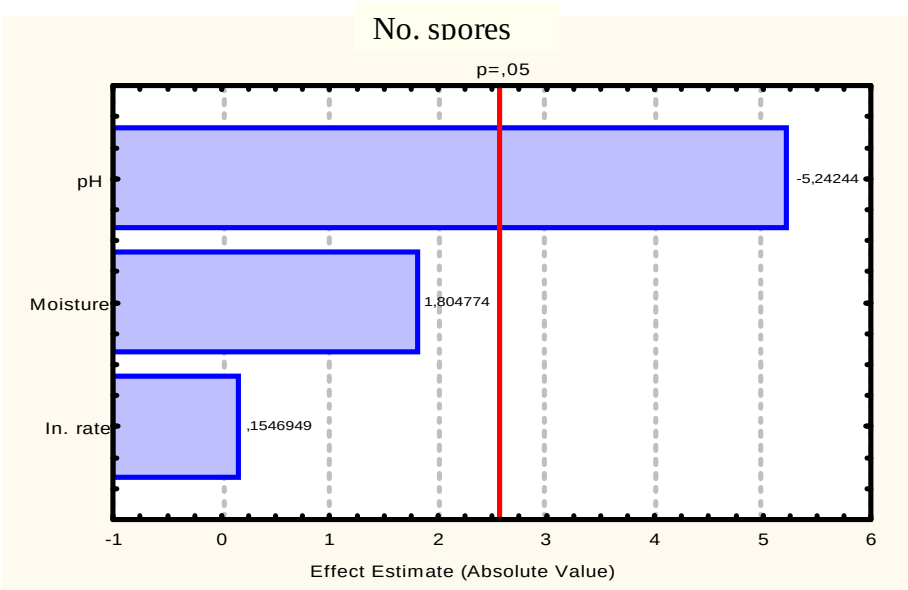

Fig. 1. Pareto Chart of effect for experimental design (2^{3-1}) in the optimization step.

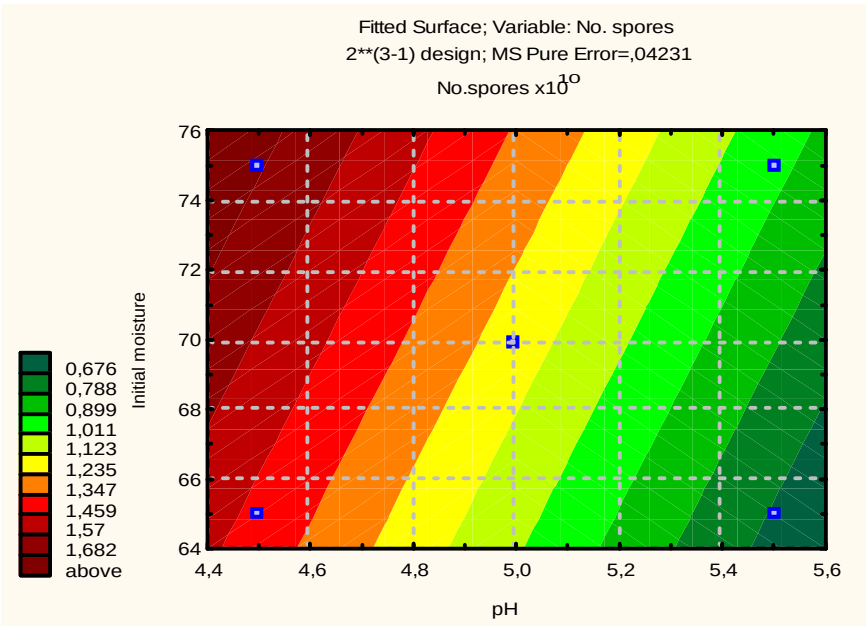

Fig. 2. Contour graph of the experimental design (2^{3-1}) for spore production of the strain of *Paecilomyces lilacinus* LPB-Pl-01 on a substrate consisted of 2:1 cassava bagasse and shrimp wastes. This graph provides the tendencies for a better spore production in relation to pH (significant factor at 5% level) and moisture content. pH values lower than 4.5 should be tested in another experiment (Darker red zone in the left side of the graph may lead to a greater response in lower pHs). Values in the left side represent the spore production ($x10^{10}$).

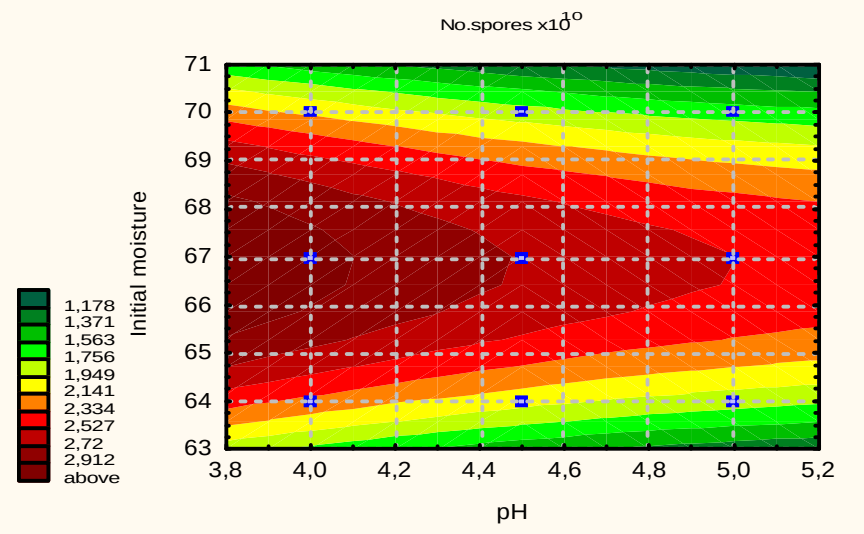

Fig. 3. Contour graph of the experimental design (3^2) for spore production of the strain of *Paecilomyces lilacinus* LPB-Pl-01 in relation to pH and initial moisture content..The left middle darker red zone in the graph corresponding to initial moisture content of 67% and pH 4.0 were the best conditions for spore production as it leads to a circular zone. Values in the left side represent the spore production ($x10^{10}$).

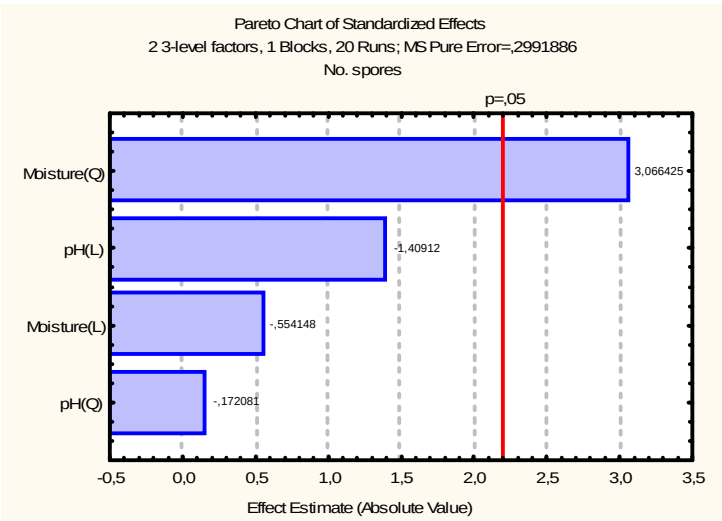

Fig. 4. Pareto chart of effects for experimental design (3^2) in the optimization step for spore production of the strain of *Paecilomyces lilacinus* LPB-Pl-01 on a substrate consisted of 2:1 cassava bagasse and shrimp wastes.

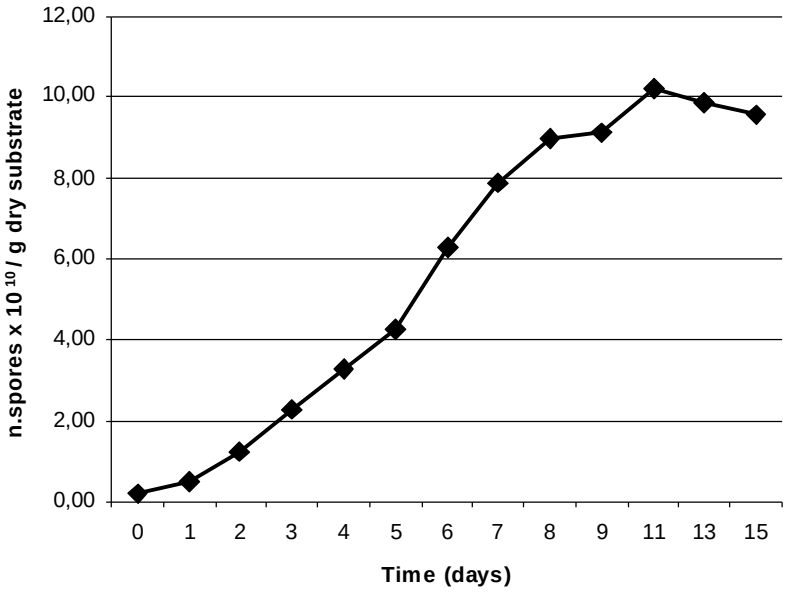

Fig. 5. Spore production of the strain of *Paecilomyces lilacinus* LPB-Pl-01 on 2:1 cassava bagasse and shrimp wastes (average of two replicates).

4.4. Capítulo IV:

Desenvolvimento de um bionematicida com *Paecilomyces lilacinus* para o controle de *Meloidogyne incognita*

ARTIGO 4 - Desenvolvimento de um bionematicida com *Paecilomyces lilacinus* para o controle de *Meloidogyne incognita*

Resumo

A doença de plantas provocada por *Meloidogyne incognita* é uma questão de grande importância pois afeta várias plantas cultivadas de importância econômica. A fermentação no estado sólido (FES) pode ser utilizada para a elaboração de formulações eficazes com fungos utilizados em controle biológico contra os nematóides. Vários substratos de baixo custo, resíduos da agro-indústria, foram testados como substrato para a produção de esporos de *Paecilomyces lilacinus* com uma conhecida capacidade nematicida. As cascas de café, o bagaço de mandioca e o farelo de soja foram utilizados como substrato, e o bagaço de cana-de-açúcar foi empregado como suporte. As fermentações no estado sólido (FES) foram realizadas em frascos de vidro cobertos com papel de filtro, a 28º C durante 10 dias. Os produtos obtidos por FES foram avaliados quanto à sua capacidade nematicida através de experimentos em vasos com a planta *Coleus* inoculados com o nematóide *Meloidogyne incognita* raça 1. As plantas foram avaliadas após 2 meses de inoculação. Os produtos obtidos por FES mostraram uma redução no número de fêmeas de nematóides. Os melhores resultados foram obtidos com o produto de FES com farelo de soja, que demonstrou uma redução de aproximadamente 100% no número de fêmeas de nematóides; a redução do produto com cascas de café foi de 80% e com o bagaço de mandioca de aproximadamente 60%.

Brand, D., Roussos, S., Pandey, A., Zilioli, P.C., Pohl, J., and Soccol, C.R. Development of a Bionematicide with *Paecilomyces lilacinus* to control *Meloidogyne incognita*. *Applied Biochemistry and Biotechnology* 118, 2004, 81-88.

Development of a Bionematicide with *Paecilomyces lilacinus* to Control *Meloidogyne incognita* in Pot Experiments

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Key words: Biocontrol, *Paecilomyces lilacinus*, *Meloydogyne*, coffee husks, cassava bagasse, defatted soybean cake, sugar cane bagasse, Solid state fermentation.

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ABSTRACT

Root-knot disease caused by *Meloidogyne incognita* is a matter of grave concern as 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 biological 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 sugar-cane bagasse was used as support. Fermentations were carried out in glass flasks covered with filter paper at 28º 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 *Meloidogyne 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%.

Introduction

Root-knot nematodes of the genus *Meloidogyne* are important 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 years. 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 nematodes 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 any commonly used nematicide. *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 nematodes galls and increase plant yields (*8-10)*

Solid state fermentation (SSF) has shown tremendous potential in effective utilisation and value addition of agro-industrial by-products (*11-13*). Application of agro-industrial residues in bioprocesses, on one hand, provides an alternative for a sustainable equilibrium of natural organic material in the planet, and on 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 by product originated from the dry method of coffee processing, is rich in organics and nutrients. Recent attempts have focused on its application 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 include protein enrichment with strains of *Rhizopus (*19*),* production of aroma compounds (*20*), and citric acid synthesis (*21).*Defatted soybean cake, the residue obtained after oil

extraction of soybean, is used for the production of proteases owing to its high protein content (*22).* Sugarcane bagasse is one of the largest cellulosic agro-industrial by-product, containing appox 50% cellulose and 25% each of hemicellulose and lignin. Extensive studies have been carried out on its utilization as support 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 substrates (carbon and energy source) and sugar cane bagasse was added as inert solid support. The fermentation products were tested for their nematicide activity in pot experiments containing the plant *Coleus* infected with eggs and juveniles of the nematode *M. incognita*.

Material and Methods

Microorganism, Culture Medium and Inoculum

The strain *P. lilacinus* (LPB-Pl-01) was maintained on potato dextrose agar (PDA) in agar slants at 4ºC. Culture was grown at 28ºC 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) and magnetic stirring. 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 Ltda- Paranavaí-PR, Brazil) were dried in an air oven at 55º C for 24 h, milled manually and sieved to obtain the fractions between 0.8-2.0 mm particle size. Sugar cane bagasse (obtained from local market) was washed three times to remove all the sugar and other solid 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 and mixed with 20 mL of distilled water. The contents were well mixed and sterilized in autoclave at 121º C for 20 minutes. After sterilization, the flasks were inoculated with 2.0 E+07 spores.g^{-1} of dry

substrate. The initial moisture content was made 65% by adding more sterile water and also considering the water coming from the inoculum. Flasks were incubated at 28º C for a period of 10 days. Each assay was done with two replicates.

In a second experiment, the substrate constituted of defatted soybean cake, coffee husk and the mixture of sugar cane bagasse with the two substrates as shown in Table 2. The content of substrate in each flask varied owing to the addition of sugar-cane bagasse as support, which occupies a great volume because of its light weight. Total content of the substrate was of 20 g for assays 1,2 and 5. Assays 3 and 4 were of 12 g. The initial moisture content was of 65% when no sugar cane bagasse was added and 75% with the addition of 2g of the support. The contents were mixed, autoclaved, inoculated and incubated as just described.

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

The spores in fermented matter were harvested by adding 30 mL of water and three drops of Tween-80 to 1g of fermented product and mixing on a magnetic stirrer for 30 minutes. Proper dilutions were made and spore concentration was assayed in 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 greenhouse without thermal, or illumination controls and subject to environmental conditions. Two pots containing good quality sterilized soil were prepared for the tests of the selected fungi and two more pots were utilized as control treatments (in which no fungi was inoculated). All 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 in wet basis (65 % humidity) and the concentration of the nematodes was of 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

hour. The roots were cut in 1 cm portions and 2 subsamples of 1g were weighed for female count under stereomicroscope (magnification: 45X).

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 test greater C/N ratios. This also evaluated wheter 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 augmented in the mixed substrate. When coffee husk was used alone, the final production was of 2.60×10^9 spores/g of dry substrate and for cassava bagasse was 7.60×10^9 spores/g of dry substrate. Thus, cassava bagasse alone used as substrate was much superior than coffe 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 (approximately 40%). St Leger et al. (24) reported that the virulence of the fungi against nematodes is related to the production of enzymes, probably proteases that can destroy the eggshell of the nematode and penetrated it 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 verify whether this had any beneficial effect on spore production. The presence of sugarcane bagasse facilitated higher aeration possibilities in the substrate and retains 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 enhanced the enzyme production (25). Defatted soybean cake demonstrated a spore production of 4.27×10^{10} spores/g of dry substrate which when mixed with sugarcane bagasse (17%) reduced to1.53×10^{10} spores/g of dry substrate (Table 2) 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 as objective to verify the nematicide activity of the fermented products obtained with cassava bagasse, coffee husks and their mixtures. The plant roots were dissected under stereomicroscope for female counting. The results obtained for this experiment are presented in Figure 1. When the quantity of cassava bagasse augmented the composition of the substrate, the number of nematode females fell, even when the the quantity of spores of the fermented product inoculated (see Table 1)was higher. Probably the virulence of the fungi was related to the nature of the substrate employed in fermentation. Most of the studies found in literature combine the utilisation of *Paecilomyces lilacinus* developed in commercial media. When inoculated in pot experiments its ability to control the nematodes increased with the integration of organic matter. The decomposition of organic matter released nematicidal principles and the organical 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 sugar cane bagasse was added as support. Best results were achieved when defatted soybean cake was present in the substrate (see Figure 2), the root galling was practically absent. The reduction in female nematodes was 99.6% and 99.3% respectively, for defatted soybean cake and defatted soybean cake mixed with sugar cane 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 as it possibly produced enzymes capable of degrading the nematodes eggshell. When coffee husks were utilized alone the reduction was in order of 80% and this confirms the results obtained in the first experiment. The addition of sugarcane bagasse to coffee husks was slightly less effective, reducing the number of female nematodes by about 70%.

Conclusions

The results proved the feasibility of using the strain of *Paecilomyces lilacinus* for spore production in SSF and its application as biocontrol agent against the nematode *Meloidogyne incognita* in pot experiments by utilizing the plant *Coleus.* The best spore production achieved was 4.27×10^{10} spores/g^{-1} of dry substrate, which permitted a reduction of almost 100% the number of nematode females in pot experiments. Probably, the virulence of the fungi is

related to the nature of the substrate employed in fermentation, as the addition of 50% defatted soybean cake to coffee husk improved the efficacy of nematode control by 10%. The fermented products should be studied by its application in different ratios of nematode population and fungi concentrations and also in other plants.

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Table 1. Different proportions of coffee husks and cassava bagasse and spore concentration obtained in experiment 1.

Assays	% Coffee husk	% Cassava bagasse	Number spores g dry substrate x 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. Different proportions of coffee husks, defatted soybean cake and sugar-cane bagasse and spore concentration obtained in experiment 2.

Assays	% Coffee husk	% Defatted soybean cake	% Sugar-cane bagasse	Number spores g dry substrate x 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

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

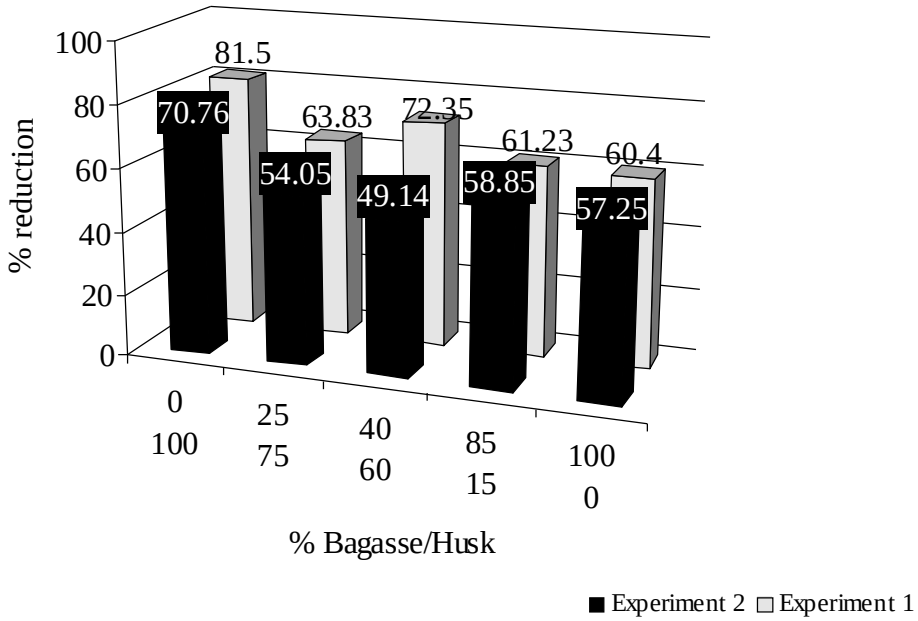
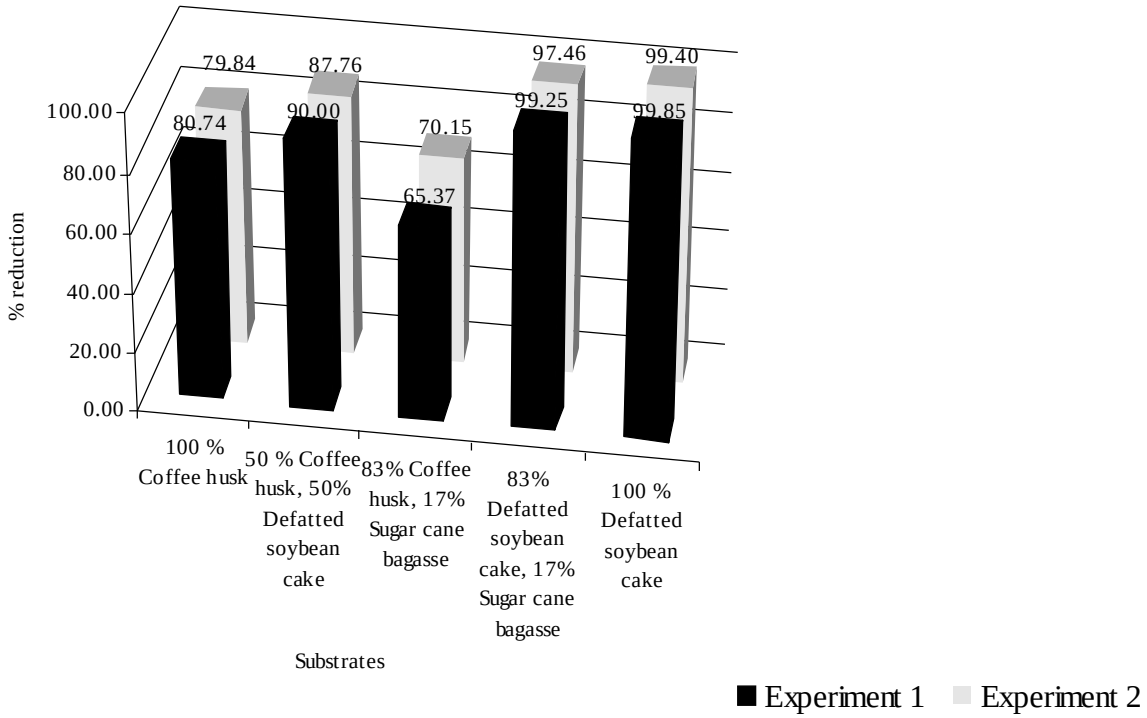


Figure 2 Reduction in the number of nematode females by employing fermented products obtained with coffee husks, soybran and sugarcane bagasse.



4.5. Capítulo V:

Produção de esporos de *Paecilomyces lilacinus* em fermentação no estado sólido e aplicação em vasos para o controle de *Meloidogyne incognita*

ARTIGO 5- Produção de esporos de Paecilomyces lilacinus em fermentação no estado sólido e aplicação em vasos para o controle de Meloidogyne incognita

Resumo

O controle biológico contra os nematóides é uma alternativa muito atrativa à utilização de pesticidas químicos que são perigosos para a saúde humana e para o meio ambiente. O fungo filamentoso *Paecilomyces lilacinus* é conhecido por sua capacidade de parasitar os ovos de nematóides e também por controlar seu desenvolvimento no solo. Diferentes experimentos foram realizados para utilizar os sub-produtos agrícolas como a casca de café e o farelo de soja como substratos para a produção de esporos de *P. lilacinus* por fermentação no estado sólido (FES). Os experimentos foram realizados em frascos de vidro contendo 20 g de substrato sólido seco. Os substratos utilizados se constituíam de farelo de soja, cascas de café ou a mistura dos dois em diferentes proporções (25:75, 50:50 e 75:25). A quantidade inicial de água no substrato era de 67%, o pH de 4,5 e taxa de inoculação de 2.0 E+07 esporos.g^{-1} de substrato seco. Após 10 dias de incubação a 28°C, o índice de esporulação, o pH e a umidade foram determinados. A FES foi igualmente conduzida em um dispositivo úmido contendo bandejas perfuradas com 50, 75 e 100g de substrato (50% casca de café e 50% farelo de soja). Os efeitos do tratamento de secagem e a conservação do produto obtido por FES sobre a viabilidade dos esporos foi determinado após 15, 30, 60, 90, 120 e 150 dias de estocagem. Os produtos obtidos por FES foram testados em experimentos conduzidos em vasos com a planta *Coleus blumei* e nematóide *Meloidogyne incognita* raça 1. O primeiro experimento foi realizado com 7 tratamentos: os 5 produtos de FES com nematóides, dois tratamentos controle com e sem nematóides. O segundo experimento foi realizado em tomate utilizando-se diferentes concentrações do produto de FES obtido em dispositivo úmido. A melhor produção de esporos (1,57 E+10 esporos .g^{-1}) foi obtido quando *P. lilacinus* foi cultivado em dispositivo úmido a 28°C após 10 dias de FES. Todos os produtos de FES testados reduziram o desenvolvimento de nematóides e demonstraram efeitos sobre o peso das raízes das plantas.

Spore production of *Paecilomyces lilacinus* by Solid State Culture and application in Pot Experiments to control *Meloidogyne incognita*

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Key words: Solid State Culture, *Paecilomyces lilacinus*, *Meloidogyne incognita*, Biological Control.

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Abstract

Biological control of nematodes is one of the greatest alternatives to chemical nematicides that are hazardous to men and environment. The fungus *Paecilomyces lilacinus* is well known for its capacity to colonize nematodes eggs and control nematode development in the soil. Attempts were made to utilize the agricultural by-products coffee husk and defatted soybean cake as substrate for the spore production of the nematophagous fungus *P. lilacinus* by solid state cultures (SSC). Experiments were carried out in glass flasks containing 20 g of dry substrate. The substrates consisted of defatted soybean cake, coffee husk and different percentages of their mixtures (25:75, 50:50, and 75:25). Initial water content of the substrate was 67% and pH 4.5, inoculation rate was of 2.0 E+07 spores.g^{-1} of dry substrate. After 10 days of incubation at 28°C spore concentration, pH and water content were evaluated. SSC was also conducted in a humid chamber containing sieves with 50, 75 and 100 g of substrate (50% coffee husks and 50% defatted soybean cake). Effects of air dry on viability of spores was assayed after 15, 30, 60, 90, 120 and 150 days. Pot experiments were accomplished with the plant *Coleus blumei* and the nematode *Meloidogyne incognita* race 1. The first experiment was realized with 7 treatments which consisted respectively to the fermented products obtained with defatted soybean cake, coffee husks and their mixtures, one control treatment and other realized only with nematodes. The second experiment was done with different concentrations of the fermented product obtained in sieves in tomato plants. Best spore production was achieved when *P. lilacinus* was cultivated in a humid chamber at 28°C and resulted in 1.57 E+10 spores.g^{-1} dry substrate after 8 days of SSC. All tested fermented products reduced the development of nematodes, and showed effects in root weight.

Introduction

Biological control is gaining much more attention due to environmental, economical and technical factors which affects important crop cultures, however it has been known for a long time although not well explored. The selection of a biological control agent (BCA) is not easy, due to a high number of available antagonists with diverse mechanisms of action and the consequences of its introduction in the environment should be well evaluated as the attack to non target species, though BCA should demonstrate high host specificity. Along with specificity the utilization of BCA species that are compatible with chemical, physical or other practices utilized to reduce a pathogens population should be considered (*1,2,3*). Another important point but certainly not the least is the economical analysis of implementing a biocontrol program, when the costs and benefits should be well evaluated (*4*).

The plant parasitic nematodes explore all vascular parts of the plants, but the species that cause great economic damage in agriculture are mainly parasites of the roots. Total losses atributted to nematodes are estimated in 10 to 20% for some cultures (*5*). The nematodes of the genera *Meloidogyne* or root-knot nematodes are so denominated due to their interaction with the plants. The nematodes develop specialized feeding structures (root-knots) in the plant root to satisfy their nutritional demands for development and reproduction (*6*). The most employed and efficent method of nematodes control is still through chemical nematicides, which are being repraised or withdrawal from the market due to environmental concerns (*7,8*). The use of resistant varieties is also recent subject of investigation, and several resistance genes were identified and cloned (*9, 10, 11*).

Microorganisms such as filamentous fungi offer great potential as BCAs of nematodes, for their constant association in the rhizosphere of almost all agricultural soils (*12*). One of the most studied fungus for the biological control of root-knot nematodes is the soil Hyphomycete *P. lilacinus*, the strain PL251 was patented and is current produced by two companies and is registered for sale in several countries (*13*). For commercial purposes this strain was assayed for paecilotoxin production and other toxins with anti-microbial activities showing no detectable levels (*14*). *P. lilacinus* is known as a egg-parasite of nematodes, but this event is not simple, it involves the presence of virulence factors that are able to destroy the eggshell and the embryo inside. This virulence factors are extracellular enzymes produced by nematophagous fungi, which correspond to the main constituents of the nematodes cuticle and eggshell(*15*). A serine protease and chitinases from *P. lilacinus* PL 251 were purified and

characterized, by inducing its production with the addition of egg yolk and chitin in a culture liquid media (*16*). This enzymes were applied in the eggshell of *Meloidogyne javanica* reducing the hacthing of juveniles (*17*).

The production of fungi BCAs by fermentation processes must generate a virulent product with high yields of spores, as it is mainly applied in this form in field conditions. Studies of ultrastructure and properties of *P. lilacinus* spores produced in liquid and solid media showed that aerial spores are more resistant to environmental UV radiation, and also viability after storage was better with aerial spores, although both showed similar nemathophagous ability (*18*). The best method for producing BCAs is solid state fermentation (SSF), considering that fungal strains are well adapted to the process in reason of its growth mecanism and that aerial spores demonstrates better stability and viability. SSF is an adavantageous method to produce aerial conidia showing high productivities, extended stability of products and low production costs, which say much about such an intensive biotechnological application (*19)*. This fermentation technique may be accomplished with natural substrates as wheat bran, rice, sugar beet as well as natural supports as sugarcane bagasse, vermiculite supplemented with culture media. Several attempts have been made with the utilization of agricultural by-products as fermentation substrate, giving also perspectives for reducing environmental problems that their disposal may cause (*20*). One of the challenges of SSF is its scale up but increasing progress and application of rational methods in engineering is leading to a more standardized and reproducible method (*21*). Different bioreactors have been designed to overcome the problems that a SSF problems may pose and they may be employed for the production of fungal biopesticides (*22,23*).

The aim of the present work was to produce high yields of *P. lilacinus* spores in substrate consisting of the agricultural by-products coffee husk and defatted soybean cake and also the application of the fermented products in plant pot experiments to verify their action against to the nematode *M. incognita* race 1.

2. Material And Methods

2.1. Microorganism and spore production

The strain of nematophagous fungus utilized in this work was *Paecilomyces lilacinus* LPB-Pl-01. This strain was maintained in potato dextrose agar (PDA) at 4ºC and periodic transferred to agar slants. For inoculum preparation 50 mL of PDA was sterilized at 120°C for 20 minutes in 250 mL Erlenmeyer flasks, before solidification of the media 1 mL of a spore suspension was added to each flask. The flasks were incubated at 28º C for 7 days. Spores were harvest by magnetic stirring and glass beads with 30 mL of distilled water and drops of tween 80.

2.2. Substrate pre-treatment

Coffee husks and defatted soybean cake were dried in an air oven for 48 hours at 55º C. The material was then milled and classified to obtain particle size between 0.8 and 2.0 mm. Coffee husks were gently donated by Café Damasco, Curitiba, Brazil, which cultivates *Coffea arabica* in the northeast region of Parana state, and utilizes the dry process for obtention of the grains ready for torrefaction (*24*). Defatted soybean cake was given by CEPPA (Food Reasearch and Processing Center) , Curitiba, Brazil.

2.3. Solid state cultures (SSC) accomplished with different percentages of defatted soybean cake and coffee husks

Coffee husks and defatted soybean cake were applied alone and with the following mixture percentages (25:75, 50:50 and 75:25; w:w) as substrates for spore production of the fungal strain. SSC were carried out in glass flasks covered with filter paper to allow gas exchanges. To each flask containing 20 g of dry substrate was added half of the volume of the water necessary to reach an initial water content of 67%. The substrate was homogenized and sterilized in autoclave for 20 minutes at 121º C. The spore suspension was added to sterile water in enough quantity to reach the desired water content The substrate was subsequently inoculated with 2.0 E+07 spores.g^{-1} of dry substrate of *P. lilacinus* in asseptic conditions under laminar flow chamber. pH was adjusted to 4.5 with HCl 1N. Incubation was at 28º C for 10 days. The fermentations were done with 4 replicates. Spore production was evaluated in the fermented products as well as its nematicide capacity.

2.4. Spore production

The spore concentration in the fermented products were assayed by taking 1 g of wet substrate magnetic stirred for 30 min with 30 mL of distilled water and three drops of Tween 80. After serial dilutions the spores were counted in Malassez cell under microscope (*22*). The sporulation index is expressed by grams of dry substrate as it was taken in account the final water content of the fermented product (*25*).

2.5. Kinetics of spore production in substate consisted of 50% of coffee husk and 50% defatted soybean cake

The spore production was verified along 12 days of fermentation in substate consisted of 50% of coffee husk and 50% defatted soybean cake. Two flasks were withdrawal each day, from which two samples were removed for the evaluation of the growth as well as the pH and moisture content along fermentation.

2.6. SSC carried out with 50, 75 and 100 g of substrate

The substrate (50% coffee husks and 50% defatted soybean cake) was weighed in a recipient, the addition of water, sterilization and pH adjustement was carried out equally as mentioned above. After inoculation of the solid substrate, the content of each recipient containing respectively 50, 75 and 100g of dry substrate was transferred to sieves, that had been placed inside a device, with 1L sterile water at the bottom for air saturation and covered at the top exit with cotton to allow gas exchanges as demonstrated in Figure 1. The device has approximately capacity for 8L, and internal diameter of 25 cm and 22 cm height. The sieves had a diameter of 15 cm with 3 cm of height and were separate by perforated disks situated at 7 cm from each other. The thickness of the substrate in the sieves for the weights of 50, 75 and 100g (dry basis) were respectively of 1.4, 2.1 and 2.8 cm.

After 8 days of incubation at 28°C, the sieves were removed from the device, under laminar flow chamber and asseptic conditions. After proper homogeneization of the substrate, spore count was realized. Studies of viability and drying of the product were carried out as described below.

Figure 1. Sieves containing substrate incubated in humid chamber at 28°C

2.7. Drying studies

Drying of the fermented products was accomplished with the air passage (100 mL. min^{-1}) held at different temperatures: room temperature (20 – 26° C), 28, 32 and 35° C. In asseptic conditions the fermented product was transferred to glass columns and had been placed in between cotton, which also served as a sterilization agent for the air. An air pump was conected to each column for 30 hours. The dry fermented products were stored at room temperature in the dark.

2.8. Viability of dry fermented products in PDA culture media

The viability of the fermented products was assayed after 15, 30, 60, 90, 120 and 150 days of stockage of the dried products. 1g of the dry product was weighed in 250 mL Erlenmeyer flask, to which was added 99 mL of sterile distilled water, 3 drops of Tween 80 and porcelain fragments to facilitate the dissolution of the femented dry material and the release of the spores. The Erlenmeyer flasks were agitated for 30 minutes at 150 rpm, after that serial dilutions were done until the concentration of 10^{-9}. 1mL dilutions 10^{-7} till 10^{-9} were plated with three replicates to which were added 20 mL of PDA at approximately 40° C. The plates were incubated upside down at 28° C for 4 days, when the number of colonies were counted. Viability of the dry fermented products was determined and compared to the total spore count.

2.9. Pot experiments

2.9.1. Pot experiments realized with fermented products consisted of coffee husk, defatted soybean cake and different proportions of their mixtures

The nematicide activity of the fermented products against nematodes of the genera *Meloidogyne* was evaluated by pot experiments. The first experiment was conducted with the

products obtained after fermentation of the substrates consisted of defatted soybean cake, coffee husk and different proportions of their mixtures (25:75, 50:50, 75:25). The nematodes were reared in *Coleus* plants (*Coleus blumei*). A nematodes suspension was made by taking roots visually infected with nematodes, with great number of galls. Roots were cut in 2 cm pieces, added of water and shaken 3 times for 15 s in a blender (Wallita model Rome). Juveniles and eggs were counted under microscope (Zeiss, Standar 25) in order to establish the inoculation rate for each treatment. The experiments conducted with 4 replicates, consisted of 5 treatments (fermentation products), 1 control (with no fungi nor nematodes) and only nematodes.

Each pot (with capacity for 3 kg) received good quality sterile soil, 1 seedling of *Coleus* and had the same disposition, with layers of soil, fermented product and nematodes suspension alternated around the root of each plant, depending on the case. The concentration of spores was of 10^9 spores.g^{-1} in wet basis (approximately 65 % humidity) and the concentration of the nematodes was of approximately 5000 eggs and juveniles per pot. The experiment was conducted in a greenhouse at Curitiba, Brazil, without thermal neither illumination controls. After 3 months the plants were removed from pots, the roots were isolated, washed and dried at room temperature for 1 hour. The roots were cut in 1 cm portions and 2 sub samples of 1g were weighed for female count under stereomicroscope Wild M8 (magnification of 45X). The reminiscent root was used for determination of dry weight, which was dried at 60º C for 24 hours.

2.9.2. Pot experiments with different concentrations of fermented products consisted of 50% defatted soybean cake and 50% coffee husk

As the fermented products consisted of 50% defatted soybean cake and 50% coffee husk demonstrated more expressive results in pot experiments with *M. incognita* race 1, another experiment was conceived in order to test different quantities of the fermented product (5, 15 and 25 g). The conditions employed in this experiment were: 3 treatments, 1 control and a treatment with only nematodes. At this time, the experiment was conducted in tomato plants (*Lycopersycum esculentum* cv. Ruetgers), and the conditions employed in the experiment were the same as described for *Coleus* plants.

3. Results and Discussion

3.1. Production of *P.lilacinus* spores on coffee husk and defatted soybean cake and their mixtures (75:25, 50:50 and 25:75)

Fermentations were carried out with coffee husks and defatted soybean cake and different proportions of the substrates with initial pH of 4.5 and water content of 67%. The spore production of *Paecilomyces lilacinus* LPB-Pl-01 is demonstrated in figure 2. Each experiment was realized with 4 replicates and figure 2 shows the average of the sporulation index with the respective standard deviations. The pH of the fermented products were all alkaline around 8.05 , showing a great utilization of the substrates by the fungus. The best spore production achieved was with 100% defatted soybean cake reaching 8.7E+09 spores.g^{-1} dry substrate followed by the mixture containing 50% of both substrates that resulted in 4.52 E+09 spores.g^{-1}. All fermented products were tested in pot experiments to investigate their activity against the nematode *Meloidogyne incognita* race 1.

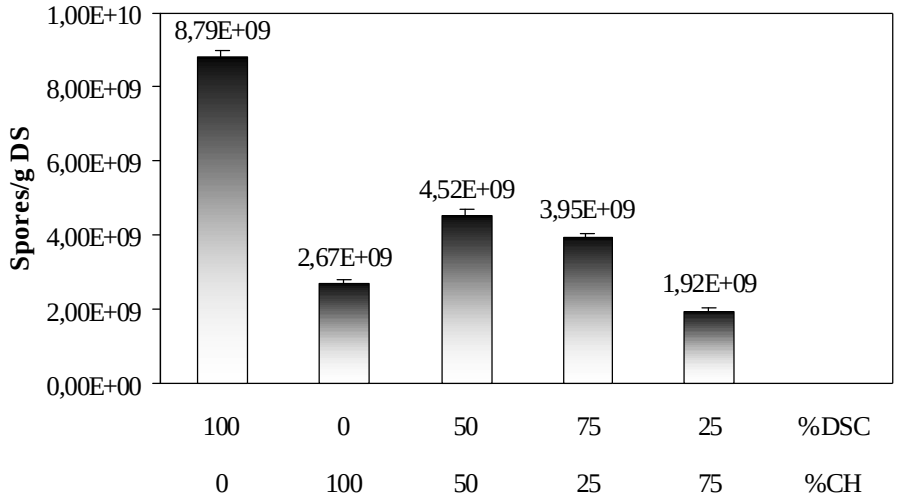

Figure 2. Spore production of *P.lilacinus* in coffee husks (CH) and defatted soybean cake (DSC) and different proportions of their mixture

Defatted soybean cake (DSC) was assayed for the production of proteases from a strain of *Penicillium* sp. in SSF (*26*), this substrate is rich in proteins with percentage composition around 40%, carbohydrates 20%,around 6% of mineral matter, 0.36% of calcium and 0.5% of potassium, on the other side coffee husks has an average of 26.5% carbohydrates and only 4.8% of protein. As it is known the virulence of the fungi against nematodes is related in certain amount to the enzymes, mainly proteases secreted by the fungus (*16)*. In this way

defatted soybean cake was utilized as substrate for spore production and later utilized in pot experiments. Sun and Liu, 2006 tested different carbon sources in the sporulation of nematophagous and entomopathogenic fungi, demonstrating differences between isolates of *P.lilacinus* that were isolated from root-knot and cyst nematodes, spore yields also varied between the carbon sources tested (*27*). Coffee husks presents a reasonable quantity of carbohydrates that can be utilized by the fungus, it was utilized in this work to enhance the concentration of C substances, and because showed positive effects when applied as fermented product in pot experiments against *M. incognita* (*28*).

3.2. Kinetics of spore production in substate consisted of 50% of coffee husk and 50% defatted soybean cake

The substrate consisted of 50% defatted soybean cake and 50% coffee husk was chosen because the best results in pot experiments were achieved with this substrate as will be later discussed. Also the fermented product presented less compactation when compared to defatted soybean cake alone, being this an important characteristic when considering scale up strategies, compactation can lead to problematic heat and mass transfers. In the Table 1, the results of spore production, pH and moisture content of the fermented products are demonstrated along 12 days of fermentation. The results express the average of two flasks that were removed daily and from each flask two samples were withdrawal.

During 12 days of fermentation it was observed that the maximum spore production (6.65E+09 spores.g^{-1} dry substrate) achieved occured in the 8^{th} day, the pH followed the same pattern, it reached it's maximum at the 4^{th} day being 8.7 and remained constant till the 8^{th} day, when spore production was greater, after this period of exponential growth, pH decreased a bit as well as the number of spores. The moisture content from 67% in the beginning of the experiment raised till 70% and was almost constant untill the 8^{th} day of fermentation and afterwards it declined, probably due to the fungus metabolism and dryness of the air. Figure 3 demonstrates the kinetics of spore production along 12 days of fermentation. Growth was intense in the first days, from the 4^{th} till the 8^{th} day the production was more constant (exponential growth phase) and after the maximum production there was a slight decline tending to a stationary phase.

Table1. *Paecilomyces lilacinus* spore production, moisture content and pH during 12 days of fermentation on substrate consisted of 50% coffee husk and 50% defatted soybean cake

Time (days)	Moisture content (%)	pH	N.spores.g^{-1} DS
0	67.02	4.5	4.83E+07
2	68.41	6.2	1.87E+09
3	70.32	7.8	3.47E+09
4	69.11	8.7	3.86E+09
5	68.88	8.7	4.46E+09
6	68.01	8.7	5.00E+09
7	68.52	8.7	5.70E+09
8	68.9	8.7	6.65E+09
9	66.96	8.6	6.32E+09
10	66.18	8.6	6.26E+09
11	64.46	8.2	5.99E+09
12	62.82	8.2	6.02E+09

DS= Dry substrate

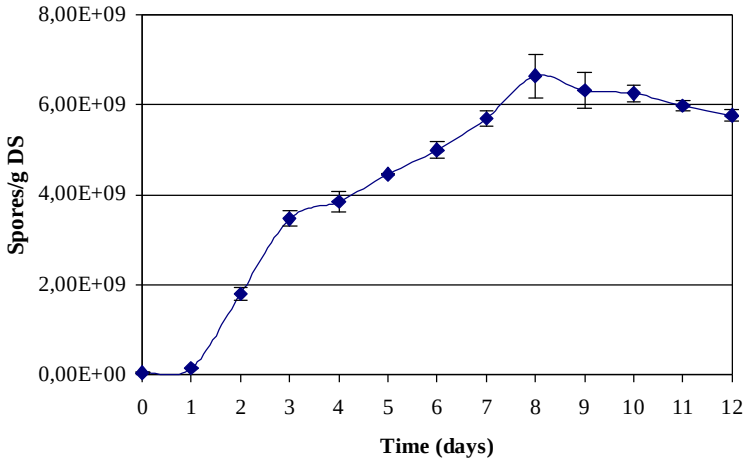

Figure 3. Kinetics pattern of *P.lilacinus* spore production during 12 days of fermentation at 28ºC

There is lack of information about the production of P. lilacinus spores, the most common method used is by the utilization of wheat kernels, resulting in a productivity of 3.5 E+09 spores.g^{-1} after 21 days (29, 30) and rice (31) but there isn't much information of the fermentation parameters utilized, once importance is given to strains activities against nematodes. Kinetics of BCA such as fungi and in this case *P.lilacinus* spore production is an important factor for scale up strategies of a fermentation process and may vary between isolates and substrates used for its production. Also the virulence of an isolate may be related

to the substrate used for production of spores or biomass and in consequence its efficacy as a BCA may differ (21). The fungus BCA *Coniothyrium minitans* was produced in packed bed bioreactor with a support and its growth and sporulation was followed with temperature control over 14 days of fermentation. Dried, non dried and spores produced in PDA were compared for germination and parasitism assays (32).

3.3. SSC carried out with 50, 75 and 100 g of substrate

The fermentation carried out in sieves are demonstrated in Figure 4. After 8 days of fermentation the fermented products were homogeneized and the spore concentration was determined. Two samples of each sieve were removed. The higher spore concentration was achieved with the sieve containing 100 g of substrate (in dry basis), followed by the one with 75 and later the one with 50g, being their average of spores of 1.57E+10, 1.45E+10 and 1.41E+10 spores per gram of dry substrate.

Figure 4. Fermented products obtained in sieves with 50, 75 and 100 g of substrate (dry basis).

3.4. Spore viability of dry fermented products

Spores are viable when they are able to germinate and produce biomass, several factors may affect spore viability such as UV radiation, temperature, A_w, etc... during storage, when spores are dormant. The spore viability was verified in 0 (without drying), 15, 30, 60, 90, 120 and 150 days of storage after drying process. The dried samples were stored at room

temperature in the dark. The results are expressed in percentage of spores that were able to germinate in PDA culture media and consists of the average of 3 samples of each fermented product. The results are compared to the total number of spores counted under microscope for undried product. The viability of the spores achieved for undried product was 57.8%, representing 9.06 E+09 spores.g^{-1} of fermented product (dry weight basis) when compared to the total spore count of 1.57E+10. Figure 5 demonstrates the results obtained for the different driyng systems applied to the fermented product.

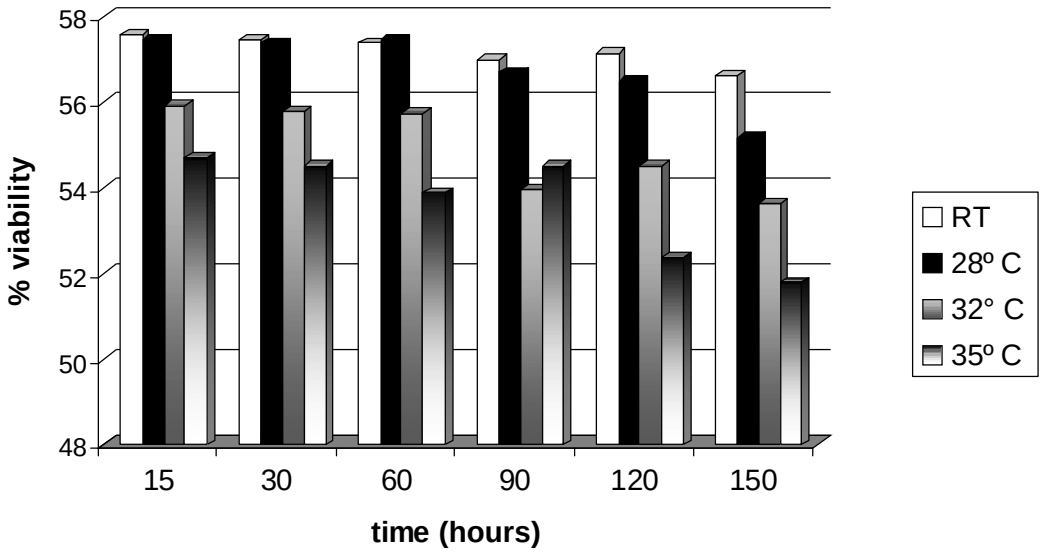

Figure 5. Spores viability of fermented products dried at 28°, 32°, 35°C and at room temperature determined after 15, 30, 60, 90, 120 and 150 days of storage.

Drying treatment didn't affect much the viability of the spores, a reduction of about 10% is observed for the fermented product dried at 32° C, and of less than 2% for treatment at room temperature. Comparing the drying treatments applied to fermented products it is observed that temperature has an effect on the viability of the produced spores. Treatments at 35° and 32°C had propitiated a negative effect on viability, as it decays faster along the time. Best treatments for spore viability were respectively at room temperature and at 28°C. When the fermented product was dried at room temperature viability was maintained at above 56%, when compared to total spore count obtained for the undried product, until 60 days and after that only a small decline was observed. For 28°C a greater decline in 150 days is identified. Water content of dried products were stable during storage and ranged between 5 and 6%. Roussos et al., 1989 studied the influence of the temperature on the viability of filamentous

fungi strains demonstrating great loss of viability when utilizing temperatures above 40°C, when drying spores in a rotavapor (*33*).

3.5. Pot experiments realized with fermented products consisted of coffee husk, defatted soybean cake and different proportions of their mixtures

This experiment was realized with 4 repetitions of each fermented product and also it was evaluated only the substrate without fermentation in female nematode counting and root weight of the plant *Coleus blumei*. The results are demonstrated in figure 6.

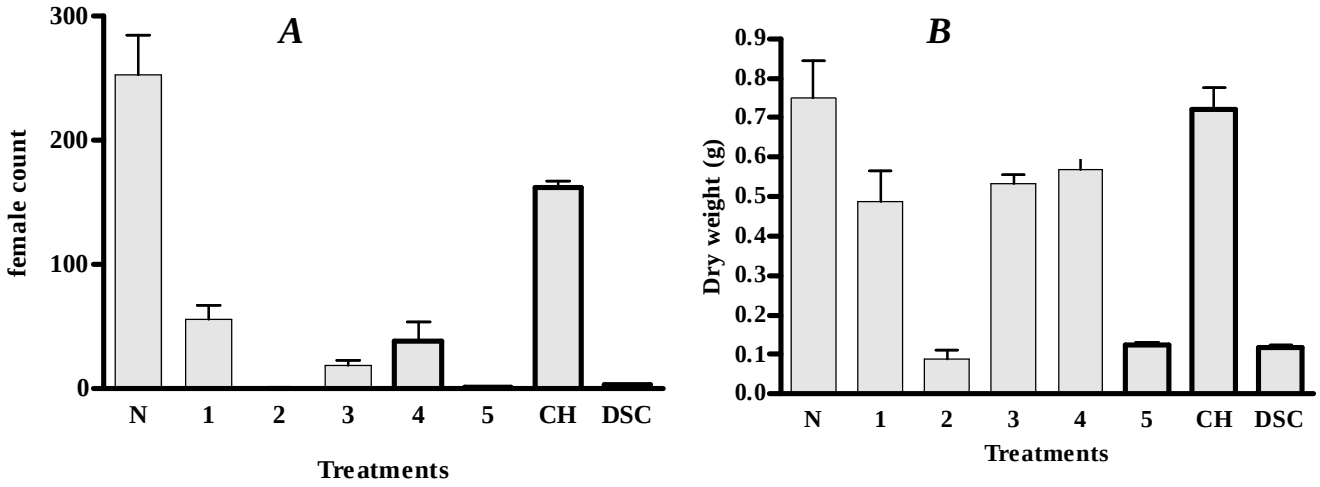

Figure 6. Female count (A) and root dry weight (B)of *Coleus blumei* plants (N = only nematodes, 1-5 fermented product, 1= coffee husk; 2 = fermented defatted soybean cake; 3 = 50% coffee husk and 50% defatted soybean cake, 4 =75% coffee husk and 25% defatted soybean cake; 5 = 75 % defatted soybean cake and 25% coffee husk; CH= coffee husk, DSC= defatted soybean cake

By observing both graphs of figure 6 it can be said that the best treatments were 3 and 4 composed respectively of the mixture 50% coffee husk, 50% defatted soybean cake and 75% coffee husk, 25% defatted soybean cake. In both of the treatments there was a significant reduction in the number of nematodes female and a good average of root weight. Control root weight showed an average of 0.63g dry weight close to the values showed by treatments 1,3 and 4. The utilization of fermented and unfermented defatted soybean cake affected drastically the development of nematodes but also the root development which possibly will affect in plant yield if this product would be utilized with crop plants. This could be due to the high N content added to each experiment. It can also be observed that unfermented coffee husk has little effect against the tested nematodes showing a reduction of only 35% in the

number of nematodes females and the mean average of root weight is higher than the control. Organic amendments were also tested for nematicide capacite when applied in treatment conducted with *M. javanica*, with or without the adition of a strain of *P. lilacinus* (*34*). Although, the fermented husk with the strain *P. lilacinus* LPB-Pl-01 demonstrated a higher reduction in the number of nematodes female, being of approximately 80% which indicated some activity of the fungus against *M. incognita* race 1. One way analyses of variance was accomplished by the Prism 4.0 software, for the number of nematodes females and for root weight, the means were significant different showing a R^2 of respectively 0.9321 and 0.8858. By Tukey's Multiple Comparison test, treatments 3 and 4 didn't differ between each other for both parameters measured. In a global evaluation of the experiment the greatest average of root weight was in treatment 4 when it was achieved a good reduction in the number of females. When only nematodes were inoculated in the plants, also it was observed a greater root weight, this is possible a response of the parasitized plant, which presented great number of galls.

3.6. Pot experiments with different concentrations of fermented products consisted of 50% defatted soybean cake and 50% coffee husk

This experiment was realized with the aim to verify the influence of different concentrations of fermented product in the control of *M. incognita* race 1 inoculated in tomato plants. The results obtained for root dry weight and nematodes female count are demonstrated in figure 7. For the factor dry weight, five experimental groups were analysed, which showed significant differences of the averages ($p=0.011$) by means of analysis of Variance with respective R^2 of 0.8161. Tukey's multiple comparison test showed that there are no differences between treatments 5 and 25 g. But significant differences were found when comparing the averages of treatment 15g with 5g and 25g.

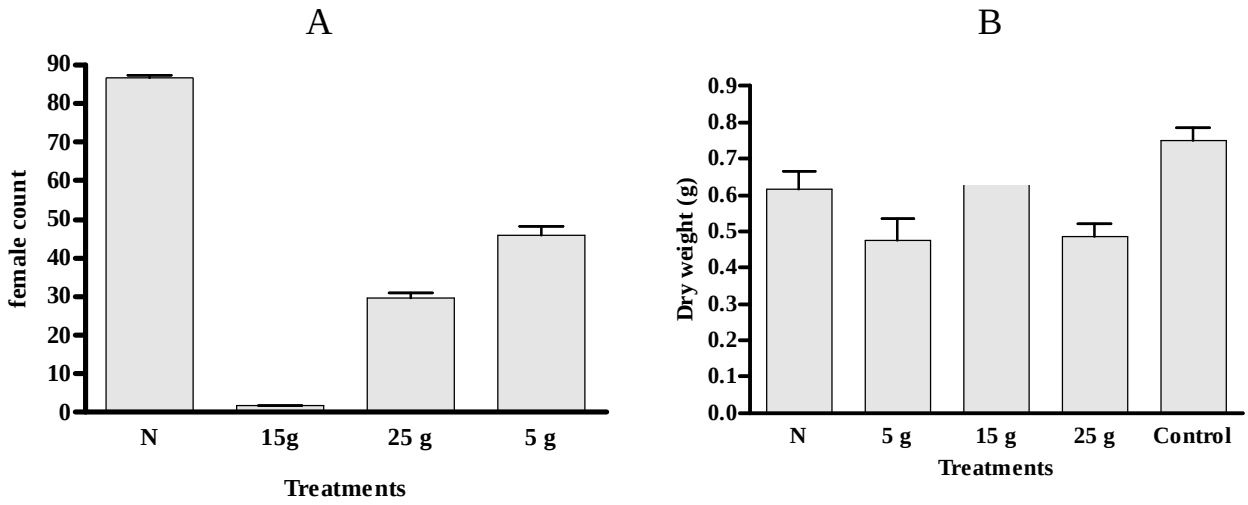

Figure 7. Female count (A) and root dry weight (B)of tomato plants treated with 5, 15 and 25 g of fermented product

In the case of female count the averages were also significant different between each other being $p<0.001$ and R^2 of 0.9865. When all treatments were compared by Tukey' s test they were significant different. The percentage of nematodes females reduction are 47.1, 65.81 and 98% respectivelly for the treatments with 5, 25 and 15g of fermented product.

In a study conducted with the fungal biocontrol agent, *Trichoderma harzianum* produced in peat bran, it was evaluated its potential to control the root-knot nematode *M. javanica*. In greenhouse experiments, root galling was reduced and top fresh weight increased in nematode-infected tomatoes following soil pretreatment with *Trichoderma* peat-bran preparations (*35*).

Conclusions

With this work it was proved that it is feasible to utilize coffee husk and defatted soybean cake as substrate for spore production of the strain *P. lilacinus* LPB-Pl-01. The best spore production achieved was with a mixture of 50% defatted soybean cake and 50% coffee husks in humid chamber, showing good perspectives for scale up. Temperature had a negative effect on the spore viability, best drying temperature was room temperature, demonstrating less viability loss. Through pot experiments it was possible to verify that defatted soybean cake fermented or unfermented showed a negative effect in the development of plant root even with the best sporulation rate achieved. The best fermented products for *M.incognita* race 1 control in *Coleus* plants were the mixture of 50% of the substrates and the mixture 75:25 of coffee husk and defatted soybean cake given a reduction of approximately 90% in nematodes female. When applying different concentrations of the fermented product consited of 50% of each substrate it was observed that with 15g of the wet product (approximately 0.1% in dry basis of total pot content) produced a better effect on root dry weight and female count in tomato plants.

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Parte 3: Respirometria

ARTIGO 6 – Efeito do modo de inoculação sobre o crescimento e a esporulação de *Paecilomyces lilacinus* determinado por respirometria

Resumo

As perdas mundiais causados por nematóides ultrapassam U$ 100 bilhões por ano, levando a uma redução no rendimento e na qualidade de culturas de grande importância econômica. O fungo filamentoso *Paecilomyces lilacinus* é um parasita efetivo do nematóide do gênero *Meloidogyne*, que causa os maiores danos na agricultura. Experimentos de fermentação no estado sólido (FES) foram realizados em fermentados de colunas com substrato constituído de 50% farelo de soja e 50% de cascas de café. Dois diferentes modos de inoculação (micélio e suspensão de esporos) foram comparados para a produção de esporos do fungo em condições otimizadas: pH 4,5, umidade inicial de 65% e fluxo de ar de 60 mL/min/coluna. As colunas foram amostradas cada 24 horas para a determinação do índice de esporulação, pH e consumo de açúcares totais. A produção máxima de esporos (8.51E+09 esporos.g^{-1} substrato seco) foi obtida com a inoculação de uma suspensão de esporos em 168 horas de fermentação. A produção de esporos quando foi inoculado o micélio foi menos significativa (3.41E+09 esporos.g^{-1} substrato seco). A partir dos valores obtidos através de experiências para o consumo de oxigênio e a evolução do CO_2, o rendimento de biomassa ($Y_{x/o}$) foi 0.415 e 0.545 g biomassa.g^{-1} O_2 consumido e coeficiente de manutenção (m) de 0.1172 e 0.1834 g O_2 consumido.g^{-1}biomassa.h^{-1}, respectivamente para a inoculação do micélio e da suspensão de esporos. A velocidade de crescimento de *P. lilacinus* em FES realizada com a inoculação do micélio é mais rápida e não existe uma fase de germinação de esporos, mas a produção de esporos foi mais expressiva quando realizou-se a inoculação de esporos.

Effect of inoculation pattern on *Paecilomyces lilacinus* growth and sporulation followed by respirometry

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Key-words: solid state fermentation; *Paecilomyces lilacinus*; respirometry; spore production.

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Summary

Annual loss attributed to nematodes are estimated in over 10 billion dollars, causing yield and quality reduction of economical important crops. The fungi *Paecilomyces lilacinus* is an effective parasite of the nematode *Meloidogyne,* which causes the greatest damages in agriculture. Attempts were made to utilize agricultural by-products for the spore production of the strain *Paecilomyces lilacinus* LPB-Pl-01 by solid state fermentation. Fermentations were carried out in column type bioreactor in substrate consisted of 50% defatted soybean cake and 50% coffee husk, with mycelium and spore inoculation and optimized conditions of pH 4.5, moisture content of 65% and 60 $mL.min^{-1}$ aeration flux. Every 24 hours, samples were withdrawal for analysis of pH, total sugar consumption, ergosterol and spore production. Maximal spore production (8.51E+09 $spores.g^{-1}$ dry substrate) was achieved with inoculum consisted of spore suspension in 168 hours of fermentation, when sugar consumption was faster pH was of 8.5. Spore production from mycelium inoculation is less effective but the patterns of sugar consumption, pH and ergosterol are similar. From the values obtained experimentally for the oxygen uptake rate (OUR) and CO_2 evolved, the system determined a biomass yield ($Y_{x/o}$) of 0.415g and 0.545g $biomass.g^{-1}$ consumed O_2 and a maintenance coefficient (m) of 0.1172 g and 0.1834 g of consumed $O_2.g^{-1}biomass.h^{-1}$ respectively for mycelium and spore inoculation. The average specific growth rates were respectively 0.11 and 0.08 for mycelium and spore inoculation. The growth velocity in fermentations conducted with inoculum consisted of mycelium is faster, as there isn't a germination phase of the spores, however spore production is greater with inoculation of spores.

Introduction

Biological control of pests is becoming a great alternative for the use of chemical substances which are pollutant and hazardous for men and environment. Such problems lead to the search of less pollutant methods to control plant diseases, which will contribute to the objective of the sustainable development in agriculture (*1*). Biological control can be defined as the action of parasites predators or pathogens in maintaining another organism's population density at a lower average than would occur in their absence (*2*).

Plant parasitic nematodes cause damage to over 100 Billion US dollars per year throughout the world, affecting economically important crop plants (*3*). Root-knot nematodes belongs to the genera *Meloidogyne,* they affect root system yield which results from invasion or feeding of root tissue due to rapid multiplication of cells in the cortical region. Plant-parasitic nematodes that are sedentary during maturation before oviposition are considered to be the most important nematode pests, attacking over 90% of the food and fiber crops (*4*).

The control of plant parasitic nematodes is usually done by a variety of methods as crop rotation, use of resistant plants and chemical control. Crop rotation is of difficult use with *Meloidogyne* due to its wide range of hosts. Resistant plants are also widely used as the recent cloned *Mi* genes in tomato, however still it was not found resistance for some types of plants and some types of nematodes are capable of overcome this monogenic resistant (*5,6*). Chemical control is still the most employed and effective method, however environmental concerns and the impending ban on methyl bromide as soil fumigant is reducing its utilization (*7*).

Nematophagous fungi are an example of natural enemies of nematodes that can reduce their populations. *Paecilomyces lilacinus* is a known soil Hyphomycete that attack nematodes eggs and females being interesting as biological control agent (BCAs). This fungus was extensively studied under field and greenhouse experiments with or without the addition of supplements to the soil (*8, 9, 10*) and in the majority of the times it showed positive results, although its production was either by utilizing wheat grain or liquid media. *Paecilomyces lilacinus* produces extracellular serine proteases that probably have an important role as virulence factor of this fungus in attacking nematodes since it must penetrate barriers presented by the host (*3*).

Solid state fermentation (SSF) offers great potential for mass production of BCAs, also presents as advantage: the utilization of agricultural by-products as substrates for fungal

growth (*11)*. The fermented products consisting of agricultural by-products, fungal biomass and spores may be directly applied in the soil for nematodes control helping to solve pollution problems that their disposal may cause (*12*). Also the utilization of SSF helps for a sustainable equilibrium of organic material in the planet and provides different value-added products such as gibberellic acid (*13*), edible mushrooms with anti-tumoral activities (*14, 15)*, enzymes such as proteases (*16*), amylases (*17*) and organic acids (*18*).

The amount of biomass in an essential parameter in kinetic studies for the determination of the conditions for optimal growth and sporulation of fungi. The estimation of biomass during a SSF process is rather difficult due to the impossibility of a direct measurement by separation of biomass from the solid matrix. In SSF, biomass should be measured by indirect methods by the determination of one or more cell components such as glucosamine, proteins, nucleic acids, proteins and ergosterol (*19-23*), although some of the methods may suffer interference from microorganisms metabolism and substrate composition. Biomass can also be measured by oxygen consumption and or carbon dioxide production (respiration) providing on line information (*24*,*25*).

The aim of the present work was to evaluate biotechnological parameters such as biomass yield, maintenance coefficient and growth velocity in SSF conducted with inoculum of the nematophagous fungi *Paecilomyces lilacinus* LPB-Pl-01 consisted of mycelium and spores in a mixture of the agricultural by-products defatted soybean cake and coffee husk, by respirometric analysis.

2. Material and Methods

2.1. Microorganism and inoculum production

The strain of nematophagous fungi utilized in this work was *Paecilomyces lilacinus* LPB-Pl-01. This strain was maintained in potato dextrose agar at 4º C and periodic transferred to agar slants. For inoculum consisted of spores: 50 mL of PDA was sterilized in Erlenmeyer flasks, before solidification of the media, 1 mL of a spore suspension was added to each flask. The flasks were incubated at 28º C for 7 days. Spores were harvest by magnetic stirring and glass beads with 30 mL of distilled water and drops of tween 80.

Inoculum consisted of mycelium: 50 mL of YM broth was sterilized in Erlenmeyer flasks, after cooling 1 mL of a spore suspension was added. Incubation was at 28º C for 72 hours.

2.2 Substrate pre-treatment

Coffee husks and defatted soybean cake were dried in an air oven for 48 hours at 55º C. The material was then milled and classified to obtain particle size between 0.8 and 2.0 mm.

2.3. Solid State Fermentation (SSF)

SSF was carried out in glass columns as described by Raimbault and Alazard (*26*). Each column had the capacity for 30 g of the substrate (dry weight basis). Columns and substrate were sterilized in autoclave for 20 min at 121°C. Substrate consisted of 50% defatted soybean cake and 50 % coffee husks. Pre-inoculated substrate was packed in the columns. Optimization step was carried out only with inoculum consisted of spores, kinetics and respiration studies were accomplished with spore and mycelium inoculation. Humidified air was passed through the columns at desired flow rates. SSF experiments were carried out at 28ºC for 10 days.

2.3.1 Optimization of initial moisture content of the substrate and aeration flux

Initially a study to verify which would be the best conditions of initial moisture content and aeration in spore production of *Paecilomyce lilacinus* in a substrate consisted of 50% defatted soybean cake and 50% coffee husk was realized in column type bioreactors. The optimized initial moisture content utilized in flasks was 67%, as in columns bioreactors the air that passes through is saturated in water, the tests were accomplished with lower values. A complete 3^2 design generated by the software STATISTICA was utilized, the studied factors were initial moisture content and aeration in three levels with a central point. All experiments were realized with two replicates, resulting in a total of 20 columns, the statistical matrix is demonstrated in Table 1.

Table 1. 3^2 design for optimization of initial moisture content of the substrate and aeration flow rate in column type bioreactor

Test	Initial moisture (%)	Aeration ($mL.min^{-1}$)
1	63	30
2	63	60
3	63	90
4	65	30
5	65	60
6	65	90
7	67	30
8	67	60
9	67	90
10 (C)	65	60

Kinetics studies of *Paecilomyces lilacinus* spore production:. Every 24 hours, samples were collected for evaluation of the spore production, ergosterol, total sugar consumption as well as pH, and moisture content during 10 days.

Growth estimation: In elapsing of the kinetic study, the respiratory metabolism of the micro-organism was evaluated by determining the O_2 consumption and the production of CO_2. This was utilized to estimate the biomass biosynthesis by the fungal culture. The exit gases from the fermenter were passed through silica gel columns to dry and then analyzed by gas chromatography to determine the oxygen uptake rate (OUR), the CO_2 evolved and the respiration quotient (RQ) during the process following the mathematical model developed by Rodriguez-Leon (*27*).

2.4. Analytical methods

Total sugar was determined after acid hydrolysis by Somogyi-Nelson method (*28).* pH was determined by potentiometry and moisture content by gravimetric method.

The spore concentration in the fermented products were assayed by taking 1 g of wet fermented substrate, magnetic stirred for 30 min with 30 mL of distilled water and 2 drops of Tween 80. After serial dilutions the spores were counted in Malassez cell under microscope.

The number of spores is expressed by grams of dry substrate as it was taken in account the final moisture content of the fermented product.

2.5. Determination of Ergosterol

1 g of the fermented product was weighed for analysis of ergosterol. The first step consisted of saponification of the samples and after ergosterol was extracted three times with n-hexane. After evaporation of the solvent by vaccum, 500μL of n-hexane was added and immediately filtered by membranes. Ergosterol was then analysed by HPLC (Varian ProStar), with C_{18} column and PDA detector at 282 nm. 10 μL of sample was used, the mobile phase was pure methanol (0 to 3 minutes), acetonitrile (3-10 minutes and again methanol (10-15 minutes with 2 $mL.min^{-1}$ elution flux. A standard solution of Ergosterol PA was used in several dilutions to obtain a standard curve as determined by De Carvalho et al (*23*).The ergosterol content in the biomass was estimated to be 4.9 $mg.g^{-1}$, as previously determined.

3. Results and Discussion

3.1. Optimization of initial moisture content of the substrate and aeration flux in spore production of *Paecilomyces lilacinus* LPB-Pl-01 in column type bioreactor

A complete factorial design 3^2 was utilized with the objective to find the best conditions for sporulation of *P. lilacinus* regarding initial moisture content and aeration in column type bioreactor. The experimental factors were distributed in three levels with one central point and the analysys of the results is based on the response surface obtained for the experiment, as showed in Figure 1. The results achieved for spore production were submitted to analysis of Variance , which is demonstrated in Table 2. From the statistical analysis accomplished for spore production of *Paecilomyces lilacinus* in column type bioreactor, the only significant factor at 5% level was the quadratic effect of the initial moisture content, being the correlation factor of 0.99823 being satisfactory for the design.

Table 2. Anova table for the 3^2 design for *Paecilomyces lilacinus* SSF optimization

Factors	Sum of the squares	Degrees of Fredom	Mean of the squares	F value	P value
(1) Moisture (L)	0.673350	1	0.673350	52.6055	0.087224
Moisture (Q)	2.582449	1	2.582449	201.7538	0.0044746*
(2)Aeration (L)	0.601033	1	0.601033	46.9557	0.092253
Aeration (Q)	1.850280	1	1.850280	144.5531	0.052828
1 L by 2L	0.435600	1	0.435600	34.0313	0.108079
1 L by 2Q	0.000300	1	0.000300	0.0234	0.903289
1 Q by 2L	0.056307	1	0.056307	4.3990	0.283235
1 Q by 2Q	0.000892	1	0.000892	0.0697	0.835729
Pure error	0.012800	1	0.12800		
Total	7.244149	9			

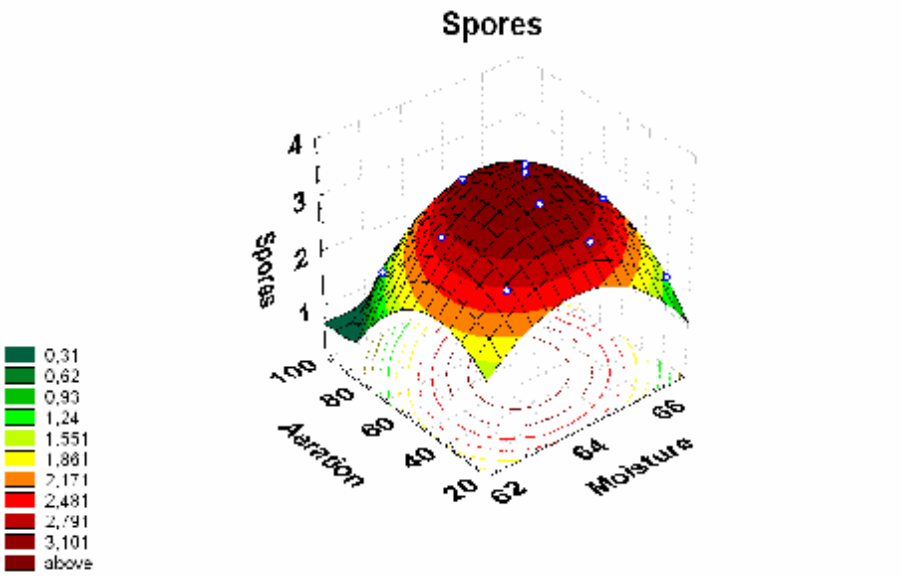

Figure 1. Surface response for design3^2 of *P.lilacinus* spore production in SSF.

Maximum spore production was achieved with 65% initial moisture content and aeration flux of 60mL.min$^{-1,}$ as it can be seen in figure 1, where it is showed a well delimited zone for a greater response of the model, the production was of 9.45 E+10 spores.g^{-1} dry substrate. At higher aeration flux , the spore production seems to be lower, probably because it leads to a greater compactation of the substrat once the quantity of water saturated air pass through the columns. At lower rates, perhaps the quantity of air that passes is not enough to cool down the heat generated by the fungal strain leading to a decrease in the metabolism. Lowest

production was 6.02 E+09 spores.g^{-1} dry substrate with 90 mL.min^{-1} and 67% of initial moisture content.

3.2. Kinetics of spore production by *Paecilomyces lilacinus* LPB-Pl-01 with inoculum consisted of biomass and spores in column type bioreactor

The kinetic study accomplished with the optimized conditions for conidia production of the strain *Paecilomyces lilacinus* LPB-Pl-01 aimed a better understanding of the growth of the fungi in the substrate consisted of 50% defatted soybean cake and 50 % coffee husk by analyzing total sugar consumption, pH and also by respirometric analysis. This substrate was chosen as it gave better results when applied in greenhouse experiments with tomato plants (*29).* Two types of inoculum were used, a traditional spore suspension and also a mycelium suspension as it could make fermentation faster and produce comparable amounts of spores to apply in biological control assays. Dry fermented products obtained by SSF using agricultural by-products is a great way to deliver the biological control agent to the soil as the inoculum used in field conditions is consisted mainly of spores which must be able to survive in the environment and to infect the nematode (*30*).

Table 3 shows the evolution of the pH, the number of spores and the consumption of total sugars along 216 hours of fermentation with inoculation of spores.

Table 3. pH evolution, spore production and total sugars consumption with spore inoculation of the fungi LPB-Pl-01.

Time (hours)	pH	n. spores/DS	Total sugar g%
0	4.5	2.3 E+07	9.8
24	5.7	9.88E+07	8.1
48	6.41	1.35E+08	6.88
72	8.31	1.55E+09	5.86
96	8.64	2.86E+09	5.6
120	8.56	3.64E+09	5.57
144	8.31	5.48E+09	4.45
168	8.51	8.51E+09	4.02
192	8.39	8.17E+09	3.78
216	8.32	7.57E+09	2.98

DS = dry substrate

Maximum spore production (8.51E+09 spores.g^{-1} dry substrate) was determined in 168 hours of fermentation, when it was observed a pH value of 8.51, being most of the sugar already consumed. After the obtention of maximum spore concentration it was verified a slight decline in the number of spores, once they could germinate as there wasn't total depletion of the carbon source present in the employed substrate. It is observed that the production of spores starts from 48 hours of fermentation, probably due to a previous biomass synthesis needed to occur before the sporulation of the fungi. The consumption of total sugars is more accentuated in this phase probably because of the needs of the fungi for biomass formation, and after this phase the velocity of sugar consumption is slower. The production of *Paecilomyces lilacinus* spores by mycelium inoculation is lower, as it can be seen in Table 4. In the figure 2 the production of spores from the two types of inoculation are demonstrated.

Table 4. pH evolution, spore production and total sugars consumption with biomass inoculation of the fungi LPB-Pl-01.

Time (hours)	pH	n. spores/DS	Total sugar g%
0	4.5	2.51E+07	9.74
28	6.5	4.45E+08	7.96
48	8.55	1.70E+09	5.68
72	8.63	2.94E+09	4.75
96	8.57	3.31E+09	4.24
120	8.54	3.41E+09	4.03
144	8.54	3.36E+09	3.98
168	8.56	3.32E+09	3.54
192	8.36	3.29E+09	3.64
216	8.34	3.12E+09	3.42

The production of spores from mycelium inoculation is lower, being maximum production reached at 120 hours of fermentation when more than half of the carbon source was consumed. The inoculation of spores is probably more suitable for SSF processes as the spore can enter within the matrix due to its size and germinate homogenously throughout the substrate.

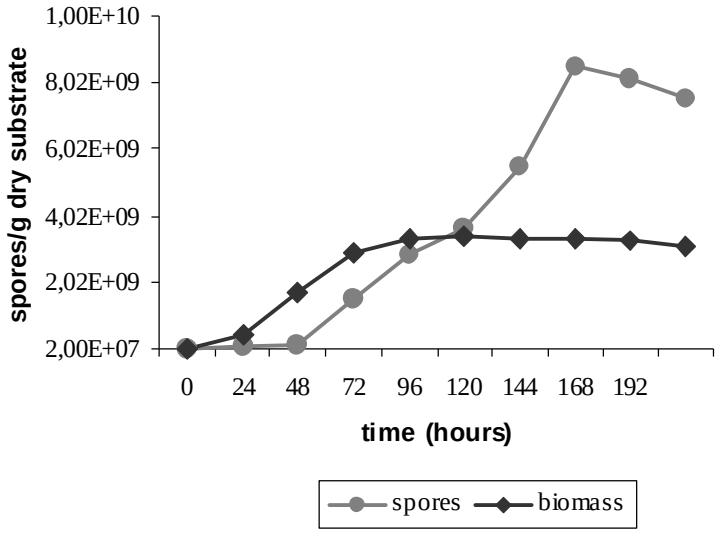

Figure 2. Production of spores of *Paecilomyces lilacinus* LPB-Pl-01 by inoculation of spores and mycelium

The pH and the sugar consumption follow practically the same pattern in both fermentations accomplished. The raise in the pH is probably due to the presence of great amounts of N substances in the substrate and perhaps one of the final products of the fungus metabolism is ammonia. The quantity of protein in the media was verified and it was constant, having even a slight decrease (data not shown). The maintenance of high amounts of protein in the substrate (approximately 30%) probably occurs due to a intense enzymatic synthesis, being in that case the biomass estimated by ergosterol and not by protein or glucosamine synthesis.

3.3. Respirometric analysis

The kinetics of SSF was determined by measuring the Oxygen Uptake Rate (OUR), the CO_2 evolved and the respiration quotient (RQ) during the process. A balance was made for the estimation of the OUR and the CO_2 evolved in terms of volumetric flow ($l.h^{-1}$), considering a initial weight of 30 g of dry matter. The exhausted air flow (F_{out}) was 0.045 $l.h^{-1}.g^{-1}$ initial dried weight and the inlet air flow (F_{in}) to the fermenter. The following equation were considered:

$V_{O2out} = (\%\ O_{2out}/100)\ Fout$

$V_{CO2out} = (\%CO_{2out}/100)\ F_{out}$

$V_{N2out} = ((100 - \%O_{2out} - \%\ CO_{2out})/100)F_{out}$

From the balance of O_2 and N_2 we obtained that:

$V_{O2uptake} = (20.9/100)\ F_{in} - (\%\ O_{2S} / 100)\ F_{out}$

$V_{N2in} = V_{N2out}$

Relating the several equations considered, the following relationship for the inlet and the outlet air flow was obtained:

$F_{in} = ((100 - \%O_2 - \%CO_2)\ F_{out})/79.1$

For the estimation of the OUR and the CO_2 evolved in mass flow units (mmoles.h^{-1}), it was considered that the air was an ideal gas, the respective volumetric flows ($V_{O2\ uptake}$ and $V_{CO2\ out}$) and the proper corrections for temperature conditions considering a temperature value of 28ºC. Figures 3 present respectively the evolution of oxygen uptake rate and CO_2 production during fermentations accomplished with spores and biomass inoculation

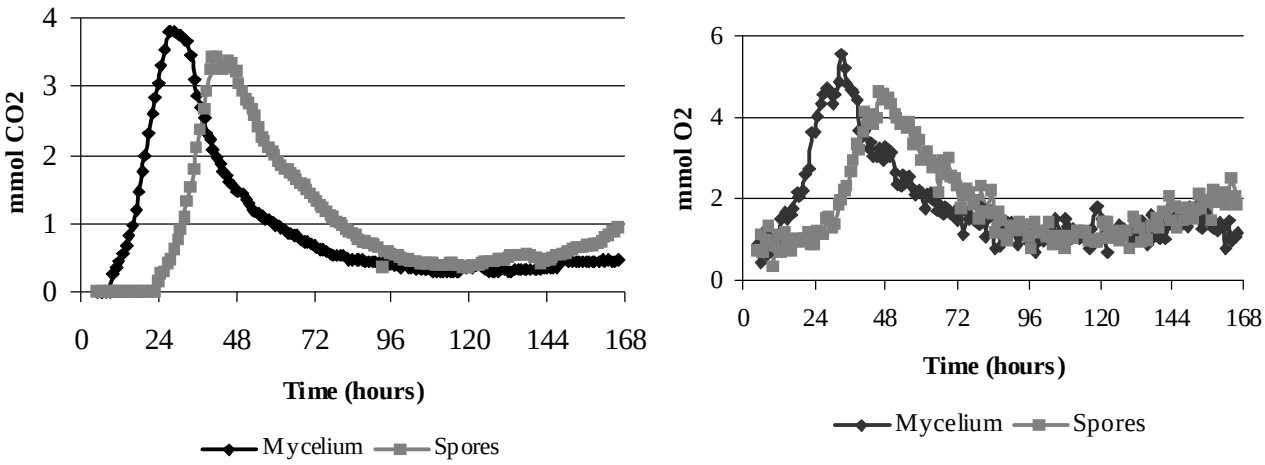

Figure 3. Kinetic pattern of the OUR and CO_2 evolved during the SSF of defatted soybean cake and coffee husk by *Paecilomyces lilacinus* LPB-Pl-01

Based on the results of OUR and CO_2 evolved, a estimation of the bioprocess parameters was realized. Considering the balance of the OUR. from this, following equation was obtained (*31*).

$$Xn = \frac{Y_{x/0}\Delta t(1/2)((dO2/dt)_{t=0} + (dO2/dt)_{t=n}) + \sum_{i=1}^{i=n-1} dO2/dt)_{t=i}) + 1 - (a/2))Xo - a\sum_{i=1}^{i=n-1} Xi}{1 + a/2}$$

where: a = m ($Y_{x/o}$)Δt

The procedure to estimate biomass content in a certain time (X_n) consisted in make a trial and error estimation, assuming values for the biomass yield based on oxygen consumption ($Y_{x/o}$) and maintenance coefficient (m) using FERSOL (*27*).The biomass in the fermented products were determined in times 0, 12, 24 and 48 hours as earlier described. By successive approximations, the software allowed to determinate the equation coefficients. The values determined by the system for biomass yield ($Y_{x/o)}$, maintenance coefficient and growth velocity (μ) for both inoculum types are demonstrated in the following table.

Table 5. Biotechnological parameters estimated

Parameter	Inoculum type	
	Biomass	Spores
($Y_{X/O}$)	0.415	0.545
m	0.1172	0.1834
μ	0.11	0.08

Biomass yield was a little higher during exponential phase of fermentation with spore inoculation being respectively of 0.545 and 0.415 g of biomass.g^{-1} consumed O_2 for spore and biomass inoculation. The cellular maintenance coefficient (m) followed the same pattern showing values of 0.1834 and 0.1172 for spore and biomass inoculation, respectively. Although , the specific growth velocities showed values of 0.11 h^{-1} and 0.08 h^{-1} for biomass and spore inoculation. The growth velocity in the fermentation conducted with biomass inoculation is faster, as it doesn't exist a phase of spore germination and adaptation to the media to initiate biomass synthesis. As it can be observed at the anterior figures, in the kinetics realized with biomass inoculation the exponential growth phase occurs a bit more advanced then it was utilized spore inoculation, consequently a smaller adaptation phase. Still by the software Fersol the concentrations of biomass and the specific growth velocities in determined time relative to the exponential growth phase of each fermentation were calculated and are demonstrated respectively by figures 4 and 5.

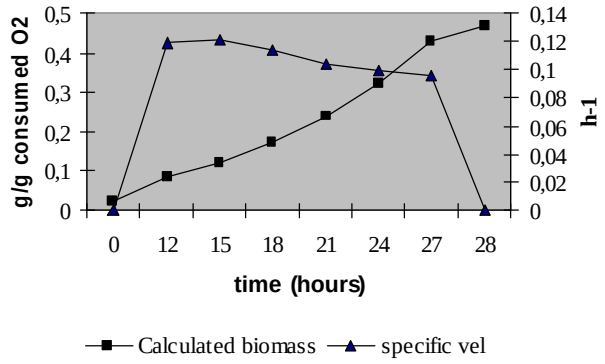

Figure 4. Estimate biomass and specific growth velocity during exponential growth phase SSF with mycelum inoculation

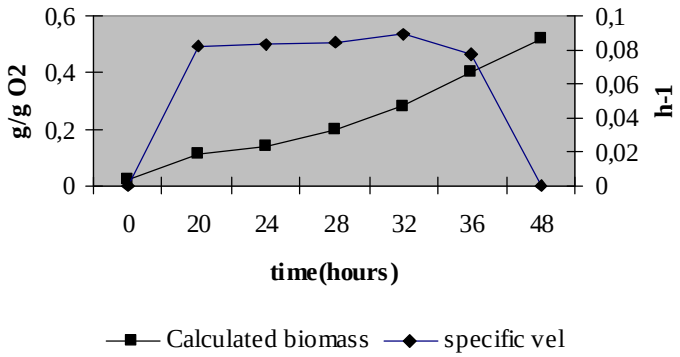

Figure 5. Estimate biomass and specific growth velocity during exponential growth phase SSF with spore inoculation

The time of exponential growth phase was of 12 to 28 hours for biomass inoculation and of 20 to 48 hours for spore inoculation verifying that the time for spore germination to occur is of 8 hours after the beginning of exponential growth phase for biomass inoculation.

4. Conclusion

The strain of *Paecilomyces lilacinus* LPB-Pl-01 produced greater amount of spores when spore inoculation was performed in substrate consisted of 50% defatted soybean cake and 50% coffee husk. Maximum spore production reached was 8.51E+09 spores.g^{-1} dry substrate in 192 hours of fermentation for spore inoculation while the maximum for mycelium

inoculation was 3.36E+09 spores.g^{-1} dry substrate in 144 hours of fermentation. When analyzing the biotechnological parameters it is concluded that the biomass yield and the maintenance coefficient are higher for spore inoculation just the specific growth velocity is smaller when comparison to biomass inoculation leading to a longer time of fermentation but producing higher spore yields which is important for the development of a biological control agent.

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5. CONCLUSÕES E PERSPECTIVAS

Os nematóide fitoparasitas do gênero *Meloidogyne* ocasionam perdas importantes em um grande número de culturas e seu impacto sobre a economia agrícola é considerável. O controle destes nematóides a muito tempo baseou-se na utilização de pesticidas químicos, que apresentam um certo número de inconvenientes: poluição de lençóis freáticos, desequilíbrio da microflora e microfauna dos solos, custo elevado, eficácia limitada e toxicidade. A substituição total ou parcial dos pesticidas químicos por agentes de controle biológico suscita cada vez mais interesse face as preocupações ambientais e de saúde pública atuais. Dentre estes organismos, os fungos filamentosos nematófagos dos gêneros *Paecilomyces* e *Verticillium* são conhecidos após longa data devido a sua eficácia contra os nematóides fitoparasitas.

Neste contexto, o objetivo deste trabalho foi de estudar e de utilizar um sistema economicamente adaptado para a produção de um biocomposto contendo uma cepa de fungo nematófago para o controle biológico dos nematóides fitoparasitas. A Fermentação no Estado Sólido (FES) é um processo de baixo custo, adaptado ao crescimento de fungos filamentosos e que permite obter um produto virulento com uma quantidade importante de esporos. Ainda, os sub-produtos agrícolas, que causam freqüentemente problemas ecológicos sérios de poluição, podem substituir os meios onerosos que são normalmente utilizados para a produção de biopesticidas.

A fisiologia de crescimento e a esporulação de cepas de fungos nematófagos foi estudada com o objetivo de se compreender melhor as fases de crescimento e conidiogênese. Primeiramente, dois diferentes modos de inoculação, em profundidade e em superfície, foram estudados em meio PDA, para se avaliar seus efeitos no crescimento e na esporulação das cepas. A inoculação em profundidade é a mais adequada pois o crescimento do micélio é mais homogêneo e os esporos são produzidos de maneira sincronizada. Os índices de esporulação obtidos são também mais importantes quando comparados com a inoculação em superfície. A estimativa de biomassa e a velocidade de crescimento radial das cepas de *Paecilomyces lilacinus* Pl-01, *P. lilacinus* Pl-02 e *P. lilacinus* Pl-03, e de *Verticillium chlamydosporium* Vc-01 foram determinadas, para se acompanhar o comportamento de cada cepas em meio PDA e MEA. As composições respectivas dos meios PDA e MEA não demonstraram possuir uma influência sobre o crescimento radial das cepas. O valor máximo (0,24 mm/h) deste último foi

observado para as cepas de *P. lilacinus* Pl-01 e *P. lilacinus* Pl-02 seguido da cepa de *P. lilacinus* Pl-03 (0,23 mm/h) e de *V. chlamydosporium* Vc-01 (0,17 mm/h). A produção de biomassa em meio PDA foi 1,4 vezes superior que em meio MEA em 166 horas de cultura para as quatro cepas estudadas. A cepa de *P. lilacinus* Pl-02 foi particular, pois desde o início a produção de biomassa foi mais elevada em meio PDA. Ao contrário, as outras cepas de *P. lilacinus* produziram mais biomassa em meio MEA, embora o meio PDA possa ter uma influência positiva na esporulação destas cepas. A cepa mais performante foi *P. lilacinus* Pl-2 e produziu 402,7 µg de biomassa/hora, seguida da cepa *P. lilacinus* Pl-01 com 315,9 µg/h em meio PDA, durante 7 dias de crescimento. A produção comparativa de esporos foi seguida em meio PDA em três dispositivos: frascos Erlenmeyer, fermentador de discos e colunas de Raimbault quando se utilizou o bagaço de cana-de-açúcar impregnado com meio PDB. Os índices de esporulação obtidos foram mais elevados em frascos Erlenmeyer seguido pelas colunas de Raimbault e por último em esporulador de discos. Embora a concentração de substratos carbonados presentes no ágar estava mais elevado do que quando se utilizou o bagaço impregnado. Portanto outros parâmetros como a relação C/N, a aeração e a temperatura de incubação devem ser igualmente otimizados com o objetivo de se obter uma produção máxima de esporos.

Vários resíduos agroindustriais diferentes foram testados como substratos para a produção de conidiosporos de fungos nematófagos As cascas de café foram escolhidas pois possui uma composição adequada para o crescimento de fungos e representam um enorme fonte de poluição ambiental. A utilização das cascas de café, pode também ter uma influência na obtenção de esporos virulentos, uma vez que elas são ricas em compostos específicos. A utilizaçào de um meio contendo extrato de cascas de café (100 g/l), foi primeiramente testado para verificar se sua utilização seria adequada como substrato, através da determinação do crescimento radial e da produção de biomassa neste meio. A cepa *P. lilacinus* Pl-01 demonstrou uma velocidade de crescimento apical mais elevada 0,20 mm/h com uma produção de biomassa de 440 µg/h em 12 dias de crescimento. A utilização dos produtos de FES em cascas de café com as quatro cepas de fungos nematófagos em experimentos em vasos com a planta *Coleus blumei* contaminadas com o nematóide *Meloidogyne incognita* raça 1, permitiu a seleção da cepa *P. lilacinus* Pl-01 uma vez que a mesma é capaz de reduzir em 80% o número de fêmeas do nematóide. As outras cepas utilizadas são menos eficazes e permitiram uma redução de 15 à 20%.

O segundo substrato testado era constituído de uma mistura de resíduos de camarão com o bagaço de mandioca (1:2). Esta mistura foi utilizada com o objetivo de verificar a influência de um substrato contendo a quitina para estimular a virulência da cepa *P. lilacinus* Pl-01. As quitinases podem exercer um papel no parasitismo do nematóide pelo fungo, pois a parede dos ovos de nematóides contêm quitina e proteínas. Com este substrato a produção de esporos foi muito elevada 3,49 $x10^{10}$ esporos/g de substrato seco em condições otimizadas de pH 4,5 e umidade inicial de 67% em 11 dias de cultura. Com as concentrações testadas no solo em plantas de tomate, a redução no número de fêmeas de nematóides foi de 60,5%. Igualmente, uma influência positiva foi observada nos pesos das raízes. A utilização dos resíduos de camarão sem outro substrato amiláceo em FES não é desejável devido a problemas de compactação do substrato.

A utilização do produto de FES em cascas de café como bionematicida através de ensaios em vasos, aportou um resultado mais encorajador. Então com o objetivo de aumentar a produção de esporos utilizando este substrato, o mesmo foi utilizado em mistura com diferentes concentrações de bagaço de mandioca, aumentando-se a relação C/N. As cascas de café como o bagaço de mandioca são abundantes no Brasil, sempre disponíveis e de baixo custo. O índice de esporulação de *P. lilacinus* Pl-01 cultivado nestas misturas em FES aumentou em função da quantidade de bagaço de mandioca presente. Deste modo, o rendimento da produção de esporos passou de 2,6 x 10^{9} esporos/g SPS com 100% de cascas de café à 7,4 x 10^{9} com uma mistura contendo 85% de bagaço de mandioca. Observou-se igualmente que a virulência dos esporos obtidos em cascas de café (100%) foi mais significativa. Se a quantidade de bagaço aumenta na mistura o índice de esporulação aumenta igualmente, embora a uma perda significativa na virulência dos esporos obtidos.

Para aumentar a quantidade de nitrogênio e conseqüentemente de proteínas no substrato, o farelo de soja foi utilizado sozinho ou em mistura com cascas de café para de um lado aumentar o índice de esporulação e de outro lado para verificar a influência da mistura sobre a virulência da cepa selecionada. O rendimento da esporulação de *P. lilacinus* Pl-01 foi de 8,79 $x10^{9}$ esporos/g de substrato seco com a utilização de 100 % de farelo de soja e diminuiu (4,52 x 10^{9}) quando misturado com 50% de cascas de café. A atividade nematicida dos produtos obtidos por FES contendo estes substratos foi verificada, as reduções respectivas do número de fêmeas de nematóides foi de 99 e de 89%. Observou-se igualmente que o farelo de soja não fermentado afetou drasticamente o desenvolvimento de nematóides (99% de redução no

número de fêmeas) e as cascas de café de aproximadamente 35%. Entretanto, o farelo de soja fermentado e não fermentado possuem uma influência negativa no desenvolvimento da raiz. Assim a mistura constituída de 50% de cascas de café e 50% de farelo de soja foi escolhida como substrato para a cultura de *P. lilacinus* Pl-01 pois sua utilização como bionematicida permitiu uma redução em torno de 90% de fêmeas de *Meloidogyne* e também um bom desenvolvimento radicular Três concentrações deste produto (0,06%, 0,175% e 0,3%) em relação com a totalidade de solo utilizada em cada experimento foram aplicadas com plantas de tomate contaminadas com *Meloidogyne incognita* raça 1. O tratamento com 0,175% de bionematicida proporcionou o melhor resultado pois permitiu uma redução de 98% no número de fêmeas de nematóide favorecendo ainda um bom desenvolvimento da raiz.

Diferentes tipos de biorreatores foram utilizados para a produção de esporos de *P. lilacinus* Pl-01 em mistura de 50% cascas de café e de 50% de farelo de soja. A produção máxima ($1,57 \times 10^{10}$ esporos/g de substrato seco) obtida quando a cepa foi cultivada em condições otimizadas em um dispositivo úmido contendo 100 g, de substrato em 8 dias de cultivo. A influência da unidade inicial, da aeração e do modo de inoculação na esporulação da cepa foi verificada em biorreator tipo coluna. As condições de cultura que permitiram de se obter o índice de esporulação mais elevado ($8,51 \times 10^{9}$ esporos/g de substrato seco) são: pH 4,5, umidade inicial de 65%, fluxo de ar de 60 ml/min/coluna e inoculação com suspensão de esporos com $2,3 \times 10^{7}$ esporos/g de substrato seco). Mas o melhor resultado global em termos de esporulação foi obtido no dispositivo úmido sem controle de aeração.

Os estudos de secagem do produtos obtido por FES foram realizados em, temperatura ambiente (20-26°C) e em temperaturas controladas (28, 32 e 35°C). As temperaturas de secagem demonstraram uma influência negativa na viabilidade dos esporos. O melhor tratamento de secagem foi a temperatura ambiente pois a viabilidade dos esporos foi mantida até 60 dias de estocagem e somente 1% de perda foi observado após 150 dias. A viabilidade dos produtos secos a 28, 32 e 35°C é inferior a medida que a temperatura aumenta. Conseqüentemente a temperatura de secagem dos esporos de *P. lilacinus* Pl-01 possui uma influência negativa na viabilidade.

A respirometria (produção de CO_2 e consumo de O_2) foi acompanhada em biorreator de FES tipo coluna utilizando-se como inoculo micélio e suspensão de esporos. A partir dos valores obtidos através de experiências para o consumo de oxigênio e evolução do CO_2, o rendimento

de biomassa ($Y_{x/o}$) foi de 0,415 e 0,545 g biomassa/g O_2 consumido e coeficiente de manutenção (m) de 0,1172 e 0,1834 g O_2 consumido/g biomassa/h, respectivamente para inoculação com micélio e suspensão de esporos. O crescimento de *P. lilacinus* em FES realizada com micélio começa mais rapidamente pois não existe uma fase de germinação de esporos. Entretanto, a produção de esporos foi mais significativa quando utilizou-se uma suspensão de esporos como inóculo.

Este trabalho de tese abriu inúmeras perspectivas que devem ser realizadas para certificar a utilização dos produtos de FES como bionematicidas competitivos e eficazes. Será interessante e necessário otimizar a produção e a secagem de esporos a partir de substratos fermentados em biorreatores de grande escala levando-se em conta a viabilidade e a fisiologia de crescimento do fungo nematófago bem como a conservação e o transporte de novos biopesticidas.

A utilização do bionematicida obtido por FES deverá levar em consideração as diferentes fases e ciclo de vida do nematóide *Meloidogyne.* Igualmente deverão ser realizados outros trabalhos para evidenciar a virulência do biopesticida frente a outros nematóides do solo como *Globodera, Radophylus, Heterodera, Tylenchulus* e *Pratylenchus* em diferentes plantas como modelo. Para experimentos em campo, deverá ser feita uma avaliação da biodiversidade da população de nematóides presentes no solo antes e após a aplicação do bionematicida.

Igualmente, pesquisas devem ser orientadas para se colocar em evidência as enzimas implicadas na penetração do fungo nos ovos e na cutícula dos nematóides que são essencialmente compostas de proteínas e de quitina. Será importante estudar as enzimas produzidas durante a FES e também demonstrar o papel dos metabólitos secundários produzidos durante a etapa de esporulação. As micotoxinas podem ter uma ação significativa ao nível de virulência das cepas e também servir como marcadores moleculares para a seleção específica de cepas nematófogas utilizadas no controle biológico

Um dos problemas freqüentemente encontrados na utilização de um agente de controle biológico no solo é a falta de meios de controle para a certificação da sobrevida e da atividade das cepas com o tempo. As ferramentas moleculares devem ser utilizadas para certificar a traçabilidade das cepas de fungos nematófagos no solo. A análise quantitativa pode ser feita por PCR através de primers específicos e a análise quantitativa pode ser realizada por RT_PCR.

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