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PRODUÇÃO DE BIOHERBICIDA POR PROCESSOS FERMENTATIVOS A PARTIR DO FUNGO *Phoma* sp.

DISSERTAÇÃO DE MESTRADO

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Santa Maria, RS, Brasil
2014

PRODUÇÃO DE BIOHERBICIDA POR PROCESSOS FERMENTATIVOS A PARTIR DO FUNGO *Phoma* sp.

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Dissertação apresentada ao Curso de Mestrado do Programa de Pós-Graduação em Engenharia de Processos, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de
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PRODUÇÃO DE BIOHERBICIDA POR PROCESSOS FERMENTATIVOS A PARTIR DO FUNGO *Phoma* sp.

elaborada por
Rodrigo Klaic

Como requisito parcial para obtenção do grau de
Mestre em Engenharia de Processos

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“Aquele que conhece o inimigo e a si mesmo lutará cem batalhas sem perigo de derrota; para aquele que não conhece o inimigo, mas conhece a si mesmo, as chances para a vitória ou para derrota são iguais; aquele que não conhece nem o inimigo e nem a si próprio será derrotado em todas as batalhas”

- *Sun tzu*

RESUMO

Dissertação de Mestrado
Programa de Pós-Graduação em Engenharia de Processos
Universidade Federal de Santa Maria

PRODUÇÃO DE BIOHERBICIDA POR PROCESSOS FERMENTATIVOS A PARTIR DO FUNGO *Phoma* sp.

AUTOR: RODRIGO KLAIC
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COORIENTADOR: RAQUEL C. KUHN
Data e Local de Defesa: Santa Maria, 07 de março de 2014.

As plantas daninhas são um dos principais problemas no cultivo de produtos agrícolas e o principal método de controle é o uso de herbicidas químicos. Embora eficiente, os herbicidas trazem consequências diretas e indiretas que superam os benefícios em muitos casos, abrindo assim caminho para o desenvolvimento de bioherbicidas. Uma barreira significativa na produção de muitos bioherbicidas é o desenvolvimento de um processo economicamente viável, logo, os objetivos deste trabalho foram produzir um bioherbicida a partir do fungo *Phoma* sp. por fermentação submersa e fermentação em estado sólido, otimizar o meio de fermentação em ambos processos e avaliar a atividade herbicida dos extratos das fermentações no controle de plantas-teste. Na fermentação submersa foi otimizado o meio de cultivo e monitorado o crescimento celular como variável resposta. A composição otimizada do meio sintético foi 20 gL^{-1} de glicose e peptona, 7,5 gL^{-1} de extrato de levedura e pH inicial de 6.0. Para o meio industrial, a composição otimizada foi 20 gL^{-1} de sacarose, 8 % de água de maceração de milho (AMM) e pH inicial de 6.0. Nessas condições a máxima biomassa obtida foi de 22 e 33 gL^{-1}, para o meio sintético e industrial, respectivamente, com uma produção de biomassa 50 % maior para o meio industrial e ainda possibilitou a redução do custo deste meio de fermentação. Na fermentação sólida foi otimizada a composição do meio de cultivo para obter a máxima ação do bioherbicida em plantas de pepino. Bioensaios foram realizados e o teste de Tukey mostrou que a altura da planta, massa verde da parte aérea, massa seca da parte aérea e principalmente os sinais de fitotoxidade foram significativos, entretanto, o comprimento do sistema radicular, a massa verde do sistema radicular, massa seca do sistema radicular e número de flores não apresentam resultados significativos. Os resultados obtidos nos bioensaios demonstraram que o bioherbicida apresenta atividade em plantas de pepino e a intensidade do efeito foi influenciado pela formulação do meio de fermentação. A condição otimizada para produção do bioherbicida foi 70 % de umidade, 30 % (p/v) de farelo de soja e 20 % (p/v) de AMM, nessas condições o nível de injúria obtida foi moderada com recuperação. O bioherbicida produzido mostrou um possível modo de ação baseado na inibição de biossíntese de carotenóides.

Palavras chave: Bioherbicida, *Phoma* sp. fermentação submersa, fermentação em estado sólido.

ABSTRACT
Thesis for the degree of Master of Science
Graduate Program in Process Engineering
Federal University of Santa Maria

PRODUCTION OF BIOHERBICIDE BY PROCESS FERMENTATION FORM FUNGAL *Phoma* sp.
AUTHOR: RODRIGO KLAIC
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Date and Local of defense: Santa Maria, March, 07, of 2014.

Weeds are one of the major problems in the agricultural products cultivation and the main strategy of control is the use of chemical herbicides. Although is efficient, the herbicides bring direct and indirect consequences that overcome its benefits and this opened the way for bioherbicides. A significant barrier in the bioherbicide production is the development of an economically viable fermentation process. This way the aim of this work was to assess the production of a bioherbicide from the *Phoma* sp. fungus by submerged fermentation and solid-state fermentation, optimizing the fermentation medium in both cases and evaluating the herbicidal activity from the fermentation extracts in the control of the target plants. The culture medium was optimized and the cell growing was monitoring as response in the submerged fermentation. The optimum composition for the synthetic medium was glucose and peptone at 20 gL^{-1}, yeast extract 7.5 gL^{-1} and an initial pH of 6.0. For the industrial medium, the ideal composition was 20 gL^{-1} of sucrose, 8 % CSL and initial pH of 6.0. Under these conditions, the maximum biomass obtained was 22 and 33 gL^{-1}, for the synthetic and industrial medium, respectively. Therefore it was possible to obtain a 50 % higher biomass production in the industrial medium, reducing the cost of this fermentation medium. In the solid fermentation the composition of the culture medium was evaluated for obtaining the maximum herbicide action on target plants (cucumber). Bioassays were realized and the plant height, fresh weight, dry weight and phytotocixity mainly were the responses evaluated. The results obtained in the bioassays demonstrate that the bioherbicide presented activity towards the target plant and the intensity of the effect was influenced by the formulation of the fermentation medium. The optimized condition for bioherbicide production was moisture at 70 %, soybean bran of 30 wt% and CSL of 20 wt%, being possible to obtain a moderate injury, usually with recovery. The bioherbicide produced showed a mode of action based on the inhibition of carotenoids biosynthesis.

Keywords: Bioherbicide, *Phoma* sp., Submerged Fermentation, Solid-State Fermentation.

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1. INTRODUÇÃO

As plantas daninhas são um dos principais problemas e um dos fatores mais limitantes no cultivo de produtos agrícolas (CHARUDATTAN e DINOOR, 2001), podem causar perda de produtividade através da concorrência em plantas cultivadas por luz, água, energia térmica, dióxido de carbono e espaço (PENARIOL *et al.*, 2008; BOYETCHKO *et al.*, 2002). As perdas também podem ser causadas pela produção de compostos inibidores de crescimento, um fenômeno designado alelopatia (ROSSKOPF *et al.*, 1999). As plantas daninhas também podem causar perdas pela redução de nutrientes disponíveis no solo, bem como o aumento do custo de preparação da terra e na colheita (BOYETCHKO, 2002; ROSSKOPF, 1999).

Existem procedimentos para o controle de plantas daninhas como a rotação de culturas, métodos mecânicos, químicos e biológicos, sendo o uso de herbicidas químicos o principal método (ROSSKOPF, 1999). Ao longo das últimas décadas, o uso de herbicidas químicos tem dominado as estratégias de manejo de plantas daninhas, principalmente nos países em desenvolvimento (BOYETCHKO, 2002). A utilização de herbicida químico é muito eficiente no controle de ervas daninhas, porém gera consequências diretas e indiretas ao meio ambiente que superam os benefícios em muitos casos. A contaminação de fontes de água e solos é um dos principais problemas originados do consumo desregrado de herbicidas químicos. Estes fatores, juntamente com a proibição de muitos herbicidas químicos, registro e regulamentação mais rigorosa e, a necessidade de alternativas ambientalmente corretas, abriu caminho para ascensão do controle biológico de ervas daninhas. Neste tipo de estratégia, os micro-organimos são utilizados como agentes de controle biológico em áreas estritamente definidas de aplicação (ZHOU *et al.*, 2004). Logo, micro-organismos utilizados para combater ervas daninhas são caracterizados como bioherbecidas (CHARUDATTAN e DINOOR, 2001).

Embora o controle de plantas daninhas usando bioherbicidas seja atraente, a pesquisa e comercialização destes produtos é baixa. Desde os primeiros relatos do uso de bioherbecidas com *Phytophthora palmivora* e *Colletotricum gloeosporioides* f. sp. *Aeschynomene* (TEBEEST *et al.*, 1992), pelo menos 11 produtos foram comercializados (BAILEY *et al.*, 2011; ASH, 2010). Alguns dos principais produtos e patentes de sucesso comercial que existem no mercado atualmente são: DeVine® é utilizado para controlar *Morrenia odorata* em citros e foi o primeiro bioherbicida registrado e comercializado nos EUA. Sua formulação é a base do fungo *Phytophthora palmivora*; Collego® é utilizado para controlar ervas daninhas de folhas largas em arroz, sua formulação é a base do fungo *Colletotrichum gloeosporioides*

aeschynomene (BOYETCHKO *et al.*, 2002). Além destes produtos comercializados, Charudattan (2001) listou mais de 50 exemplos de combinação de patógenos que apresentam potencial para serem utilizadas como bioerbicida.

Uma barreira significativa na produção de muitos bioherbicidas é o desenvolvimento de um processo economicamente viável (MITCHELL, 2003). Na produção de bioherbicidas por processos fermentativos, a obtenção do metabólito ativo é estritamente relacionada à formulação do meio e do tipo de fermentação. Os principais tipos de fermentação utilizados são a fermentação submersa e fermentação em estado sólido.

A fermentação submersa é o sistema mais utilizado industrialmente para a produção de uma variedade de bioprodutos. Este processo é idealmente favorável para o cultivo de bactérias. Jacson (1998) reportou o cultivo de *Xanthomonas campestris* em fermentação submersa, a qual é o ingrediente ativo de um bioherbicida para *Poa annua*. No caso de fungos filamentosos, a produção de esporos por fermentação submersa é limitada (GIBBS *et al.*, 2000). Porém, alguns fungos considerados como bioherbicidas produzem conídios ou outras formas de propágulos em culturas líquidas, de maneira que a fermentação submersa é o método ideal para o cultivo (ASH, 2010), viabilizando a produção de bioherbicida a partir do processo de fermentação submersa.

A fermentação em estado sólido é um processo cujo crescimento microbiano ocorre na ausência de água livre, sendo mais indicado para o cultivo de fungos e leveduras. O material sólido pode atuar como suporte inerte ao crescimento do micro-organismo ou como fonte de carbono. Os substratos usados em fermentação em estado sólido são, na grande maioria das vezes, compostos por resíduos agroindustriais, como bagaço de cana, casca de arroz, farelo de trigo, farelo de soja, entre outros. Em muitos casos, após a colonização, os substratos são moídos e aplicados em formulações de herbicidas granulares (SHABANA et al., 2010). Outros pesquisadores (WEAVER et al., 2012) usaram o extrato obtido a partir de grãos fermentados para aplicação como bioherbicida. Hoagland et al. (2007) utilizaram grãos de arroz para cultivar *Myrothecium erraria* para controlar kudzu em alguns produtos agrícolas. Eles verificaram que os extratos obtidos dos grãos de arroz fermentado foram mais eficientes que o inóculo aplicado no solo.

A produção desses compostos pode ser realizada pelo emprego de fermentação submersa ou fermentação em estado sólido. A sua viabilidade técnica e econômica dependerá da quantidade de produto excretado pelo micro-organismo, bem como pela possibilidade de

aumento de escala do processo fermentativo, o que implica na formulação do meio e no projeto de biorreatores. De acordo com a literatura, ocorre uma maior produção de metabólitos por fermentação em estado sólido, porém o seu escalonamento é complicado. Por outro lado, a fermentação submersa apresenta maior facilidade de escalonamento, enquanto a produção normalmente é menor, uma vez que o metabólito produzido encontra-se diluído em um grande volume reacional. Nesse sentido, a escolha da melhor forma de condução do processo é fundamental para a viabilidade da produção do bioherbicida (MITCHELL, 2003).

Existem muitas variáveis que podem interferir em processos fermentativos e este trabalho tem por objetivo avaliar a produção de um bioherbicida a partir do fungo *Phoma* sp. por fermentação submersa e fermentação em estado sólido, otimizar ambos os meios de fermentação e posteriormente avaliar a atividade herbicida dos extratos das fermentações sobre a planta alvo.

Dessa forma, esta dissertação se divide em cinco capítulos. O capítulo 1 trata da introdução do tema a ser desenvolvido. O capítulo 2 consiste em uma revisão bibliográfica sobre os principais temas e, é composto pelo artigo 1 de intitulado "An overview regarding bioherbicide and their production methods by fermentation" é um artigo de revisão e se refere a uma busca na literatura específica de bioherbicidas. Neste estudo foi realizada uma análise dos principais micro-organismos utilizados como bioherbicidas, tipo de fermentação mais utilizado, a importância da formulação do meio de cultivo para produção de um bioherbicida e estudos de casos envolvendo fermentação submersa, fermentação em estado sólido e biorreatores.

O capítulo 3 coresponde aos resultados deste trabalho e é composto por dois artigos. O artigo 2 intitulado "Optimization of fermentation media for the growth of a fungus used as bioherbicide" é o primeiro artigo de resultados e reporta um estudo para produzir um bioherbicida a partir de *Phoma* sp. por fermentação submersa. Este trabalho tem por objetivo otimizar o meio de fermentação sintético e industrial para maximizar a produção de biomassa deste fungo e reduzir custos de cultivo. O artigo 3 intitulado "Bioherbicide activity of fermented extract of *Phoma* sp. obtained by solid-state fermentation" é o segundo artigo de resultados e relata um estudo para produzir um bioherbicida a partir de *Phoma* sp. por fermentação em estado sólido. Este trabalho tem por objetivo otimizar o meio de fermentação para obter a máxima ação do bioherbicida em planta alvo.

O capítulo 4 é a discussão dos artigos e relata de forma geral a idéia principal de cada artigo e a importância deste estudo como um todo. O capítulo 5 corresponde à conclusão deste trabalho de dissertação, onde são retomados os principais resultados obtidos e são apresentadas sugestões para trabalhos futuros.

1.1. Objetivo

1.1.1. Objetivo geral

Estudo da produção de um bioherbicida por processo fermentativo a partir do fungo *Phoma* sp.

1.1.2. Objetivos específicos

i) Estudo da produção do bioherbicida por fermentação submersa;

ii) Estudo da produção do bioherbicida por fermentação em estado sólido;

iii) Otimizar o meio de fermentação em ambos os processos fermentativos;

iv) Avaliar a atividade herbicida do extrato fermentado no controle de plantas de pepino.

2. REVISÃO BIBLIOGRÁFICA

2.1. Artigo I: An overview regarding bioherbicide and their production methods by fermentation

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Abstract: Biological control of weed is an alternative to overcome some shortcomings of the chemical herbicides. As a consequence, there has been a growing interest in the development of biological control agents to niche markets, in principle considered smaller, for example, organic food production. Some researches looking for bioherbicides have appeared since the 1960s and nowadays there are 11 registered bioherbicide products in the market. Therefore, this review aims to present the state of the art regarding the main types of biological control strategy, the most studied microorganisms with bioherbicide action, and to provide an overview on the actual related market and possible future applications of these compounds. Techniques concerning production methods, performed by solid state and submerged fermentation, formulation and application of these products are also reviewed. Formulation aiming to increase the shelf life, hence increasing product efficiency, and application methods are also an important variable and when poorly executed, undermines the efficiency of bioherbicide. Strategies for scaling-up and the main types of bioreactors used in bioherbicides production were also commented in this review.

Keywords: Bioherbicide, Biological Control; Submersa Fermentation; Solid-State Fermentation; Scale up.

2.1.1. Introduction

Weeds are one of the factors which contribute to crop losses in agriculture. There are approximately 30,000 species of weeds distributed throughout the world and 1,800 species of weeds correspond to a crop reduction of 9.7% in agricultural production annually (Li et al., 2003). Weeds cause losses in the form of yield reductions through competition with crop plants for light, water, nutrients, heat energy, carbon dioxide, and space (Penariol et al., 2008). Reductions may also be caused by the production of growth-inhibiting compounds, a phenomenon referred to as allelopathy (Rosskopf et al., 1999). Weeds also cause losses in food, feed, and fiber quality, as well as increase the cost of land preparation or harvesting.

There are tactics for weed control such as crop rotation, mechanical methods, chemical and biological, however, the chemical methods such as the use of chemical herbicides are the most used (Rizzardi et al., 2003). Over the past decades, chemical herbicides have dominated weed management strategies in developing countries (Rosskopf et al., 1999). Thus, chemical

herbicides account for 60 and 70% of the total pesticide sales in the USA and Canada, respectively (Boyetchko et al., 2002).

Since the introduction of chemical herbicides in the 1940s (Templeton et al., 1984), some challenges have became evident such as: herbicide-resistant weeds, reductions in the quality of soil and water, residues of herbicides, and harmful effects on non-target organisms (Li et al., 2003). Thus, there is a continuing need for new herbicides or new technologies to overcome these problems (Templeton et al., 1984). Biological control weeds approach which has been exploited throughout the world (Boyetchko et al., 2002), the microorganisms are used as biological control agents in a strictly defined area of application (Zhou et al., 2004). The uses of pathogenic microorganisms from plants are called bioherbicides (Charudattan, 2000).

Research in the late 1960s through the 1980s culminated in the successful registration of two plant pathogens as bioherbicides and their subsequent use in commercial agriculture for nearly two decades (Rosskopf et al., 1999). *Phytophthora palmivora* and *Colletotricum gloeosporioides* f.sp. *aeschynomene* were the first bioherbicides used (Tebeest et al., 1992; Rosskopf et al., 1999; Charudattan, 2001) and studies reported the existence of 11 registered or commercially available bioherbicides, many of these developed from fungi (Baley et al., 2011). Besides these commercial products, Boyetchko et al. (2002) also listed more than 100 examples of pathogen combinations, which presented potential as bioherbicides. Despite approximately only 11 bioherbicide products being made available in the marketplace, a further search of the literature using the ISI Web of Science database has revealed 509 papers published which mention bioherbicides or mycoherbicides (as of February 2009) since 1987. The majority of these papers were from USA (36%), Canada (20%) and Australia (7.8%). During the same period there were over 17,000 papers published which mentioned synthetic herbicides. Up to 15,000 compounds per year were screened for herbicidal activity for each herbicide that successfully reached the market in the 1980s (Ash, 2010).

Ash (2010) defined the successful registration and commercialization of bioherbicides as low. There are many barriers to efficiently produce bioherbicides, one of the main barriers is the development of a process which is economically viable and able to produce enough quantity of microbial spores (Mitchell, 2003). In the production of bioherbicides by fermentation, obtaining the active ingredient is strictly related to the medium composition and the type of fermentation. The main kinds of processes used are solid state fermentation (SSF) and submerged fermentation (SMF). Li et al. (2003) defined the requirements for

bioherbicides to achieve commercial success: be reproduced by a biological technique; grow fast after spraying to initiative or be capable of killing weeds within a defined time; be suitable for an industrial production process; and be suitable for packaging, transport and use.

There are many variables that influence the commercial application of a bioherbicide. Therefore, this review aims to generally show the main biological control strategies types, describe the most studied microorganisms with bioherbicide action, review the state of the art in production, formulation and application and the main bioreactors used to scale-up, besides providing an overview on the market and possible future of bioherbicides.

2.1.2. The bioherbicides and opportunities for the development

Pesticide is a generic name given to substances that aim to kill, control and/or inhibit unwanted organisms, and can be classified at herbicides, fungicides and insecticides according to the target groups of organism, respectively, weeds, fungi and insects. Biopesticides have emerged as a viable alternative to chemical pesticides to solve the problems caused by the chemical counterparts. Similar to the classification of chemical pesticides, biopesticides can be classified into bioherbicides, biofungicides and bioinsecticides, however, in this review only bioherbicides will be examined.

Due to the high costs associated with chemical herbicide discovery and production, big companies have focused on their production for use in the cultivation of agricultural products in a large scale. This enabled the development of herbicides for niche markets that are considered minor, as for the production of organic food (Charudattan, 2001). The use of bioherbicides is compatible with the philosophy of organic foods, as long as there was no genetic modification and any carriers or adjuvants are natural products with a narrow host range (Rosskopf and Koenig, 2003).

About 50% of bioherbicides are used on horticultural trees and crops, 30% on grazing, with the remaining 12% in field crops (Glare et al., 2012). In terms of land area dedicated to growing organic foods, a growth of 20% annually in many western countries is estimated (Bruinsma et al., 2003). This increase will continue for a long period, due to continued demand for organic food and farm subsidies provided by governments (Rosskopf, 2003; Bruinsma et al., 2003). The use of bioherbicides helps to minimize eventual environmental impacts. This provides new opportunities to be explored. Moreover, the market of

bioherbicides may be larger than the market for synthetic herbicides due to the increasing of organic food globalization (Ash, 2010; Glare et al., 2012; Clarke et al., 2008).

2.1.3. Biological control of weeds with pathogens

There are two strategies by which microbial agents are often used for biological control of weeds: classical and inundative approaches. Classical biological control is characterized by the importation, introduction and release of a natural enemy from the same geographic origin of the weed into an area where the weed is a problem. Following release, the natural enemy (insect or fungal pathogen) is allowed to self-perpetuate, survive, and establish, thus providing long-term weed control over a period of several years (Boyetchko et al., 2002). Classical biological control is often more appropriate in rangeland where site disturbance is minimal.

The inundative approach is also known as the bioherbicide approach (Boyetchko et al., 2002). The bioherbicide is defined as a plant pathogen used as a weed control agent through inundative and repeated applications of its inoculum (Charudattan, 2000). The pathogens are often indigenous, artificially mass-produced and applied during the growing season. Weed control is short-term, compared to classical biocontrol agents, and the microbes are not expected to persist in the environment. The majority of microbes used as bioherbicides are predominantly fungal pathogens, while there are a growing number of examples of foliar and soil-applied bacterial agents being explored and developed as bioherbicides as well (Boyetchko et al., 2002). Table 1 presents the studies reported in the literature on pathogens under evaluation or commercialized as bioherbicides.

Table 1: Some fungus and bacteria used as bioherbicides.

Types	Pathogen	Weed	Fermentation	Time (Days)	Temperature (°C)	Agitation (rpm)	Reference
Fungi	*Coniothyrium minitans*	*Sclerotinia sclerotiorum*	SMF	12	24	200	Cheng et al., 2003.
Fungi	*Colletotrichum truncatum*	*Sesbania exaltata*	SMF	10	28	250	Jackson and David, 1995.
Fungi	*Colletotrichum truncatum*	*Sesbania exaltata*	SMF	5	25	150	Silman et al., 1993.
Fungi	*Colletotrichum coccodes*	*Abutilon theophrasti*	SSF	10	28	-	Meir et al., 2009.

Fungi	*Colletotrichum gloeosporioides*	*Aeschynomene virginica*	SMF	7	27	100	Templeton et al., 1984.
Fungi	*Colletotrichum gloeosporioides*	*Malva pusill*	SSF	7	25	-	Ruddick and Mortensen, 1995.
Fungi	*Colletotrichum graminicola*	*Sorghum bicolor*	SMF	6	24	120	Michell et al., 2008.
Fungi	*Colletotrichum orbiculare*	*Xanthium spinosum*	SMF	9	24	-	Auld and Say, 1999.
Fungi	*Colletotrichum sp.*	*Sorghum bicolor*	SSF	10	24	-	Zhang et al., 2003.
Fungi	*Colletotrichum truncatum*	*Sesbania exaltata*	SMF	9	nd	nd	Jackson, 1997.
Fungi	*Dactylaria higginsii*	*Cyperus rotundus*	SSF	10	27	-	Shabana et al., 2010.
Fungi	*Fusarium tumidum*	*Ulex europaeu; Cytisus scoparius*	SSF	7	25	-	Morin et al., 2000.
Fungi	*Fusarium avenaceum*	*Calamagrostis canadensis*	SSF	15	28	-	Winder, 1999.
Fungi	*Fusarium oxysporum*	*Striga sp.*	SMF	7	24	100	Elzein et al., 2004.
Fungi	*Fusarium oxysporum*	*Orobanche sp.*	SMF	6	25	100	Shabana et al., 2003.
Fungi	*Helminthosporium gramineum*	*Rhizoctonia solani*	SSF	7	27	-	Duan et al., 2007.
Fungi	*Colletotrichum gloeosporioides f.* sp. *malvae*	*Malva pusila*	SMF	5	28	200	Cunningham and Kuiack, 1989.
Fungi	*Microsphaeropsis amaranthi*	*Amaranthus*	SSF	nd	27	-	Shabana et al., 2010.
Fungi	*Myrothecium verrucaria*	*Cassia obtusifolia*	SSF	7	28	-	Weaver and Boyette, 2012.
Fungi	*Phoma betae*	*Beta vulgaris*	SMF	7	28	nd	Monte and Garcia., 1988.
Fungi	*Phoma macrostoma*	*Cirsium arvense*	SMF	nd	28	150	Baley et al., 2011a.
Fungi	*Phoma macrostoma*	*Taraxacum officinale*	SSF	nd	28	-	Baley et al., 2011b.
Fungi	*Phoma macrostoma*	*Cirsium arvense*	SMF	14	25	nd	Zhou et al., 2004.
Fungi	*Phoma macrostoma*	*Cirsium arvense*	SMF	nd	25	150	Graupner et al., 2003.
Bacteria	*Pseudomonas syringae*	*Pueraria lobata*	SMF	3	25	nd	Zidack et al., 1992.
Fungi	*Puccinia thlaspeos*	*Isatis tinctoria*	SMF	nd	nd	nd	Kropp and Darrow, 2006.
Fungi	*Ascochyta caulina*	*Chenopodium album*	SMF	15	26	40	Vurro et al., 2012.

Fungi	*Corynespora cassiicola f.* sp. *lantanae*	*Lantana camara*	SSF	15	25	-	Pereira et al., 2003.
Bacteria	*Rhizobacteria*	*Abutilon theophrasti*	SMF	9	27	100	Bergonia and Kremerr, 1994.
Fungi	*Septoria polygonorum*	*Polygonum* sp.	SMF	7	24	240	Michell, 2003.
Fungi	*Fusarium nygamai*	*Striga hermonthica*	SMF	8	25	120	Abbasher and Sauerborn, 1992.
Fungi	*Valdensinia heterodoxa*	*Gaultheria shallon*	SSF	14	26	-	Shiguang et al., 2006.
Fungi	*Ulocladium atrum*	*Botrytis cinerea*	SMF	7	25	100	Frey and Magan, 2001.
Bacteria	*Xanthomonas campestris*	*Poa annua*	SMF	3	30	100	Jackson et al., 1988.

nd: not determined; SMF: submerged fermentation; SSF: solid state fermentation.

Only 3 articles (9%) report on bacteria as agent of weed biological control, whereas 33 (91%) articles are focused in fungi. Although most microbial agents under development as bioherbicides are fungal pathogens, there are examples of bacterial agents that are currently being explored for their weed biological control potential.

The most cited genus in scientific research on bioherbicides was *Colletotrichum* sp. (30%). The next most cited are *Fusarium* sp. (15%) and *Phoma* sp. (15%) However, there is a wide variety of microorganisms that can be used for biological control, so that 40% of studies used several other species of microorganisms. This diversity of microorganisms shows that there is a great biotechnological potential to be explored characterizing a promising area of research.

2.1.3.1. Fungal agents

The first documented deliberate release of a fungus for classical biocontrol of a weed occurred in 1971, when *Puccinia chondrillina* was introduced into Australia for the control of *Chondrilla juncea* (Barton, 2004). Already, the first registered bioherbicide in the U.S. was DeVine®, a liquid formulation comprised of chlamydospores of the soilborne fungus *Phytophthora palmivora* for control of stranglervine (*Morrenia odorata*) in citrus (Boyetchko et al., 2002). This product provides consistent weed control of over 90% and control can

persist for at least 2 years, is recommended for use in orchards and gardens (Li et al., 2003). The first registered bioherbicide in Canada was BioMal®, a product containing spores of *Colletotrichum gloeosporioides* f sp. *malvae* for control of round-leaved mallow (*Malva pusill*) (Boyetchko et al., 2002). However, the small market size and technical difficulties associated with mass-production were two major considerations that deterred commercialization of the product (Li et al., 2003; Boyetchko et al., 2002; Ash et al., 2010). Since the first studies many researches have been conducted regarding the use of various microorganisms for weeds control. Some of these studies are showed in Table 1 and some will be discussed below.

Colletotrichum coccodes was one of the first fungus studied and characterized as biocontrol agent of weeds and many other innovative researches can be performed using this microorganism. For example, Meir et al. (2009) used *Colletotrichum coccodes* for the biological control of *Abutilon theophrasti*, using genetic engineering. These authors increased the virulence of microorganism modifying the gene *Oxaloacetate acetylhydrolase* (*oahA)* to increase the oxalate production and inhibit the defense system of the plant. Further optimization might be obtained through gene stacking, or the addition of multiple virulence genes to this potential biocontrol agent. Mitchell (2008) used the combination of *Colletotrichum graminicola* (Cg) and *Gloeocercospora sorghi* (Cs) for biological control of weeds. After testing several combinations and mixture rates, they found an ideal combination of Cg = $1x10^7$ and Cs = $4x10^4$ conidia/mL added by a sub-lethal dose of glyphosate (6.6 μg/mL).

Bailey et al. (2011a) studied the effects of *Phoma macrostoma* on target and non-target plant weed species and found that this fungus was pathogen to 94 plant species in 38 botanical families, with 57 species of 28 families identified as resistant. *Phoma macrostoma* was pathogen to all species of dicotyledonous plants, but did not present pathogenicity to monocotyledonous. Commercial applications for weed management in lawn, agriculture, horticulture and forestry seems probable, while internally managing weeds in lawns, ornamental and transplanted annual flowering species may provide alternative markets.

Morin et al. (2000) studied the relationship between trichothecene production and pathogenicity was investigated for 29 isolates of *Fusarium tumidum*, a potential bioherbicide for gorse (*Ulex europaeus*) and broom (*Cytisus scoparius*) in New Zealand. Two isolates from gorse were highly aggressive towards both weeds. These isolates offer prospects for the

development of a safe bioherbicide that could target two major weeds in New Zealand, as trichothecenes were not detected from them at the higher concentrations**.**

Abbasher et al. (1992) studied *Fusarium nygamai,* a potential bioherbicide for *Striga hermonthica* control in sorghum. Striga incidence was decreased up to 100% when the fungus was incorporated into the soil preplanting. Emerged Striga plants at different stages of growth up to the flowering stage were killed by the fungus when the fungus was applied postemergent. In root-chamber trials none of the Striga seeds germinated when 10 ml inoculum suspension of 8×10^6 spores/mL of *F. nygamai* was applied on seeds of the parasitic weed sprinkled on the surface of filter paper. *F. nygamai* has potential as a bioherbicide for Striga control.

2.1.3.2. Bacterial agents

The quantity of fungus as control agents of weeds is relevant when compared to the amount of bacteria (Boyetchko et al., 2002), although there are many bacteria used as bioherbicides, as for example *Bacillus* (Bettiol et al., 2012). Most of the bacteria with an ability to produce toxins are Gram-negative bacteria such as *Pseudomonas*, *Erwinia*, *Xanthomonas*, but there are a few Gram-positive bacteria such as *Streptomyces*, *Corynebacterium fasciomonads* and some are non-fluorescent *Pseudomonas* (Kremer et al., 1990).

The first bacteria studied as a weed biological control agent was the genus *Xanthomonas*. Johnson (1994) studied *Xanthomonas* as bioherbicide for controlling annual bluegrass (*Poa annua L.*) in bermudagrass (*Cynodon dactylon L. Pers*). Other studies with bacteria for the production of bioherbicides were developed by Jackson et al. (1998) that studied *Xanthomonas campestris* for control of *Poa annua L.*; Zidack et al. (1992) studied the effect of *Pseudomonas syringae* on the control of *Pueraria lobata*; Begonia (1994) studied the effect chemotaxis of deleterious rhizobacteria to velvetleaf seeds and seedlings. Camperico® is an example of commercial product that was registered and commercialized in Japan, is an isolate of *Xanthomonas campestris* pv. *poae* that causes systemic wilting of annual bluegrass *(Poa annua).*

Therefore, although there are few bacteria with potential to develop bioherbicides, there are studies in the literature and even some commercial products registered.

2.1.3.3. Phytotoxins

Fungi and bacteria are capable of producing toxic metabolites. The metabolites are biosynthesized and excreted through of a set of metabolic pathways, but are not essential for the growth and survival of the microorganism (Betina, 1989). These compounds are present in the culture medium or substrate where fungi are growing, originating from primary or secondary metabolism.

Primary metabolites are small molecules found in all living cells. They are intermediate or end products of intermediary metabolism, building blocks for essential macromolecules, or coenzymes. The primary metabolites most industrially important are the amino acids, nucleotides, vitamins, solvents, and organic acids (Demain, 2000).

The group of secondary metabolites includes antibiotics, toxins and pesticides (Li et al., 2003; Betina, 1989; Demain, 2000). The secondary metabolites produced by filamentous fungi which demonstrate toxic properties in animals are generically called mycotoxins, when those that demonstrate toxic properties in plants are called phytotoxins. It is observed that some fungi and bacteria can procuzir phytotoxins thus can be used for weed control.

Fungi, such as *Alternaria*, *Fusarium*, *Colletotrichum* and *Phoma* can produce phytotoxin (Li et al., 2003). The fungi *Phoma macrostoma* produce phytotoxins known as macrocidins (macrocyclic 3-acyltetramic acids), which are molecules worthy of synthesis given their potential as a template for herbicide design (Schobert and Barnickel, 2010). Graupner et al. (2003) studied the effect of macrocidins produced by the fungus *Phoma macrostoma* and found that when applied to broadleaf plants, it induces bleaching of the leaves and the inhibition of root growth followed by plant death. Studies performed by Bailey et al. (2011b) with *Phoma macrostoma* showed that the mortality reached 95% in field trials.

There are many fungi that produce phytotoxins with bioherbicide action, some known are AAL-toxin, cornexistin and tentoxin. The AAL-toxin and its analog in structure can suppress cearmide synthetase and results in sphingol accumulation that induces membrane rupture. Cornexitin is an inhibitor of metabolism and the mechanism of action of this compound is similar to aminoacetic salt. It inhibits one isoenzyme of asparagine aminotransferase, but once an acid from tricarboxylic acid cycle, such as aspartic acid and glutamic acid is added, the activity of toxin will disappear. Tentoxin has two different mechanisms of action under different conditions. One is interrupting the formation of chloroplast by blocking synthesis of coding nucleocytoplasmic protein and the other is energy

transferase inhibitor of ATPase's coupling factor for controlling photophosphorylation (Li et al., 2003).

Regarding bacteria, *Pseudomonas syringe pv. phaseolicola* is a bacterial plant pathogen which causes halo blight disease and localized death on common bean (*Phaseolus vulgaris*) and kudzu (*Pueraria lobata*), whose toxin is called phaseolotoxin. Once it infects the plant roots, it will spread to shoot terminus, then causes stunting, chlorosis and even foliage necrotic lesions (Li et al., 2003). Another bacterial that also causes diseases in crop plants and weeds is *Pseudomonas syringae* var. *tabaci*, which produces the phytotoxins tabtoxin, the causal agent of wildfire disease in tobacco (Hoagland et al., 2007). Phaseolotoxin, a tripeptide from *Pseudomonas syringe* pv. *phaseolicola*, inhibits arginine synthesis by competing with carbamoyl phosphate for the binding site on ornithine carbamoyl transferase (Li et al., 2003). *Xanthomonas campestris* is another bacterial bioherbicide and has been registered to control annual bluegrass (Hoagland et al., 2007). The isolation and characterization of a phytotoxin from *Xanthomonas campestris pv. retroflexus* was studied by Mingzhi et al. (2007). They identified molecular compounds including organic acids and cyclo-(proline-phenylalanine). The greenhouse cultivation test was used to determine the influence of the isolated fractions on the growth of target weed redroot pigweed (*Amaranthus retroflexus L*). Further, greenhouse and field test was processed, and the results showed that the use of the toxin seemed to have a potential to be developed further as a bioherbicide to control weedy grasses.

2.1.4. The state of the art regarding bioherbicides

2.1.4.1. Production of bioherbicides

The production of bioherbicides can be accomplished by fermentation processes. Fig. (1) shows a relationship of scientific research presented in Table 1, comparing studies using SMF e SSF.

Figure 1 shows a tendency for using submerged fermentation for bioherbicides production, as approximately 65% of the articles report studies using SMF, representing 22 of 34 articles. The submerged fermentation is more widespread both in academia and in industry due to some advantages provided by this type of fermentation, such as ease of scale-up and temperature control.

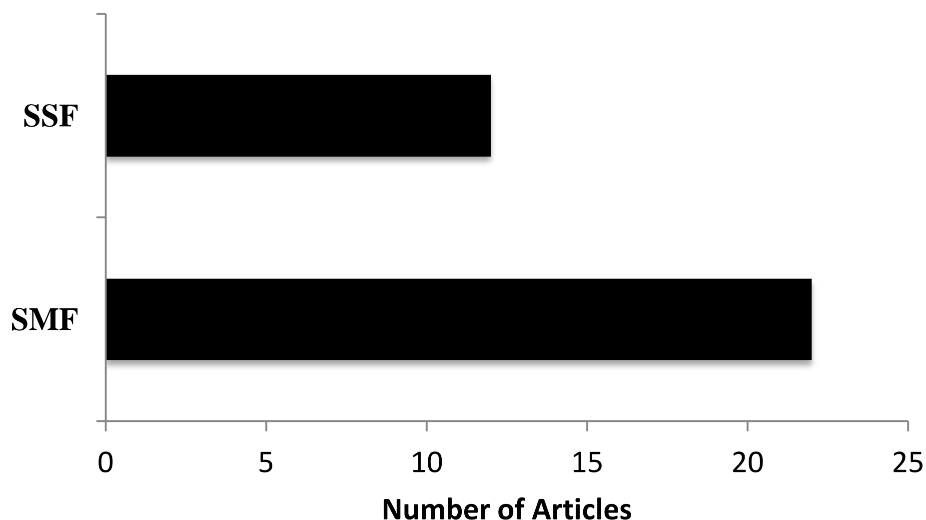

Fig. (1). Data from the literature regarding submerged (SMF) versus solid state fermentation (SSF) to produce bioherbicides.

2.1.4.1.1. Submerged fermentation

Submerged or liquid fermentation is the system most widely used industrially for the production of a variety of microbial products (Gibbs et al., 2000). It is easy to scale-up and to realize the control of operating variables (Huang and McDonald, 2009a). This process is ideally suited for bacteria, since in the case of filamentous fungi, the production of spores in this kind of fermentation can be problematic (Gibbs et al., 2000; Cheng et al. 2003). However, some fungi considered as bioherbicide candidates will produce conidia or other forms of propagules in liquid culture and hence submerged fermentation is an ideal method for their production, also known to produce secondary propagules in liquid culture such as microsclerotia or chlamydospores from hyphae or from conidia which can be variously formulated and applied as a bioherbicide (Ash, 2010; Cheng et al., 2003; Gibbs et al., 2000). Thus submerged fermentation is an excellent option for bioherbicides.

Analyzing the studies cited in this review, it is observed that processes of submerged fermentation with fungi have fermentation time of 5 to 12 days, with a temperature around 24-28 °C and agitation of 100-150 rpm were most frequent. For fermentation using bacteria, the average time ranged from 1 to 3 days with system temperature around 26-30 °C and

agitation of 100 rpm. Regular fermentation temperature for fungi and bacteria is observed, but the fermentation time is lower for bacteria, whereas fungi require milder agitation compared to bacteria cells, since high agitation rates increases the shear stresses in the bioreactor impairing the growth of the mycelium, and consequently reducing fungal biomass.

Another important factor in fermentation is the culture medium. There are studies that use synthetic culture medium (Gibbs et al., 2000; Zhang et al., 2012; Selbman et al., 2002; Yu et al., 1988) and other studies use industrial medium (Para et al., 2005; Singhania, 2010). Synthetic medium uses biotechnological reagents to develop the culture medium, for example: yeast extract, peptone, glucose and several micronutrients, but use of these compounds increases the production cost. Industrial medium using agro-industrial residues or subprodutс as substrates in the fermentation and some of the residues most widely used in submerged fermentation are: corn steep liquor, soybean oil, cottonseed oil, sugar cane molasses, soya milk and sorghum.

The use of industrial medium represents a good alternative to produce bioherbicide at low cost, since the production may improve or at least remain the same, as that obtained using a synthetic medium. In addition, the search for the most suitable substrate is not only dictated by costs and availability of the substrate but by other factors such as complexity of unwanted reactions that affect not only upstream, midstream but downstream activities. On the other hand, industrial residues may contain insoluble substances and other components that can affect the growth of the microorganism (Singhania et al., 2010).

2.1.4.1.2. Solid state fermentation

Solid-state fermentation is defined as the fermentation involving solids in absence (or near absence) of free water. However, the substrate must have enough moisture to support growth and metabolism of microorganism (Pandey, 2003). The solid material can act as inert support for the growth of microorganism or as a support and carbon source, the latter being most widely used in the production of bioherbicides.

SSF offers numerous opportunities in processing of agro-industrial residues. This is partly because solid-state processes have lower energy requirements and produce less wastewater (Pandey, 2003)**,** mreover, in most cases to obtain the final concentrate product and a better product recovery. However, the disadvantage is pricipal carry out the scaling up.

The substrates used in solid state fermentation are, in most cases, composed of agro-industrial residues such as bagasse, rice husk, wheat bran and soybean meal. In many cases, after colonization, the substrates are ground and applied granular formulations of herbicides. Alternatively an extract made from fermented grains may be used as a bioherbicide. Hoagland et al. (2007) used rice grains to cultivate *Myrothecium erraria* to control kudzu in some agricultural products; they found that extracts of fermented rice grains were more efficient than the inoculum applied to the soil.

Shabana et al. (2010) showed the production of bioherbicide by SSF from fungal *Dactylaria higginsi* and 19 solid substrates were tested. Conidial yields were highest when the fungus was grown on purple nutsedge hay, but conidia produced on this medium had low virulence. Conidia produced on sorghum sudangrass and cogongrass hays were slightly larger and thicker walled than those produced on other substrates. Conidia produced on sorghum sudangrass were the most virulent on nutsedge seedlings. Purple nutsedge was more susceptible to *D. higginsii* than yellow nutsedge. Cogongrass and sorghum sudangrass hay substrates offer a suitable and easy medium for large-scale production of *D. higginsii*.

Winder et al. (1999) studied various substrates and temperatures for their effect on the sporulation and virulence of *F. avenaceum*, a potential biological control agent for marsh reed grass (*Calamagrostis canadensis*). The best temperature to sporulation was 20 °C. On substrate the inoculum produced on puffed wheat was twice as virulent as that produced on marsh reed grass straw amended with malt extract. Mesoconidial production was maximal using a 1:1 mixture of puffed wheat and wheat straw with malt extract. Straw cultures without malt extract produced the most virulent inoculum. Straw cultures generally produced a higher proportion of macroconidia but virulence was not a directly related to conidial morphology. While conditions favouring abundant sporulation did not favour virulence, bag cultures were a suitable method for production of *F. avenaceum* conidia.

Valdensinia heterodoxa is a potential fungal bioherbicide for control of *Gaultheria shallon* (Zhao et al., 2006). This study showed the effect of culture media, substrates and relative humidity (RH) on growth, sporulation and conidial discharge of *V. heterodoxa*. Culture media significantly affected the growth, sporulation, and conidial discharge of *V. heterodoxa*. Of the 12 solid substrates used, the greatest numbers of discharged conidia were observed from wheat bran and wheat bran–salal within 14 days of sporulation. Relative umidity (RH) significantly affected the sporulation and conidial discharge for both isolates across all solid substrates tested. No conidia were produced or discharged below 93 % RH on

wheat bran–salal and millet. With an increase of the RH from 93 to 97 %, sporulation and the number of discharged conidia increased significantly for both isolates on wheat bran–salal, but not on millet.

No process with bacteria was found in the studies about solid fermentation. On the often a wide range of microorganisms were found in the studies with fungi. Some relevant process variables are: fermentation time, which was 7 to 15 days in most of the studies, slightly higher than submerged fermentation; temperature, ranging around 24 to 28 °C; and relative humidity on growth of microorganisms. The selection of the substrate also is important, since it affects the growth, sporulation, and conidial discharge (Zhao et al 2006; Shabana et al. 2010; Hoagland et al., 2007).

2.1.4.2. Formulation of bioherbicides

Formulations of bioherbicides contain the active ingredient (spore or secondary metabolites), a carrier (largely inert material) and adjuvants, which may contain compounds such as nutrients and/or chemicals, which aid in the survival of the pathogen or help in protecting the active ingredient from adverse environmental conditions (Hynes and Boyechko, 2006). The adjuvants may also aid in the infection of the host. There has been a dearth of detailed information on formulation of bioherbicides in the literature until recently, which could be attributed to the proprietary nature of the research (Hynes and Boyechko, 2006; Fravel et al., 2005) or the lack of understanding of the basic microbial processes being affected. As such, the formulation of bioherbicides is often viewed as an art rather than a science. Many believe that improvement in formulation of bioherbicides may provide the key elements to increasing success of the agents in the field (Brar et al., 2006) and as the formulation may have a great effect on the shelf life of the product, it will also affect the development, registration and commercialization (Ghobani et al., 2005).

For the successful implementation of bioherbicides into a weed management system, practical approaches for their application require an understanding of formulation, delivery and methods for assuring consistent field performance. Thus, the active constituent is combined with a solvent or surfactant in order to make its delivery and dispersal convenient, which is the broad definition of formulation (Rosskopf, 1999).

Development of effective formulations can provide micro-environments that will ensure adequate propagule survival and facilitate the infection process into the target weed

species (Hoagland et al., 2007). DNA technologies are also providing tools to enhance the pathogenicity or virulence of the pathogens and to also facilitate monitoring of the organisms in order to assess risk of introducing them into the environment (Boyetchko et al., 2002). Effective formulations also can provide a great effect on the shelf life of the product, that affect the development, registration and commercialization (Ash, 2010).

Green et al. (2007) reports the use of surfactants, which are thermodynamically stable suspensions of bioherbicide liquid or dissolved in surfactant micelles, which causes the appearance of micro-emulsions to improve the efficiency of bioherbicide. Surfactants can be classified into groups based on their ionization in water and this may give some clue as how a new or untried surfactant will behave in a biological control setting. The commonly used surfactants, Tween 20, Tween 80 Triton, X-100 and Tergitol are all nonionic surfactants. These compounds have been the most commonly used for initial experimentation. One drawback with this type of nonionic surfactant is that it helps to break the cuticle and may cause the plant to produce resistance-inducing compounds or promote entry of microorganisms capable of inducing cross-protection, both of which can retard the growth of the pathogen. Other surfactants that act as emulsifying agents used with more complex formulations such as those that involve oil-based spore suspensions, are now more commonly used (Rosskopf et al., 1999).

The Pesta formulation is also an away to deliver microorganisms in the formulated material. This material consists of wheat gluten mixed with the fungus to prepare sheets of material that are then dried and crumbled. Shabana et al. (2003) studied the Pesta formulation with *Fusarium oxysporum* for biocontrol of *Orobanche cumana*. These authors tested the Pesta formulations using two different types of fungal spores and eight types of coadjuvants used alone or together to examine the viability over time (shelf life) and efficiency under greenhouse conditions. Regarding the shelf life, it successfully reduced the loss of viability at 40 and 100% and longer shelf life was obtained when the granules were stored at 3 °C. The best results yielded reductions in emissions of 80 and 76% *Orobanch cumana* weed. Elzein et al. (2004) studied the effect of inoculums type and concentration on the viability of the encapsulated propagules of *Fusarium oxysporum* (Foxy 2), in Pesta granules during storage. Pesta granules were made with different inoculums of Foxy 2, including: conidia, a mixture of mycelia and conidia, and fresh as well as dried chlamydospore-rich biomass, each with three deferent inoculums concentrations. All granular preparation was stored at 4 °C or room temperature (21 ± 3 °C) for 1 year. Throughout the year, the viability of fresh and dried

chlamydospore-rich biomass was not significantly affected by the concentration of chlamydospores in the formulation at 4 °C or room temperature. Thus, 85–100% viability of Foxy 2 propagules can be achieved in Pesta granules for at least 1 year by formulating chlamydospore-rich biomass and storing at a temperature of 4 °C. This information has significant implications in enhancing shelf-life of Foxy 2 products thereby helping us to overcome this obstacle to commercialization.

Product formulations are becoming increasingly complex when are the active compounds they formula (Boyette et al., 2008). In some situations, both formulations and active ingredients need to be stabilized. Microencapsulation and particle coating are controlled release technologies that are increasingly being used to protect actives and stabilize formulations. Ultraviolet light absorbing compounds, anti-oxidants and other compounds are also used to stabilize complex formulations (Green and Beestman, 2007).

2.1.4.3. Application technology

The application of the bioherbicide when poorly executed can directly affect the efficiency of bioherbicide. Some researches in this area were carried out by Hiltz et al. (2013) and Carlsen et al. (2006), who studied the influence of adjuvant usually based on polymers, which reduce the size of spray droplets and improves the covering of the sheet thus increasing the efficiency of bioherbicide (Green and Beestman, 2007).

The findings of Doll et al. (2005) that severe disease caused by the fungus *Microsphaeropsis amaranthi* on *Amarathus* sp. is affected by droplet size and coverage is not surprising, but also indicates that more attention needs to be paid to application technology of bioherbicides in the future (Ash, 210). Byer et al. (2006) also found a greater efficacy of *Colletrotrichum truncatum* with smaller droplet sizes on scentless chamomile. Therefore, the diameter of spray droplets directly influences bioherbicide efficiency, and besides the droplet diameter, the sheet surface, velocity and angle of impact are also important parameters. However, more studies are needed in this area to better understand this mechanism.

2.1.5. Scale-up at bioherbicides production

It is observed that there are few published articles in literature dealing with scale-up of fermentation processes for production of biological control agents at large volumes, since most studies use experimental laboratory reactors. Microbial fermentation development is

usually carried out in three steps: shake flask cultures and bench-top fermenters are initially used, pilot scale for optimization of operating conditions, and plant scale for satisfaction of industrial yields and economy (Hosobuch and Yoshikawa, 1999).

Scale-up of the culture in a large scale bioreactor is the key challenge toward commercial exploitation (Banque et al., 2012). The improvement of potential control agents often depends on an adequate mass-production method for producing the infective propagules on a suitably large scale (Issaly et al., 2005). Physical, chemical, and process factors may need to be compensated as scale increases. Scale-up is generally more practical with submerged than solid cultures (Cheng et al., 2003).

Observing the difficulty of scaling-up biotechnological processes, a search in literature was realized to verify how is this issue developing in bioherbicide production by SMF and SSF.

2.1.5.1. Bioreactors for SMF

Submerged fermentation processes are more widespread industrially, because ease scale-up in bioreactors of SMF and ease operation of this type of reactor. Within this group there are some possible reactor configurations that can be used to produce bioherbicides and biopesticides. Huang and McDonald (2009a) listed some of the main types of bioreactors used for scale-up fermentation processes. Stirred tank and pneumatic bioreactors are commonly applied for microbial fermentation.

2.1.5.1.1. Stirred tank bioreactors

The main advantages of stirred-tank bioreactors (STR) are their flexibility and ability to provide high volumetric mass transfer coefficients (Huang and McDonald, 2009b). In mechanically agitated bioreactors, the stirrer is the main gas dispersing tool and stirrer speed and design have both a pronounced effect on mass transfer (Ochoa and Gomez, 2009). Suitable impellers can provide high volumetric mass transfer coefficients and a homogeneous environment enabling suspended plant cell growth and foreign protein production to be controlled consistently (Huang and McDonald, 2009a, 2009b, 2012). Some studies using STR bioreactors for production of bioherbicides and biopesticides are commented below.

Vurro et al. (2012) studied the optimization of the production of herbicidal toxins by the fungus *Ascochyta caulina* for biocontrol of the weed *Chenopodium album*. The fungus

produced in liquid culture three main metabolites proposed as possible natural herbicides. It proved to grow well and to produce up to 230 mg of toxins L^{-1} culture when grown for 5–10 days in shaken conditions, provided the initial inoculum was at least 10^5–10^6 conidia mL^{-1} of culture. Toxin production was improved by the addition of yeast extract to the medium. Attempts were made to assess the suitability of the fungus to be mass produced in a stirred fermentation system and to test its ability to produce the toxins in those conditions. Therefore, a fermenter (BIOSTAT 30 L– Sartorius Stedim Biotech) was used in 40 rpm, 26 °C and 15 days of fermentation. Every 2 or 3 days the rotation speed was increased to around 200 rpm for two or three minutes, and then restored to 40 rpm to reduce adhesion of the mycelia lumps to the fermenter walls and blades.

Boyette et al. (2008) provided an example of submerged culture mycelial formulation of the fungus *Myrothecium verrucaria* (IMI 361690) containing 0.20% Silwet L-77 surfactant, which was found to be highly effective in controlling the exotic invasive weed kudzu. Scale-up production in soy flour–corn meal medium using laboratory fermenters (10 – 25 L) resulted in a mycelial formulation that caused 90% mortality of naturally-occurring mature kudzu within 48 h after application in field experiments. The cultivation time was 7 days at 28 °C and agitation of 200 rpm.

Dokken (2007) studied the submerged fermentation of *Colletotrichum truncatum* a potential bioherbicide for biological control of *Scentless chamomile* using STR reactor of 20 L. The medium production was optimized for C/N ratio around 20:1, temperature of 20 °C, pH of 7.5 and agitation of 200 rpm. The preliminary results were satisfactory, showing that growth and sporulation are possible in production scale.

Mendizábal et al. (2012) studied the production of the *Bacillus subtilis* CPA-8 using low cost commercial products and by-products for biological control of postharvest fruit diseases. After bench experimental testing, the production was scaled-up in a 5 L bioreactor, while pH and oxygen consumption were optimized. There was a 5-h lag phase before growth, after which exponential growth occurred and maximum production was $3x10^9$ CFU/mL after 20 h (CFU - colony forming units). Results showed a disease reduction of 95% and could be used to provide a reliable basis for scaling up the fermentation process to an industrial level.

This review shows that there are few studies on biopesticide production and bioherbicide used bioreactors, however, most used reactors have scale between 20 to 30 L (Vurro et al., 2012; Boyette et al., 2008, Dokken, 2007). These studies have aimed to optimize

the process conditions such as agitation, aeration, temperature, pH and fermentation time (Vurro et al., 2012; Boyette et al., 2008, Dokken, 2007, Mendizábal et al., 2012), besides using agro-industrial waste to reduce operating costs (Boyette et al., 2008, Faye Dokken) or optimizing the synthetic medium (Vurro et al., 2012). The amount of inoculum is also important and should be optimized, but depends on the characteristics of each microorganism (Vurro et al., 2012, Mendizábal et al., 2012). These variables are of great importance to maximize production and reduce costs and achieve a workable process.

2.1.5.1.2. Pneumatic bioreactors

A pneumatic bioreactor is a type of gas–liquid dispersion bioreactor consisting of a cylindrical vessel where compressed air or gas mixture is introduced at the bottom of the vessel through nozzles, perforated plates or a ring sparger, for aeration, mixing and fluid circulation, without moving mechanical parts (Hung and McDonald, 2009b). Bubble columns and airlift bioreactors are two main types of pneumatically agitated reactors (Hung and McDonald, 2009a, 2009b; Ochoa and Gomez, 2009).

Airlift bioreactors are considered one of the most promising designs in the direction of increasing oxygen transfer rates and at the same time minimizing energy consumption. In this type of device, all of the energy needed is invested in the injection of air at a convenient pressure and flow rate (Hung and McDonald, 2009a). The air circulation in the device has the double role of delivering the oxygen needed for the metabolism of the microorganisms and of creating enough turbulence in the liquid phase to obtain an acceptable oxygen transfer rate. Compared to stirred tanks, airlift bioreactors are considered low-shear systems with more evenly distributed energy dissipation and shear stress throughout the reactor (Hung and McDonald, 2009b; Znad et al., 2006).

Bubble columns bioreactors have some advantages as easy construction and scaling up, low operational cost, contamination risk, shear stress and no heat generation from mechanical agitation. But it also has some disadvantages such as poor oxygen mass transfer ability, poor fluid mixing in highly viscous cultures compared to stirred-tank bioreactor and serious foaming under high aeration conditions (Hung and McDonald, 2009a, 2009b; Znad et al., 2006; Ochoa and Gomez, 2009).

No articles were found for bioherbicides production in pneumatic bioreactors in literature, but there were some articles regarding biopesticide production, which can serve as

an analysis for future studies regarding bioherbicides. For example, Micheloud et al. (2011) studied the production of biopesticide with the formulation based on the fungus *Anticarsia gemmatalis multiple nucleopolyhedrovirus* and optimization production in airlift and stirred bioreactor.

Visnovski et al. (2011) studied the influence of bioreactors in cell growth rates for the production of biopesticides. They compared the cell growth in shaker-flask, STR and air-lift reactors for production of *Anticarsia gemmatalis* multiple nucleopolyhedrovirus (AgMNPV) for biocontrol of *Anticarsia gemmatalis*. The cell line was able to adapt to conditions that can be used in industrial scale, both in an airlift and a stirred reactor, although the former was better than the latter to support the cell growth. The infection with AgMNPV in the airlift reactor produced a high yield of occlusion bodies, with very low production of budded virus (the progeny used as inoculum). On the other hand, infection in the stirred reactor yielded high concentrations of budded virus. These results suggest that a feasible strategy for scaling-up the production of AgMNPV might involve the use of airlift reactors for cell suspension cultures and the final production of inclusion bodies, while the scaling-up of the viral inoculum would be carried out in stirred reactors.

Visnovoski et al. (2003), the influence of reactor configuration and superficial gas velocity in the cultivation of insect cells in airlift reactors for biopesticide production was studied. The reactors used were 500 mL and the cultivation was performed at 28 °C. The cells were cultivated in three different concentric tube airlift reactors that differed in their geometrical parameters. Modifying the reactor design, the cellular growth rate could be improved from 0.019 h^{-1} to 0.031 h^{-1}, while the maximum viable cell density varied from $9x10^5$ to $2.4x10^6$ cells/mL. Once reactor configuration was selected, the influence of gas flow rate was determined to find an optimal value of superficial gas velocity that renders sufficient oxygenation of 0.091 cm^3/min.

2.1.5.2. Bioreactors for SSF

Initially, is important to analyze which forms of SSF process are used before starting the discussion about the bioreactors that are commonly employed. Typically, the SSF is a batch process, the culture medium is added to the reactor, then going to inoculation and incubation of the substrate. The product can be extracted by means of the suspension with water, solutions or solvents, or simply dried and stored.

The last decade has witnessed an unprecedent increase in interest in solid state fermentation for the development of bioprocesses (Pandey et al., 2000). This increase is due to some advantages that SSF provides on the SMF, including the reduction of production cost. However, SSF processes are difficult to scale-up, which represents a challenge to be overcome. Therefore, SSF processes are being widely used and this is also observed in the production of bioherbicides, although, in a smaller proportion.

Breukelen et al. (2011) studied the cultive of *Metarhizium anisopliae* in bioreactor for solid-state fermentation. The spores of this microorganism are a promising alternative to chemical insecticides against malaria mosquitoes. For such application, fungal spores need to be produced in large quantities. Cultivation of the fungus on several solid substrates and in two bioreactor types for solid state fermentation was studied. Experiments showed that *M. anisopliae* cannot withstand mixing; therefore aerated static packed beds are the most suitable reactor type. Pilot-scale fermentation demonstrated the feasibility of scale-up of the aerated packed bed with impregnated hemp, showing its potential for large scale production of *M. anisopliae* spores. The cylindrical, jacketed, stainless steel packed bed reactor was used. The inner diameter was 200 mm and the bed height 600 mm, corresponding to a bed volume of approximately 20 L.

2.1.6. The business and future of bioherbicides

If the gauge of success of a biopesticide is its commercialization, then there are many aspects of business and marketing which determine the success or otherwise of the venture (Ash, 2010). The market for biopesticides remains relatively small, while the cost of research and development is high. The Organization for Economic Co-operation and Development (OECD) has estimated that US$ 5 billion has been spent worldwide in public research for biological control during the past 40 years, averaging now US$500 million per year, similar to the estimated US$ 600 million spent yearly in research by the agrochemical industry (Glare et al., 2012).

It is questionable that the number of biopesticides now available justifies this investment, although it must be noted that the investment in biological control does not only include development of biopesticides. Clearly, more targeted public/private partnerships are needed. In the period 1972–2002, 72% of biopesticide business ventures were estimated to

have failed, showing the difficulties faced by small start-up companies in pest management (Glare et al., 2012).

Biopesticides have to be competitive on price in addition to efficacy and consistency (Ash, 2010). The economics of mass production, formulation and/or application have, in the past, greatly limited biopesticides reaching the market place because the sale price needed to recover costs was prohibitive. Often, the cost of fermentation is higher than the cost of making a synthetic chemical. This, so to be competitive in the market, microbial isolates must have high potency against the pest or high yielding capacity during production. By overtly considering the cost of production early in the development process, companies are avoiding developing non-profitable products. For example, during the development of Contans®, the company Prophyta considered the economics of spore mass production early in the process to ensure that the product would be commercially viable. Products such as those based on *B. thuringiensis*, Serenade® and Contans® are competing on price with synthetic pesticides, demonstrating that production cost is not always a barrier (Goeden and Andres., 1999).

With the development of sustainable agriculture and consciousness of human environmental protection, government and enterprises will pay more attention to the study and exploitation of microbial pesticide because of their potential benefits for the environment. With further study of weed control mechanisms and establishment of many models of screening weed pathogens, microbial herbicides will have a high chance to develop successfully (Li et al., 2003).

Biological control will expand to new countries and include the transfer of proven biological control agents from other areas (Boyetchko, 2002; Zhou et al., 2004; Charudattan, 2000). As in the past, the transfer and establishment of successful agents are followed by the development of new facilities, expertise, and the undertaking of new projects (Gurr et al., 2010). In developed countries, the main challenge will be to provide new biological control successes that will engage the imagination and support of research administrators and a public increasingly oriented toward high-technology solutions (Charudattan, 2000; Goeden and Andres, 1999). Attempts to develop weed project cost-benefit analyses will improve, although efforts to include ecological and aesthetic benefits will continue to pose difficulties (Gurr et al., 2010). To optimize the benefits of biological control, changes in agricultural production, resource use, and perceptions of what constitutes damaging weed populations will be required (Goeden and Andres, 1999).

Therefore, the biological control of pests and weeds has been increasing worldwide. Currently, there are already some conferences to discuss the future and prospects of new biocontrol agents. One example is the conference held in Australia and New Zealand in 2010 "Australia and New Zealand biocontrol conference: Emerging themes, future prospects", what was attended by several researchers, suppliers and practitioners (Gurr et al., 2010).

2.1.7. Concluding remarks

With the development of sustainable agriculture and consciousness of human environmental protection, government and enterprises will pay more attention to the study and exploitation of microbial bioherbicide, due to their potential benefits for the environment. Thus, this review brings concept of the biological control as a promising alternative for weed control and shows the need for scientific research on the agents and the techniques that should be mastered for bioherbicides development and production.

By the year 2009, approximately only 11 bioherbicide products were made available in the marketplace.This demonstrates the need for new biological weed control products in the market (Ash, 2010). Nevertheless, when considering the level of investments by chemical companies for the development of a chemical bioherbicide, with a success rate less than 1%, compared to a success rate of 5% on the development of bioherbicides (Ash, 2010). Investment of US$ 80 million is estimated to develop a chemical bioherbicide and US$ 0.8 to 1.6 million to develop a bioherbicide product. This shows that bioherbicides are progressing very well (Li et al., 2003).

Although research on bioherbicides has received less support than research on classic chemical herbicides, investment in long-term research coupled with the lack of support from administrators and funding agencies generally have been the major obstacles to new classic biological control agents introduction. Today, the largest centers that develop research in biological control area are the United States, Canada and Australia.

A bioherbicide should be competitive in price, in addition to efficiency and consistency, to be successful. A virulent pathogen and a suitable formulation ensure bioherbicide efficiency and consistency. A competitive price is obtained through optimization of fermentation techniques, efficient bioreactors and the use of raw materials with low added value. These conditions, associated with a suitable marketing plan, increase probability that

the final product will be success. A good example previously mentioned in the review was the development of Contans® by Prophyta (Boyetchko et al., 2002).

It would be naive to suggest that biological control will replace chemical herbicides, but the high cost related to herbicides development led to the creation of niche markets for bioherbicides. Therefore, the demand for bioherbicides has increased, but on the other hand, more research is needed to discover new biological control agents to improve the existing agents' action and also to optimize production processes.

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References

Abbasher, A.A. and J. Sauerborn. 1992. *Fusarium nygamai*, a potential bioherbicide for *Striga hermonthica* control in sorghum. *Biological Control. 2:291-296.*

Ash, G.J. 2010. The science art business of succeful bioherbicides. *Biological Control*. 52:230–240.

Auld, B.A. and M.M. Say. 1999. Comparison of isolates of *Colletotrichum orbiculare* fromArgentina and Australia as potential bioherbicides for *Xanthium spinosum* in Australia. *Agriculture Ecosystems and Environment*. 72:53-58.

Bailey, K.L., W.M. Pitt, F.L. Leggett, C. Sheedy and J. Derby. 2011b. Determining the infection process of *Phoma macrostoma* that leads to bioherbicidal activity on broadleaved weeds. *Biological Control*. 59:268-276.

Bailey, K.L., W.M. Pitt, S. Falk, and J. Derby. 2011a. The effects of *Phoma macrostoma* on nontarget plant and target weed species. *Biological Control*. 58:379–386.

Baque, M.A., S.H. Mho, E.J. Lee, J.J. Zhong and K.Y. Paek. 2012. Production of biomass and useful compounds from adventitious roots of high-value added medicinal plants using bioreactor. *Biotechnology Advances*. 30:1255–1267.

Barton, J. 2004. How good are we at predicting the Field host-range of fungal pathogens used for classical biological control of weeds? *Biological Control*. 31:99–112.

Begonia, M.F.T. and K.J. Kremer. 1994. Chemotaxis of deleterious rhizobacteria to velvetleaf *(Abutilon theophrasti* Medik.) seeds and seedlings. *Microbiology Ecology*. 15:227-236.

Betina, V. 1989. Chromatographic methods as tools in the field of mycotoxins. *Journal of Chromatography*. 477:187-233.

Bettiol, W., M.A.B. Morandi, Z.V. Pinto, T.J.P Júnior, E.B. Corrêa, A.M. Bittencourt, C.M.M. Lucon, J.C.B. Costa and J.L. Bezerra. 2012. Commercial products based agents biocontrol of plant diseases. *Brazilian Agricultural Research Corporation*.

Boyetchko, S.M., E.N. Rosskopf, A.J. Caesar and R. Charudattan. 2002. Biological weed control with pathogens: search for candidates to applications. *Agriculture and Food Production*. 2:239-266.

Boyette, C.D., M.A. Weaver, R.E. Hoagland and K.C. Stetina. 2008. Submerged culture of a mycelial formulation of a bioherbicidal strain of *Myrothecium verrucaria* with mitigated mycotoxin production. *World J. Microbiol Biotechnol*. 24:2721–2726.

Brar, S.K., M. Verma, R.D. Tyagi and J.R. Valéro. 2006. Recent advances in downstream processing and formulations of *Bacillus thuringiensis* based biopesticides. *Process Biochemistry*. 41:*323-342.*

Breukelen, F.R., S. Haemers, R.H. Wijffels and A. Rinzema. 2011. Bioreactor and substrate selection for solid-state cultivation of the malaria mosquito control agent *Metarhizium anisopliae*. *Process Biochemistry*. 46:751–757.

Bruinsma, J., N. Alexandratos, G. Bödeker, N. Scialabba, J. Schmidhuber, N. Urquia and V. Timon. 2003. Selected issues in agricultural technology. *World Agriculture*. 23:297–330.

Byer, K.N., G. Peng, T.M. Wolf, B.C. Caldwell. 2006. Spray retention and its effect on weed control by mycoherbicides. *Biological Control*. 37:307–313.

Carlsen, S.C.K., N.H. Spliid and B. Svensmark. 2006. Drift of 10 herbicides after tractor spray application.2. Primary drift (droplet drift). *Chemosphere*. 64:778–786.

Charudattan, R. 2001. Biological control of weeds by means of plant pathogens: significance for integrated weed management in modern agro-ecology. *Biocontrol*. 46:229–260.

Charudattan, R. and A. Dinoor. 2000. Biological control of weeds using plant pathogens: accomplishments and limitations. *Crop Protection*. 19:69-695.

Cheng, J., D. Jiang, X. Yi, Y. Fu, G. Li and J.M. Whipps. 2003. Production, survival and *Coniothyrium minitans* conidia produced in shaken liquid culture. *FEMS Microbiology Letters*. 227:127-131.

Clarke, N, P. Cloke, C. Barnett and A. Malpass. 2008. The spaces and ethics of organic food. *Journal of Rural Studies*. 24:219–230.

Cunningham, J.E. and C. Kuiack. 1989. Esterase activity as a marker for sporulation in *Colletotrichum gloeosporioides* f. sp. *malvae* in submerged culture *Mycological Research. 93: 236-239.*

Demain, A.L. 2000. Small bugs big business: The economic power of the microbe. *Biotechnology Advances*. 18:499–514.

Dokken, F. 2007. Submerged Fermentation of *Colletotrichum truncatum* for Biological Control of Scentless Chamomile. Head of the Department of Applied Microbiology and Food Science, University of Saskatchewan.

Doll, D.A., P.E. Sojka and S.G. Hallett. 2005. Effect of nozzle type and pressure on the efficacy of spray applications of the bioherbicidal fungus *Microsphaeropsis amaranthi*. *Weed Technology*. 19:918–923.

Duan, G., Z. Zhang, J. Zhang, Y. Zhoub, L. Yub and Q. Yuana. 2007. Evaluation of crude toxin and metabolite produced by *Helminthosporium gramineum rabenh* for the control of rice sheath blight in paddy fields. *Crop Protection*. 26: 1036–1041.

Elzein A, J. Kroschel and D.M. Stover. 2004. Effects of inoculum type and propagule concentration on shelf life of Pesta formulations containing *Fusarium oxysporum* Foxy 2 a potential mycoherbicide agent for *Striga spp. Biological Control*. 30:203–211.

Elzein, A, J. Kroschel and D. Muller-Stover. 2004. Effects of inoculum type and propagule concentration on shelf life of Pesta formulations containing *Fusarium oxysporum* Foxy 2, a potential mycoherbicide agent for *Striga spp. Biological Control*. 30:203–211.

Fravel, D.R., K.L. Deahl and J.R. Stommel. 2005. Compatibility of the biocontrol fungus *Fusarium oxysporum* strain CS-20 with selected fungicides. *Biological Control*. 34:165-169.

Frey. S. and N. Magan. 2001. Production of the fungal biocontrol agent *Ulocladium atrum* by submerged fermentation: accumulation of endogenous reserves and shelf-life studies. *Microbiol Biotechnol*. 56:372–377.

Ghorbani, R., C. Leifert and W. Seel. 2005. Biological control of weeds with antagonistic plant pathogens. *Advances in Agronomy*. 86:191-225.

Gibbs, P.A., R.J. Seviour and F. Schmid. 2000. Growth of filamentous fungi in submerged culture: problems and possible solutions. *Critical Reviews in Biotechnology*. 20:17–48.

Glare, T., J. Caradus, W. Gelernter, T. Jackson, N. Keyhani, J. Kohl, P. Marrone, L. Morin and A. Stewar. 2012. Have biopesticides come of age? *Trends in Biotechnology*. 30:250-258.

Goeden, R.D. and L.A. Andres. 1999. Andres. Biological control of weeds in terrestrial and aquatic evironments. *Handbook of Biological Control*. pp. 871-890.

Graupner, P.R., A. Carr, E. Clancy, J. Gilbert, K.L. Bailey, J.A. Derby and B.C. Gerwick. 2003. The macrocidins: novel cyclic tetramic acids with herbicidal activity produced by *Phoma macrostoma*. *Natural Products*. 66:1558-1561.

Green, J.M. and B.G. Beestman. 2007. Recently patented and commercialized formulation and adjuvant technology. *Crop Protection*. 26:320–327.

Gurr, G.M., P. Horne, G.J. Ash and L.J. Pilkington. 2010. Australia and New Zealand biocontrol conference: Emerging themes future prospects. *Biological Control*. 52:195–197.

Hilz, E. and A.W.P. Vermeer. 2013. Spray drift review: The extent to which a formulation can contribute to spray drift reduction. *Crop Protection*. 44:75-83.

Hoagland, R.E., C.D. Boyette and M.A. Weaver. 2007. Bioherbicides: Research and risks. *Toxin Reviews*. 26:313–342.

Hosobuch, M. and H. Yoshikawa. 1999. Scale-up of microbial processes. In manual of industrial microbiology and biotechnology. *ASM Press*. 236-239.

Huang, T.K. and K.A. McDonald. 2009a. Bioreactor engineering for recombinant protein production in plant cell suspension cultures. *Biochemical Engineering Journal*. 45:168–184.

Huang, T.K. and K.A. McDonald. 2009b. Bioreactor engineering for recombinant protein production in plant cell suspension cultures. *Biochemical Engineering Journal*. 45:168–184.

Huang, T.K. and K.A. McDonald. 2012. Bioreactor systems for in vitro production of foreign proteins using plant cell cultures. *Biotechnology Advances*. 30: 398–409.

Hynes, R.K. and S.M. Boyetchko. 2006. Research initiatives in the art and science of biopesticide formulations. *Soil Biology & Biochemisty*. 38: 845-849.

Issaly, N., H. Chauveaua, F. Aglevor, J. Fargues and A. Durandd. 2005. Influence of nutrient pH and dissolved oxygen on the production of *Metarhizium flavoviride* Mf189 blastospores in submerged batch culture. *Process Biochemistry*. 40:1425–1431.

Jackson, M.A. 1997. Optimizing nutritional conditions for the liquid culture production of effective fungal biological control agents. *Journal of Industrial Microbiology & Biotechnology*. 19:180–187.

Jackson, M.A. and A.S. David. 1995. Liquid culture production of microsclerotia of *Colletotrichum truncatum* for use as bioherbicidal propagules. *Mycol. Res.* 99:879-884.

Jackson, M.A., J.S. Frymier, B.J. Wilkinson, P. Zorner and S. Evans. 1998. Growth requirements for production of stable cells of the bioherbicidal bacterium *Xanthomonas campestris*. *Journal of Industrial Microbiology & Biotechnology*. 21:237–241.

Johnson, B.J. 1994. Biological control of annual bluegrass *with Xanthomonas campestris pv. poannua* in bermudagrass. *Hortic. Sci*. 29(6):659–662.

Kremer, R.J., M.T.F. Begonia, L. Stanley and E.T. Lanham. 1990. Characterization of rhizobacteria associated with weed seedlings. *Appl. Environ. Microbiol.* 56:1649–1655.

Kropp, B. and H. Darrow. 2006. The effect of surfactants and some herbicides on teliospore viability in *Puccinia thlaspeos (Schub.)*. *Crop Protection*. 25:369–374.

Li, Y., Z. Sun, X. Zhuang, L. Xu, S. Chen and L. Mingzhi. 2003. Research progress on microbial herbicides. *Crop Protection*. 22:247-252.

Meir, S, C. Larroche, A.H. Ahmad and J. Gressel. 2009. Fungal transformation of *Colletotrichum coccodes* with bacterial oahA to suppress defenses of *Abutilon theophrasti*. *Crop Protection*. 28:749–755.

Mendizábal, V.Y., I. Viñas, J. Usall, R. Torres, C. Solsona and N. Teixidó. 2012. Production of the postharvest biocontrol agent *Bacillus subtilis* CPA-8 using low cost commercial products and by-products. *Biological Control*. 60:280–289.

Micheloud, G.A., V.V. Gioria, I. Eberhardt, G. Vinovsky and J.D. Clau. 2011. Production of the *Anticarsia gemmatalis multiple nucleopolyhedrovirus* in serum-free suspension cultures of the saUFL-AG-286 cell line in stirred reactor and airlift reactor. *Jurnal of Virological Methods*. 178:106-116.

Mingzhi, L., X. Ling and S. Ziling. 2007. Isolation and Characterization of a phytotoxin from *Xanthomonas campestris pv. Retroflexus*. *Chin. J. Chem. Eng.* 15(5):639-642.

Mitchell, J.K., C.N. Yerkes, S.R. Racine and E.H. Lewis. 2008. The interaction of two potential fungal bioherbicides and a sub-lethal rate of glyphosate for the control of shattercane. *Biological Control*. 46:391–399.

Mitchell, J.K. 2003. Development of a submerged-liquid sporulation medium for the potential smartweed bioherbicide *Septoria polygonorum*. *Biological Control*. 27:293–299.

Monte, E. and I.A. Garcia. 1988. Vegetative and reproductive structures of *Phoma Betae* in vitro. *Trans. Br. mycol Soc.* 90(2):233-245.

Morin L, A.F. Gianotti, D.R. Lauren. 2000. Trichothecene production and pathogenicity of *Fusarium tumidum* a candidate bioherbicide for gorse and broom in New Zealand. *Mycol. Res.* 104(8):993-999.

Ochoa, F.G. and E. Gomez. 2009. Bioreactor scale-up and oxygen transfer rate in microbial processes: An overview. *Biotechnology Advances*. 27:153–176.

Pandey, A. 2003. Solid-state fermentation. *Biochemical Engineering Journal*. 13: 81–84.

Pandey, A., C.R. Soccol and D. Mitchel. 2000. New developments in solid state fermentation: I-bioprocesses and products. *Process Biochemistry*. 35:1153–1169.

Parra, R., D. Aldred and N. Magan. 2005. Medium optimization for the production of the secondary metabolite squalestatin S1 by a *Phoma sp.* combining orthogonal design and response surface methodology. *Enzyme and Microbial Technology*. 37:704–711.

Penariol, M.C., A.C Monteiro and R.A. Pitelli. 2008. Growth and sporulation of *Bipolaris euphorbiae* cultivated under different nutritional conditions. *Science Rural de Santa Maria.* 38:1907-1913.

Pereira J.M., R.W. Barreto, C.A. Ellison, and L.A. Maffia. 2003. *Corynespora cassicola f. sp. lantanae*: a potential biocontrol agent from Brazil for *Lantana camara*. Biological Control. 26:21-31.

Rizzardi, M.A., N.G. Fleck, D. Agostinetto and A.A. Balbinot. 2003. Herbicides action in plant defence mechanisms to pathogens. *Science Rural Santa Maria*. 33:957-965.

Rosskopf, E. and R. Koenig. 2003. Are bioherbicides compatible with organic farming systems and will businesses invest in the further development of this technology? *VI International Bioherbicide Group Workshop.*

Rosskopf, E.N. Charudattan, R. Kadir, J.B. (1999) Use of plant pathogens in weed control. *Handbook of Biological Control*. pp. 891-918.

Ruddick, B.H. and K. Mortensen. 1995. In vitro formation and survival of appressoria of a mycoherbicide agent *Colletotrichum gloeosporioides* f.sp. malvae and a benomy I-resistant strain HP4.5RR. *Mycal. Res.* 99:1103-1107.

Schobert, R. and B. Barnickel. 2010. Toward the macrocidins: Macrocyclization via williamson etherification of a phenolate. *JOCnote*. 75:6716–6719.

Selbmann, L., S. Onofri, M. Fenice, F. Federici and M. Petruccioli. 2002. Production and structural characterization of the exopolysaccharide of the Antarctic fungus *Phoma herbarum* CCFEE 5080. *Research in Microbiology*. 153:585–592.

Shabana, Y.M., D. Singh, L.M.O. Ribbing and S.G. Hallett. 2010. Production and formulation of high quality conidia of *Microsphaeropsis amaranthi* for the biological control of weedy *Amaranthus* species. *Biological Control*. 55:49–57.

Shabana, Y.M., D.M. Stover and J. Sauerborn. 2003. Granular Pesta formulation of *Fusarium oxysporum f. sp. orthoceras* for biological control of sunflower broomrape: efficacy and shelf-life. *Biological Control*. 26:189–201.

Shabana, Y.M., R. Charudattan, A.H.A. Tabl, J.P.M. Payan, E.N. Rosskopf and W. Klasse. 2010. Production and application of the bioherbicide agent Dactylaria higginsii on organic solid substrates. *Biological Control*. 54:159–165.

Shiguang, Z. and S.F. Shamoun. 2006. The effects of culture media, solid substrates, and relative humidity on growth, sporulation and conidial discharge of *Valdensinia heterodoxa*. *Mycological Research*. 110(11): 1340-1346.

Silman, R.W., R.I. Bothast and A. Schisler. 1993. Production of *Colletotrichum truncatum* for use as a micoherbicide effects of culture, drying and storage on recovery and efficacy. *Biological Control*. 11:561-573.

Singhania, R.R., R.K. Sukumaran, A.K. Patel, C. Larroche and A. Pandey. 2010. Advancement and comparative profiles in the production technologies using solid-state and submerged fermentation for microbial cellulases. *Enzyme and Microbial Technology*. 46:541-549.

Tebeest, D.O., X.B. Yang, C.R. and Cisar. 1992. The status of biological-control of weeds with fungal pathogens. *Annual Review of Phytopathology*. 30:637–657.

Templeton, G.E.. D.O. Tebeest and R.J. Smith. 1984. Biological weed control in rice with a strain of *Colletotrichum gloeosporioides* (Penz.) Sacc. Used as a mycoherbicide. *Crop Protection*. 3:409-422.

Visnovsky, G., G.A. Micheloud, V.V. Gioria, I. Eberhardt and J.D. Claus. 2011. Production of the *Anticarsia gemmatalis multiple nucleopolyhedrovirus* in serum-free suspension cultures of the saUFL-AG-286 cell line in stirred reactor and airlift reactor. *Journal of Virological Methods*. 178:106–116.

Visnovsky, G., J.D. Claus and J.C. Merchuk. 2003. Cultivations of insect cells in Airlift reactors: influence of reactor configuration and superficial gas velocity. *Latin American Applied Reserarch*. 33:207-211.

Vurro, M., A. Andolfi, A. Boari, M.C. Zonno, S. Caretto, F. Avolio and A. Evidente. 2012. Optimization of the production of herbicidal toxins by the fungus *Ascochyta caulina*. Biological Control. 60:192-198.

Weaver, M.A. and C.D. Boyette. 2012. Bioherbicidal activity from washed spores of *Myrothecium verrucaria. World Journal Microbiol Biotechnol*. 28:1941–1946.

Winder, R.S. 1999. The influence of substrate and temperature on the sporulation of *Fusarium avenaceum* and its virulence on marsh reed grass. *Mycol. Res.* 103(9):1145-1151.

Yu, X., S.G. Hallett, J. Sheppard and A.K. Watson. 1988. Effects of carbon concentration and carbon-to-nitrogen ratio on growth, conidiation, spore germination and efficacy of the potential bioherbicide *Colletotrichum coccodes* X Yu1. *Journal of Industrial Microbiology & Biotechnology*. 20:333–338.

Zhang, L., S.Q. Wang, X.J. Li, A.L. Zhang, Q. Zhang and J.M. Gao. 2012. New insight into the stereochemistry of botryosphaeridionefrom a Phoma endophyte. *Journal of Molecular Structure*. 1016:72–75.

Zhang, W., M. Wolf, K.L. Bailey, K. Mortensen and M. Boyetchko. 2003. Screening of adjuvants for bioherbicide formulations with *Colletotrichum spp.* and *Phoma spp. Biological Control*. 26:95–108.

Zhao, S. and S.F. Shamoun. 2006. The effects of culture media, solid substrates, and relative humidity on growth, sporulation and conidial discharge of *Valdensinia heterodoxa Mycological Research*. 110:1340-1346.

Zhou, L., K.L. Bailey and J. Derby. 2004. Plant colonization and environmental fate of the biocontrol fungus *Phoma macrostoma*. *Biological Control*. 30:634-644.

Zidack, N.K., P.A. Backman and J.J. Shassw. 1992. Promotion of bacterial infection of leaves by an organosilicone surfactant: implications for biological weed control: *Biological Control*. 2:111-117.

Znad, H., M. Tokumura and Y. Kawase. 2006. Axial distribution of oxygen concentration in different airlift bioreactor scales: Mathematical modeling and simulation. *Chem. Eng. Technol.* 29(9):1042–1047.

3. RESULTADOS

3.1. Artigo II: Optimization of fermentation media for the growth of a fungus used as bioherbicide

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Abstract: In this work the composition of fermentation media for the growth of *Phoma* sp. was optimized. This is a potential fungus to be used for the production of bioherbicide. For this purpose, the fungal growth was optimized, firstly, using synthetic media containing glucose, peptone, and yeast extract. In a second moment, glucose was replaced by sucrose food-grade while, peptone and yeast extract by corn steep liquor. The optimized composition of synthetic media was glucose and peptone 20 $g.L^{-1}$, yeast extract 7.5 $g.L^{-1}$, and initial pH of 6.0. For industrial media, the optimized composition was sucrose 20 $g.L^{-1}$, corn steep liquor 8%, and an initial pH of 6.0. At these conditions, the maximum fungal biomass for each medioum was 22 $g.L^{-1}$ and 33 $g.L^{-1}$, respectively. The cost of fermentation media to obtain 1000 kg of biomass decreased from US$ 41,664 to US$ 38 when synthetic media was changed by industrial media.
Keywords: Bioherbicide, experimental design, fermentation submerged, *Phoma* sp.

3.1.1. Introduction

Since the introduction of chemical herbicides in the decade of 1940 (Templeton et al., 1984), some limitations as weeds that are resistant to herbicides, contamination of soil and water resources, and chemical residues harmful to non-targets organisms (Li et al., 2003) are evident, motivating the search for new technologies or new molecules to overcome these problems. An alternative that has been used is the biological control, where the microorganism or a secondary metabolite produced by this microorganism can be employed as an agent of control in a narrow field of application (Zhou et al., 2004). Bioherbicides are a new weapon in the struggle against the resistance of plants, and products can lead to the use for organic and conventional farmers in modern agriculture.

Although the control of weeds using bioherbicides is attractive, research and commercialization of these products are yet low. Since the first reports of using bioherbicides from *Phytophthora palmivora* and *Colletotricum gloeosporioides f.* sp. *aeschynomene* (Tebeest et al., 1992), at least 11 products were commercialized (Bailey et al., 2011; Ash, 2010). Besides those commercial products, Charudattan (2001) listed more than 50 examples of pathogens combination that present potential to be used as bioherbicides.

The search for biocontrol agents of weeds is increasing. Among the microorganisms that can be used, the fungal of genus *Phoma* sp. was reported as potential agent. Bailey et al. (2011) evaluated the action of *Phoma macrostoma* as bioherbicide, whereas Zhang et al. (2012) used *Phoma* sp. to obtain antimicrobial extracts. Parra et al. (2005) optimized the

production of secondary metabolites of *Phoma* sp. with herbicide activity. Evidente et al. (2001) used *Phoma exigua var. exigua* as a potential bioherbicide for the control of *Cirsium arvense*.

However, one of the key aspects on design of industrial fermentation for production of molecules with commercial interest, including bioherbicides, is the definition of substrate employed in the media formulation. The bulk fermentation broth is often considered as one of the most important component in the cost of the fermentation products, which usually accounts for almost 50% of the whole production process (Li et al., 2013). Pure chemicals though idealistic would increase greatly the fermentation costs and would not be economically viable, unless the fermentation product is high cost and low volume. Thus, in order to lower the costs of production the search for the most cheapest and economical source of fermentation substrate will be the top most agenda in any proposed fermentation industry. Research on the selection of suitable substrates for fermentative processes has mainly been centered on agro-industrial residues, due to their potential advantages (Dhillon et al., 2013; Li et al., 2013; Treichel et al., 2012).

The use of an industrial medium represents a good alternative to produce bioherbicide at a low cost, since the production yield may improve or at least remain the same as that obtained using a synthetic medium. In addition, the search for the most suitable substrate is not only dictated by costs and availability of the substrate, but by other factors such as complexity of unwanted reactions that affect not only upstream, midstream, but downstream activities as well. On the other hand, industrial residues may contain insoluble substances and other components that can affect the growth of the microorganism (Treichel et al., 2010).

Based on these aspects, the main objective of this work was the optimization of the media composition for the growth of *Phoma* sp. in shaken flasks. For this purpose, the fungal growth was optimized, firstly using synthetic media containing glucose, peptone, and yeast extract. In a second moment, glucose was replaced by sucrose food-grade, peptone and yeast extract by corn steep liquor and the growth was optimized again. Microbial kinetics for optimized conditions was determined. In addition, the fermented broth at optimized conditions was applied for the control of a target plant.

3.1.2. Material and methods

3.1.2.1. Materials

Corn steep liquor (CSL) obtained from Corn Products Brazil (Mogi Guaçu, SP, Brazil), and sucrose food-grade (Cristal), purchased in a local market, were used in this experiment. All other chemicals, $(NH_4)_2SO_4$, $FeSO_4.7H_2O$, $MnSO_4.H_2O$, $MgSO_4$, glucose, peptone, and yeast extract were purchase from Sigma-Aldrich.

3.1.2.2. Microorganism, inoculum and fermentations

The strain of *Phoma* sp. (NRRL Y-7571) was obtained at the National Center for Agricultural Utilization Research – EUA (ARS). The culture was maintained in a potato dextrose agar (PDA) between 4 -6°C and subcultured every 15 days. Cell production for pre-inoculum was incubated on PDA in a Petri dish for 8 days at 28ºC. Then, the Petri dish was washed with 5 mL of sterile water and transferred to an Erlenmeyer for fermentation.

The fermentations were carried out in a 250 mL Erlenmeyer, which contained 50 mL of fermentation medium at 28°C and 120 rpm for 5 days, the micronutrient composition remained constant in all fermentations ($g.L^{-1}$): $(NH_4)_2SO_4$ 2.0, $FeSO_4.7H_2O$ 1.0, $MnSO_4.H_2O$ 1.0 and $MgSO_4$ 0.5 (Parra et al., 2005; Zhang et al., 2012; Selbmann et al., 2002; Yu et al., 1998).

3.1.2.3. Optimization of synthetic medium for fungal growth

The composition of synthetic media was defined combining glucose, peptone, and yeast extract at different initial pHs. For this purpose, a Plackett–Burman design with 8 runs plus 3 central points (PB8) was used to determine the effects of independent variables on the growth of *Phoma* sp. The range of variables investigated was 5-15 $g.L^{-1}$ for glucose and peptone, 5-10 $g.L^{-1}$ for yeast extract and pH 5-7. Based on the statistical analysis of PB8, a central composite rotational design for two independent variables was proposed to optimize the concentrations of glucose and peptone. The response evaluated in both experimental designs was dry cell mass, which was expressed as gram of dry biomass per liter of fermentation medium.

3.1.2.4. Optimization of industrial medium for fungal growth

The formulation of industrial medium consisted in substituting glucose for food-grade sucrose and nitrogen sources (peptone and yeast extract) by corn steep liquor. In addition, it evaluated the influence of micronutrient concentration on growth of *Phoma* sp. For this purpose, a central composite rotational design for three independent variables was proposed to optimize the concentrations of food-grade sucrose, CSL, and micronutrients. The range of variables investigated was 15-35 $g.L^{-1}$ for sucrose, 10-30 wt% for CSL, and 1-4 wt% for micronutrient.

Based on the analysis of Central Composite Rotational Design (CCRD), two independent variables were conceived to optimize the concentrations of sucrose and CSL. The variables investigated were 15-25 $g.L^{-1}$ for sucrose and 5-15 wt% for CSL. The response evaluated in both experimental designs was dry cell mass, which was expressed as gram of dry biomass per liter of fermentation medium.

3.1.2.5. Determination of fungal biomass

The fungal biomass was determined by the method of dry cell weight (Prakash and Srivastava, 2008). After fermentation, the mass of cells was separated from fermentation broth by centrifugation at 4000 rcf in 10 minutes. The cells were washed with distilled water and dried in an oven at 105°C for 24 hours. Finally, the mass of dried biomass was determined by weight.

3.1.2.6. Assay for bioherbicide activity

The activity of the bioherbicide obtained from *Phoma* sp. was demonstrated using cucumber as a target. For this purpose, a fermentation using fermented industrial media at optimized condition (sucrose 20 $g.L^{-1}$, CSL 8.0%, and pH 6 by 5 days) without fungal biomass (withdrawn by centrifugation at 4000 rcf in 10 minutes) was carried out and used in the bioassay. The bioassay consisted in the cultivation of two trays, each one containing 20 seedlings of cucumber. All plants on the first tray were sprayed with fermented broth, whereas the second one was used as a control. The application of bioherbicide was carried out after 15 days of cucumber cultivation, when the plants presented 3 to 4 leaves. The plants were then monitored every seven days to verify the action of the bioherbicide.

3.1.2.7. Statistical analysis

All statistical analysis was carried out using the software Statistica® 8.0 (Statsoft Inc., Tulsa, OK, USA), considering a significance level of 90%.

3.1.3. Results and discussion

3.1.3.1. Optimization of synthetic medium for fungal growth

Table 1 presents the concentration of fungal biomass obtained in the eleven runs of the PB8 after five days of fermentation.

Table 1. Matrix of the Plackett–Burman design to evaluate the influence of synthetic medium on concentration of fungal biomass after 5 days of fermentation.

Run	pH	Glucose ($g.L^{-1}$)	Peptone ($g.L^{-1}$)	Yeast extract ($g.L^{-1}$)	Fungal Biomass ($g.L^{-1}$)	Cost to produce Fungal Biomass (US$/1000kg)
1	7 (1)	5 (-1)	5 (-1)	10 (1)	7.25	63150.00
2	7 (1)	15 (1)	15 (-1)	5 (-1)	9.29	72143.00
3	7 (1)	15 (1)	15 (1)	5 (-1)	14.77	45181.00
4	5 (-1)	15 (1)	15 (1)	10 (1)	16.35	48758.00
5	7 (1)	5 (-1)	15 (1)	10 (1)	9.18	73626.00
6	5 (-1)	15 (1)	5 (-1)	10 (1)	5.85	98997.00
7	5 (-1)	5 (-1)	15 (1)	5 (-1)	9.29	58776.00
8	5 (-1)	15 (-1)	5 (-1)	5 (-1)	4.09	109846.00
9	6 (0)	10 (0)	10 (0)	7.5 (0)	12.57	44756.00
10	6 (0)	10 (0)	10 (0)	7.5 (0)	14.22	39563.00
11	6 (0)	10 (0)	10 (0)	7.5 (0)	11.38	49436.00

The biomass concentration ranged from 4.09 (run 8) to 16.35 $g.L^{-1}$ (run 4). This variability in the results suggests that the independent variables present significant effects on

fungal growth. To check this, data of Table 1 was used to compute the magnitude and significance of these effects, which are presented in Table 2.

Table 2. Analysis of effects of independent variables of the Plackett–Burman design on the fungal biomass.

	Effect	**Std. Err.**	**t(5)**	**p-value**
Mean	10.48	0.90	11.58	<0.0001
pH	1.23	2.12	0.58	0.5830
Glucose	4.11	2.12	1.94	0.1000
Peptone	5.78	2.12	2.72	0.0344
Yeast extract	0.30	2.12	0.14	0.8925

Initial pH and yeast extract concentration did not present significant influence on fungal growth. On the other hand, the effects for peptone and glucose were positive and statistically significant ($p<0.1$), indicating that the increase of concentration of both variables would lead to an increase in the concentration of fungal biomass in the fermentation.

For this reason, a CCRD was used to evaluate the influence of glucose and peptone on concentration of fungal biomass, maintaining the concentration of yeast extract at 7.5 $g.L^{-1}$ and pH at 6.0, respectively. Table 3 presents the results obtained in the CCRD, where it is seen that the concentration of fungal biomass ranged from 11.74 (run 1) to 22.05 (run 8). The highest concentration of fungal biomass was obtained when glucose and peptone were maintained in the “level +1” of the experimental design. However, at the positive star points for peptone and glucose (runs 6 and 8, respectively), the increase in biomass was not verified. Meanwhile, the concentration of biomass increased considerably regarding the PB8, indicating that the strategy used was efficient to improve the growth of fungal biomass.

Table 3. Matrix of the CCRD design to evaluate the influence of synthetic medium on concentration of fungal biomass after 5 days of fermentation.

Run	Glucose ($g.L^{-1}$)	Peptone ($g.L^{-1}$)	Fungal Biomass ($g.L^{-1}$)	Cost to produce Fungal Biomass (US$/1000kg)
1	10 (-1)	10 (-1)	11.74	53451.00
2	20 (1)	10 (-1)	16.99	44073.00
3	10 (-1)	20 (1)	16.51	51215.00
4	20 (1)	20 (1)	22.05	43849.00
5	8 (-1.41)	15 (0)	14.06	50660.00
6	22 (1.41)	15 (0)	20.70	42613.00
7	15 (0)	8 (-1.41)	15.47	41664.00
8	15 (0)	22 (1.41)	19.78	48020.00
9	15 (0)	15 (0)	18.19	43826.00
10	15 (0)	15 (0)	18.92	42135.00
11	15 (0)	15 (0)	18.92	42135.00

The results presented in Table 3 were used to build a quadratic model expressing the concentration of fungal biomass in function of independent variables. Eq. 1 presents the significant terms of the model ($p<0.1$).

$$FB_s = 18.68 + 2.53 \cdot G - 0.82 \cdot G^2 + 1.99 \cdot P - 0.70 \cdot P^2 \quad (1)$$

Where FB_s is the concentration of fungal biomass obtained using synthetic media ($g.L^{-1}$), G is the coded glucose concentration, and P is the coded peptone concentration. The significant terms of models can be used to discuss the effects of each term on fungal growth. Both linear terms for glucose and peptone were statistically significant, but the effect of glucose was more accentuated than peptone. The negative quadratic terms concerning glucose and peptone indicate the presence of a maximum point in the system that is similar for both variables.

The model was validated by analysis of variance (ANOVA). The calculated F-test for Eq. 1 was about 8 times greater than the standard significance at $p=0.1$, and the determination

coefficient (R^2) was 0.9649. The high value for the determination coefficient indicate good fitting of experimental data, allowing the use of such model to predict the fungal biomass. The validated model was used to optimize the fungal biomass and the results obtained are presented in Fig. 1 in the form of contour plots. Maximum concentration of fungal biomass obtained for glucose and peptone concentrations were higher than 16 g.L^{-1}. Although, not fully delimited, the optimum region in the contour plot affirmed that the optimized condition, found in this work, is glucose and peptone at 20 g.L^{-1}, yeast extract at 7.5 g.L^{-1}, and an initial pH of 6.0. At this condition, maximum fungal biomass was 22 g.L^{-1}, which is higher than that obtained in the PB8, indicating that the strategy used to increase the fungal biomass was efficient.

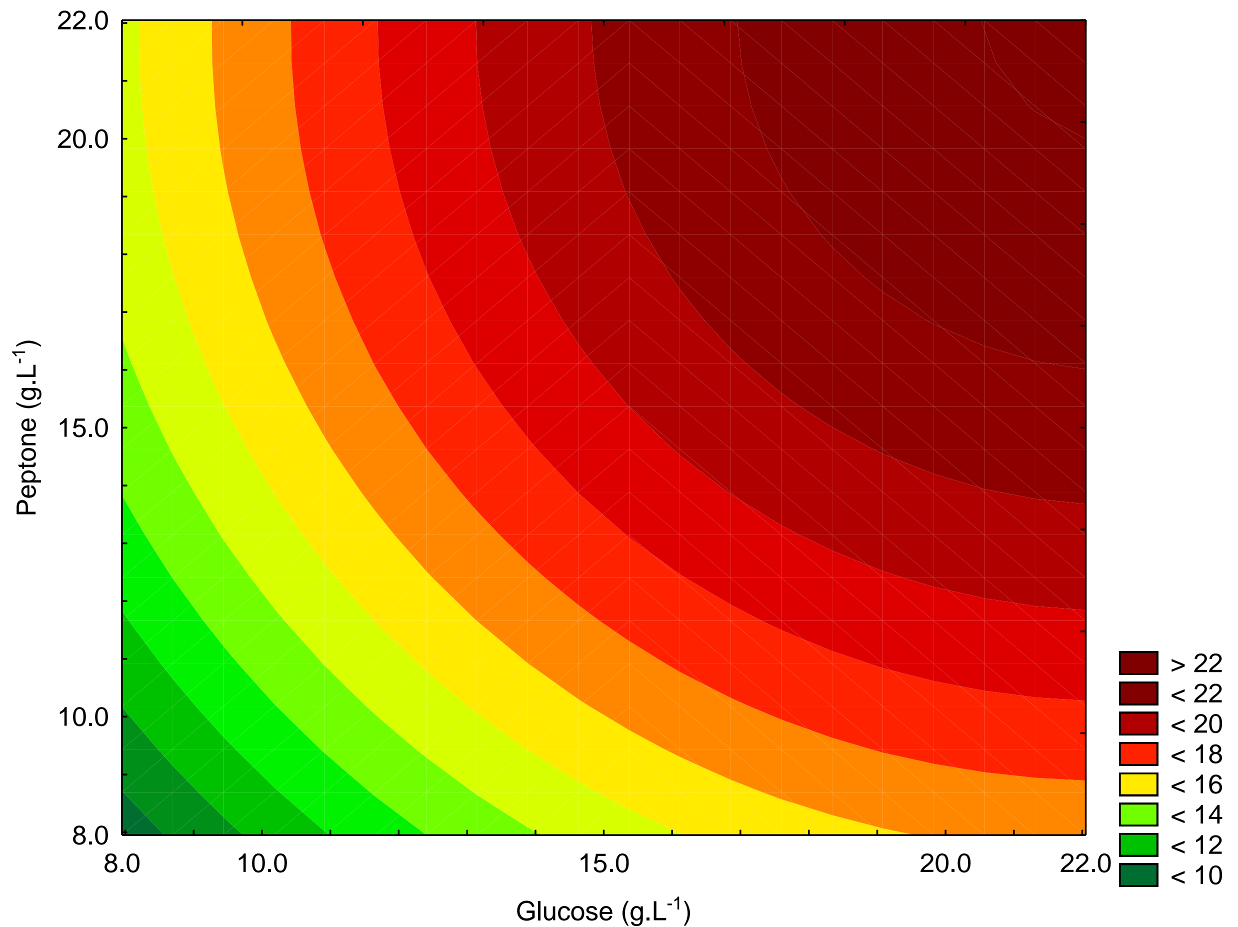

Fig. 1. Contour plot expressing the fungal biomass in function of peptone and glucose concentrations.

In order to obtain a simplified estimative of the cost to produce 1000 kg of dry fungal biomass, data of Tables 1 and 2 referring to composition of fermentation media and cell

production were used for this purpose. The price of all reagents of medium was obtained from Sigma-Aldrich. In the PB8 design (Table 1), a great variation in the production cost was observed, which ranged from US$ 39,563.00/ton (run 10) to US$ 109,846.00/ton (run 6). Obviously, the concentration of reagents influences the final cost, but the main concerning is related with biomass concentration. This is easily visualized at runs 9-11 of Table 1 (central points), where a variation of 2.8 $g.L^{-1}$ in the concentration of fungal biomass increased the production cost in US$ 9,873.00/ton. In all runs of Table 2, the biomass concentration increased in relation to Table 1, however the production cost did not suffer significant alteration, remaining around US$ 42,000.00/ton at optimized conditions because the concentration of glucose and peptone were increased. In a general way, the cost to produce fungal biomass using synthetic medium is high and unfeasible for scaling up of the process. The alternative is to change the high-price synthetic compounds by industrial media.

3.1.3.2. Optimization of industrial medium for fungal growth

The composition of synthetic medium was based on peptone, glucose, and yeast extract, which can be expensive for industrial applications, making it unfeasible for high volume bioreactor operations. For this reason, the constituents of fermentation medium were changed from sucrose (carbon source) and corn steep liquor (nitrogen and micronutrients source), which are less expensive substrates. Table 4 presents the results obtained in the CCRD for two independent variables studied, namely, concentration of sucrose and corn steep liquor©. The concentration of fungal biomass ranged from 33.29 $g.L^{-1}$ (run 5) to 3.06 $g.L^{-1}$ (run 3). One important aspect to be pointed out is the concentration of biomass obtained, which had a 51% higher fermentation rate than using the synthetic medium. This result was expected, since the microbial growth was higher using a medium considerably less expensive.

Table 4. Matrix of the CCRD design to evaluate the influence of industrial medium on concentration of fungal biomass after 5 days of fermentation.

Run	Sucrose ($g.L^{-1}$)	CSL (%)	Fungal Biomass ($g.L^{-1}$)	Cost to produce Fungal Biomass (US$/1000kg)
1	15 (-1)	5 (-1)	19.00	54.00
2	25 (1)	5 (-1)	25.50	58.00
3	15 (-1)	15 (1)	3.06	545.00
4	25 (1)	15 (1)	4.46	477.00
5	13 (-1.41)	10 (0)	33.29	38.00
6	27 (1.41)	10 (0)	29.46	64.00
7	20 (0)	3 (-1.41)	15.43	73.00
8	20 (0)	17 (1.41)	5.50	369.00
9	20 (0)	10 (0)	26.09	60.00
10	20 (0)	10 (0)	25.49	62.00
11	20 (0)	10 (0)	30.83	51.00

In the same way the fermentations using synthetic medium, the results presented in Table 4 were used to build a quadratic model, expressing the concentration of fungal biomass in function of independent variables. Eq. 2 presents the significant terms of the model ($p<0.1$).

$$FB_i = 27.50 - 6.39 \cdot CSL - 10.55 \cdot CSL^2 \quad (2)$$

Where FB_i is the concentration of fungal biomass obtained using industrial medium ($g.L^{-1}$) and CSL is the coded corn steep liquor concentration. From the significant model parameters it is seen that only linear and quadratic terms referring to CSL concentration were significant in the range evaluated. This result agrees with the data of Table 4, where one can easily visualize the influence of high concentration of corn steep liquor on fungal growth, since all the fermentations carried out at concentrations above 15 wt%, grew at very low rates.

The model was validated by ANOVA. The calculated F-test for Eq. 2 was about two times greater than the standard significance at p=0.1, and the determination coefficient (R^2) was 0.8163, validating, in this way, the model generated. The validated model was used to optimize the fungal biomass and the results obtained are presented in Fig. 2 in the form of contour plots. Maximum concentration of fungal biomass is obtained for sucrose concentration higher than 17 $g.L^{-1}$ and CSL in the range of 8-10 wt%. One aspect to be observed in Fig. 2, is the fact that increasing the concentration of sucrose also increases the optimal range of CSL. For example, sucrose at 17 $g.L^{-1}$ gives the optimal range for CSL between 8-9 wt% and sucrose at 20 $g.L^{-1}$ gives the optimal range for CSL between 7.5-9.5wt% and sucrose at 25 $g.L^{-1}$ gives the optimal range for CSL between 7-10wt%. From this finding, it is possible to conclude that the growth of fungi is dependent on the sucrose concentration and, mainly, supported by the increase in the source of nitrogen in the medium (in this work, CSL). The optimized condition for the industrial medium was sucrose at 20 $g.L^{-1}$ and CSL at 8wt%. At this condition, maximum fungal biomass was 33 $g.L^{-1}$, which is 51% higher than that obtained using synthetic medium.

In a similar way as for synthetic medium, data of Table 3 referring to composition of fermentation medium and cell production were used to obtain a simplified estimative of the cost to produce 1000 kg of dry fungal biomass. The cost of the substrates used was US$ 130.00 per ton of CSL provided by the company Corn Products Brazil and US$ 1,000.00 per ton of sucrose food-grade. The production cost ranged from US$ 38.00/ton (run 5) to US$ 545.00/ton (run 3). In this way, it is possible to conclude that the use of industrial medium is an alternative to turn economically a technically feasible the production process since it was possible to decrease 1,105 times the cost to obtain the biomass. In addition, the formulated medium enabled the increase in the biomass concentration and, mainly, its composition is simple, what is important in purification steps.

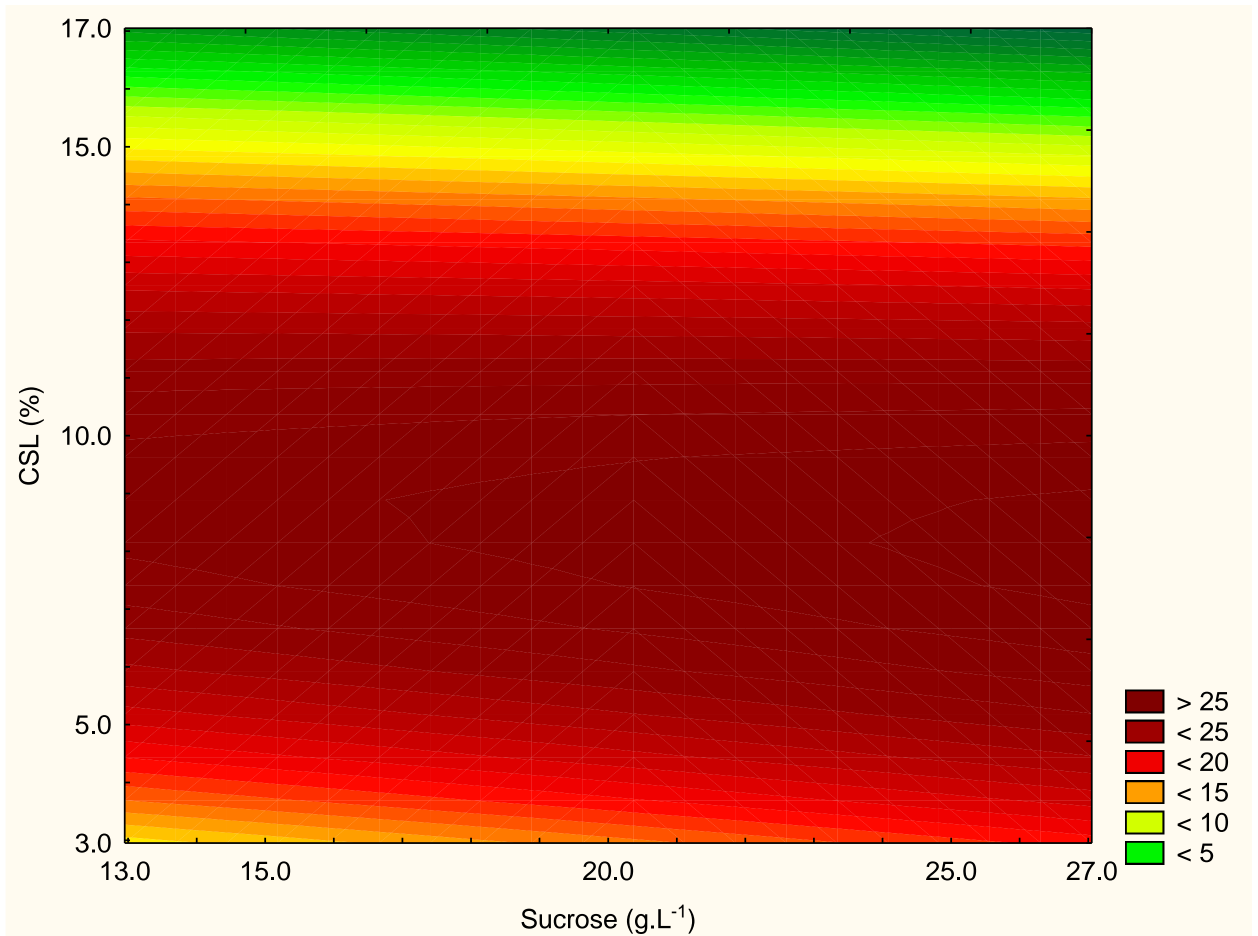

Fig. 2. Contour plots expressing the fungal biomass in function of sucrose and CSL concentrations.

3.1.3.3. Microbial kinetics for optimized synthetic and industrial medium

All fermentations evaluated were carried out by 5 days. At his moment, the kinetics of fungal growth was evaluated for the synthetic and industrial medium at conditions optimized above. Specifically, the fermentation using synthetic medium was carried out using glucose and peptone 20 g.L^{-1}, yeast extract 7.5 g.L^{-1}, and an initial pH of 6.0, whereas for industrial medium the composition was sucrose 20 g.L^{-1}, CSL 8.0%, and an initial pH of 6.0. The results obtained are presented in Fig. 3, where the maximum concentration of fungal biomass was obtained around 4-6 days of fermentation for the two types of medium. From Fig. 3, it is evident that the industrial medium promoted a more effective growth, since the concentration was about 51% higher than using synthetic medium, corroborating with results obtained in previous sections.

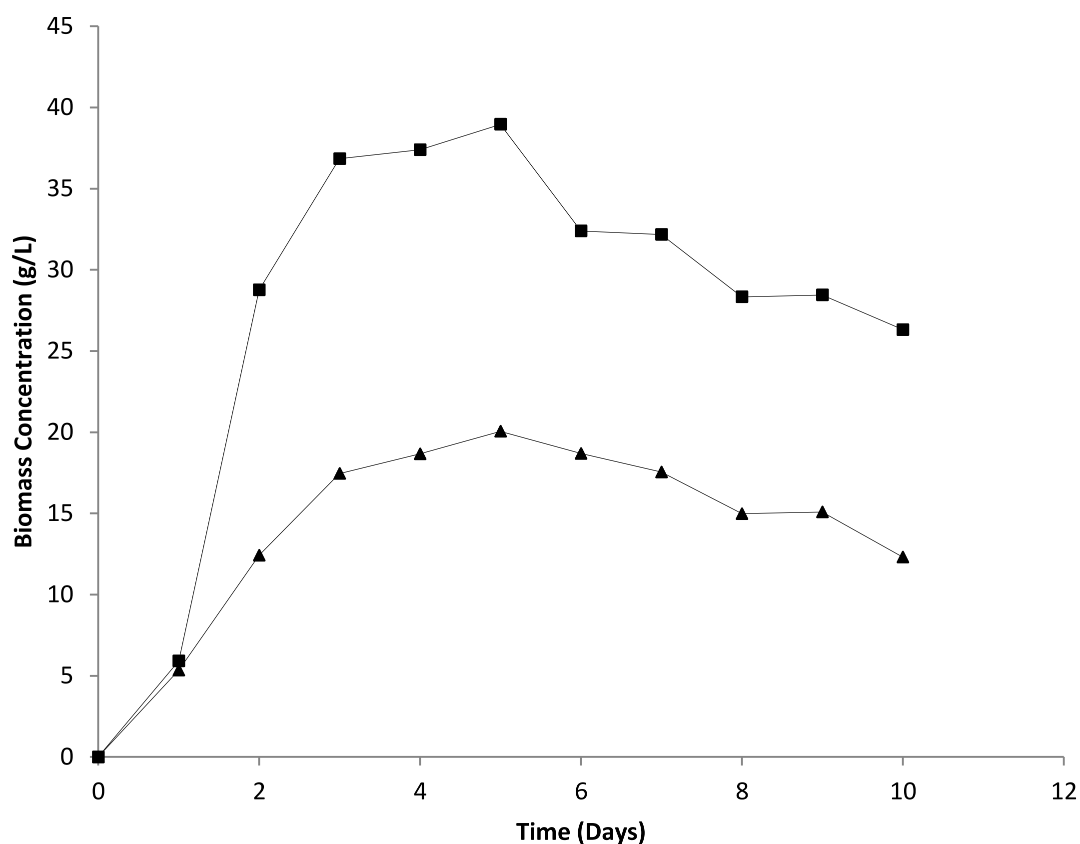

Fig. 3. Kinetic of fungal growth using synthetic (▲) and industrial medium (■) at optimized conditions.

3.1.3.4. Evaluation of bioherbicide activity

The results obtained in this section have the objective to show the potential of *Phoma* sp. to produce the bioherbicide. Fig. 4 presents the effect of fermented broth without fungal biomass after 21 days of application on the control, cucumber, which was the target plant used in this study. As it can be seen, the fermented broth presented positive results, since some cucumber plants died or had their leaves severely affected by the herbicide. In fact, the results presented here are preliminary, but they show a promising future. Additional tests to identify and quantify the molecule(s) with hebicide activity are necessary, besides defining of fermentation conditions to maximize the production of molecule(s) of interest.

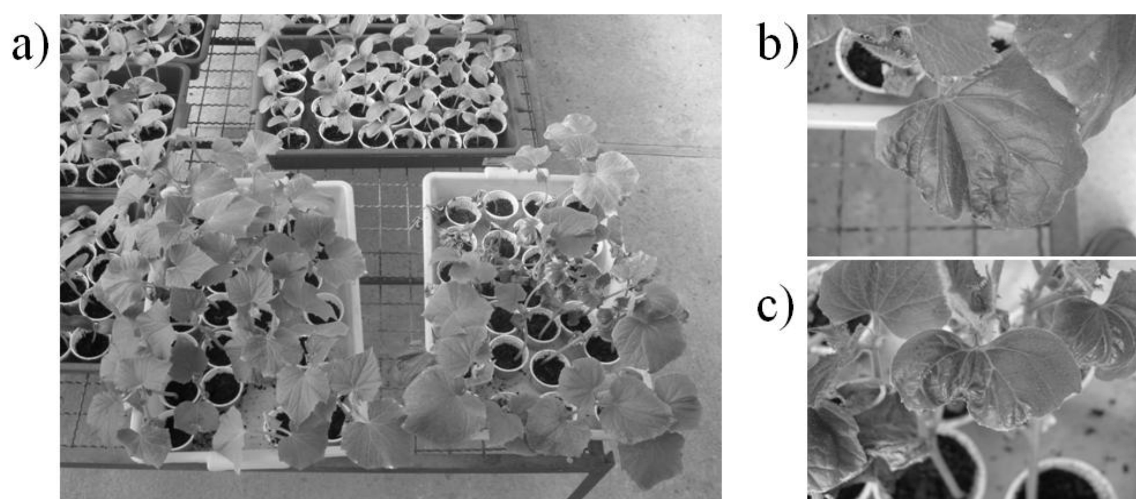

Fig. 4. Bioassay using the industrial fermented broth without cells as bioherbicide in the control of plant target. a) control plants; b,c) plants after 21 days of application.

3.1.4. Conclusions

In this work, the culture medium was optimized based on synthetic and industrial compounds for the growth of *Phoma* sp., which is a potential fungus for the production of bioherbicide. The optimized conditions for production of *Phoma* sp. by synthetic medium were glucose and peptone 20 $g.L^{-1}$, yeast extract 7.5 $g.L^{-1}$, and an initial pH of 6.0, yielding a maximum fungal biomass of 22 $g.L^{-1}$. For industrial medium, the optimized composition was sucrose 20 $g.L^{-1}$, CSL 8%, and initial pH of 6.0, obtaining maximum fungal biomass of 33 $g.L^{-1}$ that is 51% higher than for synthetic medium.

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References

Ash, G.J., (2010). The science art business of successful bioherbicides. Biol. Control, 52, 230-240.

Bailey, K.L., Pitt, W.M., Falk S., Derby, J. (2011). The effects of *Phoma macrostoma* on nontarget plant and target weed species. Biol. Control, 58, 379-386.

Boyetchoko, S.M., Rosskopf, E.N., Caesar, A.J., Charudattan, R. (2002). Biological weed control with pathogens: search for candidates to application. Agr. Food Prod, 2, 239-266.

Charudattan, R., (2001). Biological control of weeds by means of plant pathogens: significance for integrated weed management in modern agro-ecology. Biocontrol, 46, 229-260.

Evidente, A., Cimmino, A., Andolfi, A., Berestskiy, A., Motta, A. (2011). Phomachalasins AeD, 26-oxa[16] and [15] cytochalasans produced by *Phoma exigua* var. *exigua*, a potential mycoherbicide for *Cirsium arvense* biocontrol. Tetrahedron, 67, 1557-1563.

Kona, R.P., Qureshi, N., Pai, J.S. (2001). Production of glucose oxidase using *Aspergillus niger* and corn steep liquor. Bioresour. Technol., 78, 123-126.

Li, Y., Sun, Z., Zhuang, X., Xu, L., Chen, S., Mingzhi, L. (2003). Research progress on microbial herbicides. Crop Prot., 22, 247-252.

Miller, G.L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem., 31, 426–428.

Parra, R., Aldred, D., Magan, N. (2005). Medium optimization for the production of the secondary metabolite squalestatin S1 by a *Phoma sp*. combining orthogonal design and response surface methodology. Enzyme Microb. Tech., 37, 704-711.

Rodrigues, M.I., Iemma, A.F. (2005). Planejamento de Experimentos e Otimização de Processos: Uma estratégia seqüencial de planejamentos. Campinas, BRA: Casa do Pão.

Selbmann, L., Onofri, S., Fenice, M., Federici, F., Petruccioli, M. (2002). Production and structural characterization of the exopolysaccharide of the Antarctic fungus *Phoma herbarum* CCFEE 5080. Res. Microbiol., 153, 585–592.

Tebeest, D.O., Yang, X.B., Cisar, C.R. (1992). The status of biological-control of weeds with fungal pathogens. Annu. Rev. Phytopathol., 30, 637-657.

Templeton, G.E., Tebeest, D.O., Smith, R.J. (1984). Biological weed control in rice with a strain of *Colletotrichum gloeosporioides* (Penz.) *sacc.* ysed as a mycoherbicide. Crop Prot., 3, 409-422.

Treichel, H., Oliveira, D., Mazutti, M.A., Di Luccio, M., Oliveira, J.V. (2010). A review on microbial lipases production. Food Bioprocess Tech., 3, 182-196.

Yong-Lan, X., Chen, K., Dai, W., Ma, J., Zhang, M., Jiang, M., Wei, P., Ouyang, P.K. (2013). Succinic acid production by *Actinobacillus succinogenes* NJ113 using corn steep liquor powder as nitrogen source. Bioresour. Technol., 136, 775–779.

Yu, X., Hallett, S.G., Sheppard, J., Watson, A.K. (1998). Effects of carbon concentration and carbon-to-nitrogen ratio on growth, conidiation, spore germination and efficacy of the potential bioherbicide *Colletotrichum coccodes*. J. Ind. Microbiol. Biotechnol., 20, 333–338.

Zhang, H., Xiong, Y., Zhao, H., Yi, Y., Zhang, C., Cuiping, Yu, Xu, C. (2013). An antimicrobial compound from the endophytic fungus *Phoma sp*. isolated from the medicinal plant *Taraxacum mongolicum*. J. Taiwan Inst. Chem. E., 44, 177-181.

Zhang, L., Wang, S.Q., Li, X.J., Zhang, A.L., Zhang, Q., Gao. G.M. (2012). New insight into the stereochemistry of botryosphaeridione from a Phoma endophyte. J. Mol. Struct., 1016, 72–75.

Zhou, L., Bailey, K.L., Derby, J. (2004). Plant colonization and environmental fate of the biocontrol fungus *Phoma macrostoma*. Biol. Control, 30, 634-644.

3.2. Artigo III: Bioherbicide activity of fermented extract of *Phoma* sp. obtained by solid-state fermentation

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Abstract: In this work was optimized the medium composition for the production of a bioherbicide from *Phoma* sp. by solid-state fermentation. For this purpose, the production was optimized using agroindustrial residues such as bagasse, soybean bran and corn steep liquor as substrate. After the fermentation, the bioherbicide was extracted from the fermented solid and applied for the control of target plant. The responses evaluated in the bioassay were the plant height, root length, fresh weight, dry weight, number of flowers and phytotoxicity. The results obtained in the bioassays demonstrate that the bioherbicide presented activity towards target plant and the intensity of the effect was influenced by the formulation of the fermentation medium. The optimized condition for bioherbicide production were substrate moisture of 70 %, soybean bran content of 30 wt% and CSL content of 20 wt%, yielding an injury level 40. The produced bioherbicide showed a mode of action based on the inhibition of carotenoids biosynthesis.

Keywords: Bioherbicide; bioassay; solid-state fermentation; experimental design; *Phoma* sp.

3.2.1. Introduction

The weed is one of the major contributing factors to yield loss in agricultural production in developing countries [1-2]. Weeds can cause substantial losses in the form of yield reductions through competition with crop plants for light, water, nutrients, heat energy, carbon dioxide, and space [3-4-1]. Reductions may also be caused by the production of growth-inhibiting compounds, a phenomenon referred to as allelopathy [5]. Moreover the weeds increasing the cost of land preparation or harvesting [5].

While weeds significantly contribute to crop yield losses, some of the reported negative impacts of chemical pesticides such as residues in soil and water, spray drift, and weed species exhibiting herbicide resistance underscore the importance of exploring alternatives to chemical herbicides [1]. An alternative that has been used is the biological control, where the microorganism or a secondary metabolite produced by it can be employed as agent of control in a narrow field of application [6]. Development of alternative control strategies such as biocontrol can provide additional management tools as supplements to other control measures for plant diseases, for use in rotation with other control measures, or as back-up when favored control measures are withdrawn from the market or fail due to new strains or races of the pathogen [7].

The science and technology of weed control by using plant pathogens gained attention and momentum in the 1970s when some century-old concepts in weed biocontrol and plant disease epidemiology were successfully put to test and a few economically important weeds were controlled. Since then, researchers in different parts of the world have critically examined and tried to apply these strategies with the hope of solving some of the most intractable weed problems [2].

There have been a number of successful introductions of classical biological control agents, including the more notable *Puccinia chondrillina* for control of skeleton weed. In the early 1980s, DeVine® was introduced as the first registered bioherbicide in the U.S. to control stranglervine in citrus. This was followed by Collego®, which is commercially available for northern joint-vetch control. Besides these products, there are other important marketed bioherbicides and some in pre-commercial evaluation stages [1]. By the year 2010 there was only 11 bioherbicides of commercial success [8]. This shows that there is a great market to be explored. Bioherbicide is defined as a plant pathogen used as a weed-control agent through inundative and repeated applications of its inoculum.

A significant barrier to bioherbicide production is the development of an economically viable process [9]. In the bioherbicide production by fermentative process the obtaining of an ingredient is strictly related to the formulation of the medium and the type of fermentation. The main types of fermentative process are submerged fermentation and solid-state fermentation.

The solid state fermentation has shown much promise in the development of several bioprocesses and products [10] and offers an alternative fermentation method that possesses several biotechnological advantages over the conventional submerged fermentation [11]. The substrates are oftentimes agroindustrial residues as sugarcane bagasse, rice husk, wheat bran and soybean meal. In many cases, the substrates are ground after fermentation and applied in granular formulations of bioherbicides. Some researchers used the extract obtained from fermented grains as bioherbicide. Hoagland et al. (2007) [12] used rice grains to grow *Myrothecium erraria* to control kudzu in some agricultural products. They found that extracts fermented from rice grains were more efficient than the inoculum applied on soil. There are many microorganisms that can be used for production of bioherbicides. Some studies report the use of fungi from *Phoma* sp. as bioherbicides using submerged fermentation. Bailey et al. (2011) [13] evaluated the action of *Phoma macrostoma* as bioherbicide, whereas Zhang et al.

(2012) [14] used *Phoma* sp. to obtain antimicrobial extracts. Parra et al. (2005) [15] optimized the production of secondary metabolites of *Phoma* sp. with herbicide activity.

Based on these aspects, the main objective of this work was to optimize the media composition for the production of a bioherbicide from *Phoma* sp. by solid-state fermentation. For this purpose, the production was optimized using agroindustrial residues such as bagasse, soybean bran and corn steep liquor as substrate. After the fermentation, the bioherbicide was extracted from the fermented solid and applied for the control of target plant. The responses evaluated in the bioassay were the plant height, root length, fresh weight, dry weight, number of flowers and phytotoxicity.

3.2.2. Material and methods

3.2.2.1. Materials

Sugarcane bagasse was obtained in a microdistillery located at the Federal University of Santa Maria, the sample was dried, milled and sieved. The particle size distribution was determined, obtaining a mean particle size of 1.32 mm and coefficient variation of the sample was 0,86. The soybean bran was purchased in a local market and the mean particle size was 0.95 mm and coefficient variation of the sample was 0.50. Corn steep liquor (CSL) was kindly supplied by Corn Products Brazil (Mogi Guaçu, SP, Brazil).

3.2.2.2. Microorganism, inoculum and fermentations

The strain of *Phoma* sp. NRRL Y-7571 was acquired from the National Center for Agricultural Utilization Research – EUA (ARS). The culture was maintained in potato dextrose agar (PDA) at 4-6°C and subcultured every 15 days. Cell production for pre-inoculum was incubated on PDA in a petri dish for 8 days at 28ºC. Then, the petri dish was washed with 5 mL of steril water and transferred to the fermentation medium.

The inoculum was cultivated in Erlenmeyer of 250 mL containing 50 mL of fermentation medium at 28°C, 120 rpm for 5 days. The composition was ($g.L^{-1}$): glucose 20, peptone 10, yeast extract 7.5, $(NH_4)_2SO_4$ 2.0, $FeSO_4.7H_2O$ 1.0, $MnSO_4.H_2O$ 1.0 and $MgSO_4$ 0.5 [15-14-16].

3.2.2.3. Optimization of fermentation medium for fungal growth

Fermentations were carried out in conical flasks (500 mL) containing 10g of solid substrate at 28°C for 5 days in a chamber with temperature and humidity control. Then, the solid substrate was supplemented and the moisture content adjusted at specified level. Each flask was covered with hydrophobic cotton and autoclaved at 121°C for 20 min. Preliminary studies showed that no changes in moisture content of the substrate after autoclaving were detected. After cooling, each flask was inoculated using 10 mL of inoculum.

The composition of fermentation medium was defined combining sugar cane bagasse, soybean bran and corn steep liquor and different conditions initial of moisture. For this purpose, a Central Composite Rotatable Design (CCRD) with 14 runs plus 3 central points was conceived to determine the effects of independent variables on growth of *Phoma* sp. and production of phytotoxins. The range of variables investigated was (wt%): soybean bran 8-32, CSL 8-32 and moisture content 50-80. The response evaluated in the experimental design was bioherbicide activity on the target plant.

3.3.2.4. Extraction of bioactive compounds

After the end of fermentation, the bioactive compounds of each essay were extracted using 100 mL of distilled water in an orbital shaker at 100 rpm and 28 °C for 1 hour. The broth obtained from the extraction was filtered and stored to be further applied in the bioassays.

3.2.2.5. Bioassay to evaluated bioherbicide activity

The activity of the bioherbicide obtained from *Phoma* sp. was determined using cucumber (*Cucumis sativus* variety *wisconsin*) as target plant. Although it is not a weed, this species is sensitive to synthetic herbicides and is frequently used in bioassays. Moreover, this species have rapid seed germination and do not exhibit dormancy.

All experiments were conducted in a greenhouse, where each experiment of the CCRD represents a treatment containing 20 plants. For the application of the bioherbicide a garden sprayer was used and approximately 30 mL were applied in each treatment. One assay was performed to verify the influence of the culture medium and another without treatment.

The application of the bioherbicide was carried out after 15 days of cucumber cultivation, where the plants presented 2 to 3 leaves. The plants were monitored for 21 days

after application to verify the bioherbicide action. The following aspects were evaluated for each treatment: plant height, root length, fresh weight, dry weight, number of flowers and phytotoxicity. To evaluate the phytotoxicit the scale of Franz and Crowley (1986) was used [17], which is showed in Table 1.

Table 1. Phytotoxic evaluation method in plants by Frans and Crowley (1986).

%	Description of the Main Categories	Description Phytotoxicity of the Culture
0	No Effect	No injury or reduction.
10	Slight Effect	Slight discoloration or atrophy.
20		Some discoloration or atrophy.
30		Injury more pronounced, but not lasting.
40	Moderate Effect	Moderate injury, usually with recovery.
50		More lasting injury, doubtful recovery.
60		Lasting injury without recovery.
70	Severe Effect	Heavy injury, stand reduction.
80		Next crop destruction.
90		Rarely some plants remain.
100	Total Effect	Complete destruction of culture.

3.2.2.6. Statistical analysis

The statistical analysis of the DCCR was carried out using the software Statistica® 8.0 (Statsoft Inc., Tulsa, OK, USA), considering a significance level of 90%. Tukey test was carried out using the software Assistat® 7.7 (DEAG-CTRN-UFCG, Campina Grande, PB, Brazil), considering a significance level of 95%.

3.2.3. Results and discussion

Taking into account that composition of fermentation medium and moisture content influence on the growth of microorganisms as well as on the production of bioactive

compounds, a CCRD was conceived to investigate the influence of soybean bran, CSL and moisture content on the production of compounds with bioherbicide activity using the *Phoma* sp. by solid-state fermentation. Table 2 presents the results obtained in the 17 runs of the CCRD referring to phytotoxicity of bioherbicide towards the target plant. More severe phytotoxic effects were observed in runs 6, 8, 10, 15, 16 and 17. These treatments presented profound and no continuous injury, representing injury ranging from 30-40 (according to the scale used) towards to target plant. Runs 15, 16 and 17 also presented significant injury to target plant. In addition, these runs are the central points of the CCRD and the results showed good agreement among the repetitions. It is also important to point out that phytotoxicity of control and control using fermentation medium presented lower levels of injury than tests using fermented broth. Tukey test ($p<0.05$) showed differences statistically significance in 13 runs of the CCRD in comparison with the control test.

Table 2. Matrix of the CCRD to evaluate the influence of independent variables on the bioherbicide production by solid-state fermentation.

Run	Moisture content (wt%)	CSL (wt%)	Soybean bran (wt%)	Phytotoxicity
Control	-	-	-	6[h]
Medium	65 (0)	20 (0)	20 (0)	7[h]
1	50 (-1)	8 (-1)	(8) -1	26[cde]
2	80 (1)	8 (-1)	(8) -1	31[bcd]
3	50 (-1)	32 (1)	(8) -1	12[gh]
4	80 (1)	32 (1)	(8) -1	15[fgh]
5	50 (-1)	8 (-1)	(32) 1	25[def]
6	80 (1)	8 (-1)	(32) 1	40[ab]
7	50 (-1)	32 (1)	(32) 1	25[def]
8	80 (1)	32 (1)	(32) 1	36[abc]
9	40 (-1,68)	20 (0)	20 (0)	15[fgh]
10	90 (1,68)	20 (0)	20 (0)	31[abcd]
11	65 (0)	0 (-1,68)	20 (0)	18[efg]
12	65 (0)	40 (1,68)	20 (0)	38[ab]
13	65 (0)	20 (0)	0 (-1,68)	13[gh]
14	65 (0)	20 (0)	40 (1,68)	36[abc]
15	65 (0)	20 (0)	20 (0)	39[ab]
16	65 (0)	20 (0)	20 (0)	41[a]
17	65 (0)	20 (0)	20 (0)	40[ab]

* Means followed by the same letter in the column do not show statistical difference, by test Shapiro-Wilk at 5% probability.

The bioherbicide activity of the fermented extracts also was investigated in terms of plant height, root length, fresh weight, dry weight and number of flowers for the 17 runs of CCRD plus the two control tests, where the results obtained are presented in Table 3. The height of plants for runs 1, 2, 3, 4, 6, 7 and 9 showed statistically significant ($p<0.05$) differences from the with control test. The lowest height (8.27 cm) was observed in run 9 that

is about 3.2 cm lower than the control test, whereas the highest height (11.46 cm) was observed in the run 14. The mean height of target in all runs was 10.33 cm with a variation coefficient of 13%. The root system also was monitored and the results showed no statistical difference for fresh and dry weight of roots. This results indicate that the bioherbicide produced do not have effect on roots of plants. Concerning aerial part of plant, the dry weight of runs 6 and 7 was statistically significant, presenting weight about 24% lower than control test, whereas fresh weight of run 6 was statistically significant, presenting weight about 20% lower than the control.

Table 3. Statistical analysis of the influence of bioherbicide from *Phoma* sp. on different part of target plant.

Run	Height Plant	Length Root	Fresh Weight		Dry Weight		Number Flowers
			Plant	Root	Plant	Root	
Control	11.47^{ab}	12.06^{a}	1.91^{a}	1.02^{ab}	0.19^{ab}	0.0588^{a}	2.80^{ab}
Medium	11.03^{abcd}	11.03^{a}	1.71^{ab}	1.06^{a}	0.18^{abcd}	0.0653^{a}	2.60^{ab}
1	9.13^{efg}	9.70^{a}	1.63^{ab}	0.99^{ab}	0.16^{abcd}	0.0423^{a}	2.60^{ab}
2	8.87^{fg}	11.93^{a}	1.62^{ab}	1.04^{a}	0.18^{abc}	0.0568^{a}	2.50^{ab}
3	9.33^{defg}	9.53^{a}	1.80^{a}	0.97^{ab}	0.18^{abc}	0.0417^{a}	2.60^{ab}
4	9.37^{defg}	11.00^{a}	1.63^{ab}	0.95^{ab}	0.16^{abc}	0.0402^{a}	3.70^{a}
5	10.23^{bcdef}	9.63^{a}	1.86^{a}	1.02^{ab}	0.20^{a}	0.0530^{a}	2.50^{ab}
6	9.50^{cdefg}	9.60^{a}	1.45^{b}	0.96^{ab}	0.14^{d}	0.0546^{a}	2.30^{b}
7	9.73^{cdefg}	9.59^{a}	1.69^{ab}	1.04^{a}	0.15^{cd}	0.0416^{a}	2.60^{ab}
8	10.10^{bcdef}	11.87^{a}	1.78^{a}	0.99^{ab}	0.20^{a}	0.0587^{a}	3.20^{ab}
9	8.27^{g}	12.20^{a}	1.78^{a}	1.01^{ab}	0.19^{abc}	0.0594^{a}	2.90^{ab}
10	11.17^{abc}	11.90^{a}	1.72^{ab}	0.92^{ab}	0.19^{ac}	0.0524^{a}	2.50^{ab}
11	10.53^{abcdef}	10.43^{a}	1.61^{ab}	0.88^{ab}	0.15^{bcd}	0.0436^{a}	2.10^{b}
12	11.73^{ab}	10.93^{a}	1.69^{ab}	0.82^{b}	0.19^{abc}	0.0470^{a}	2.40^{ab}
13	11.13^{abc}	11.77^{a}	1.71^{ab}	0.92^{ab}	0.19^{a}	0.0522^{a}	2.40^{ab}
14	11.97^{a}	11.63^{a}	1.78^{a}	0.95^{ab}	0.19^{ab}	0.0567^{a}	2.30^{b}
15	10.60^{abcde}	11.40^{a}	1.85^{a}	0.99^{ab}	0.20^{a}	0.0570^{a}	2.80^{ab}
16	11.20^{abc}	10.83^{a}	1.86^{a}	0.96^{ab}	0.20^{a}	0.0504^{a}	3.00^{ab}
17	10.83^{abcde}	10.77^{a}	1.88^{a}	0.91^{ab}	0.18^{abc}	0.0551^{a}	2.60^{ab}
Average	**10.33**	**10.94**	**1.73**	**0.97**	**0.18**	**0.05**	**2.65**
C.V. (%)	**12.81**	**19.98**	**14.38**	**16.58**	**16.35**	**44.73**	**40.5**

* Means followed by the same letter in the column do not show statistical difference, by test Shapiro-Wilk at 5% probability.

From data of Tables 2 and 3 it is seen that the bioherbicide as activity towards target plant and the degree of injury was influenced by the independent variables studied here. However, the influence of each variable on the response is not clear in the results discussed above. For this reason, data of Table 2 were used to compute the magnitude and significance of these effects, which are presented in Table 4. The CSL did not present significant influence on herbicidal activity. On the other hand, the effects for moisture and soybean bran were positive and statistically significant ($p<0.1$), indicating that the increase of concentration of both variables would lead to an increase in the herbicide action on weeds. However, the effect of soybean bran was more accentuated than moisture. Quadratic terms for soybean bran and moisture content were statistically significant and both were negative, indicating the presence of a maximum point for these variables in the evaluated range. Interaction terms were not significant in the evaluated range.

The results presented in Table 4 were used to build a quadratic model expressing the herbicide action characterized by phytotoxicity in function of independent variables. Eq. 1 presents the significant terms of the model ($p<0.1$).

$$FB_s = 39.86 + 4.46 \cdot M - 5.57 \cdot M^2 + 5.91 \cdot S - 5.04 \cdot S^2 - 3.80 \cdot C^2 \quad (1)$$

where FB_s is the phytotocixity of bioherbicide, M, S and C are the coded moisture, soybean bran and corn steep liquor contents, respectively. The model was validated by analysis of variance (ANOVA). The calculated F-test for Eq. 1 was about 13 times greater than the tabulated ones for significance at $p=0.1$, and the determination coefficient (R^2) was 0.7121. The high value for the determination coefficient indicate good fitting of experimental data, allowing the use of such model to predict the bioherbicide activity towards the plant target.

Table 4. Analysis of the effects of independent variables of the CCRD on the bioherbicide production.

	Effect	Std. Err.	t(10)	p-value
Mean	39.87	4.07	9.80	<0.0001
Moisture (L)	8.92	3.82	2.33	0.0418
Moisture (Q)	-11.15	4.21	-2.65	0.0245
CSL (L)	-0.06	3.82	-0.01	0.9881
CSL (Q)	-7.60	4.21	-1.80	0.1012
Soybean (L)	11.82	3.82	3.09	0.0114
Soybean (Q)	-10.08	4.21	-2.39	0.0377
Moisture x CSL	-1.50	5.14	-0.29	0.7787
Moisture x Soybean	4.50	5.14	0.88	0.4099
CSL x Soybean	6.50	5.14	1.27	0.2461

The validated model was used to optimize the phytotoxicity and the results obtained are presented in Figs. 1, 2 and 3. Fig. 1 presents the influence of moisture and CSL contents on the phytotoxicity of the bioherbicide. The highest activity was obtained for moisture content ranging from 65 to 75 wt% and CSL from 14 to 26 wt%. Fig. 2 presents the influence of moisture and soybean bran contents on the phytotoxicity of the bioherbicide, and the highest bioherbicide activity was when soybean bran concentration ranged from 20 to 34 wt% at moisture content ranging from 65 to 78 wt%. Fig. 3 presents the influence of CSL and soybean bran contents on the phytotoxicity of the bioherbicide and the results presented corroborates with findings of Figs. 1 and 2.

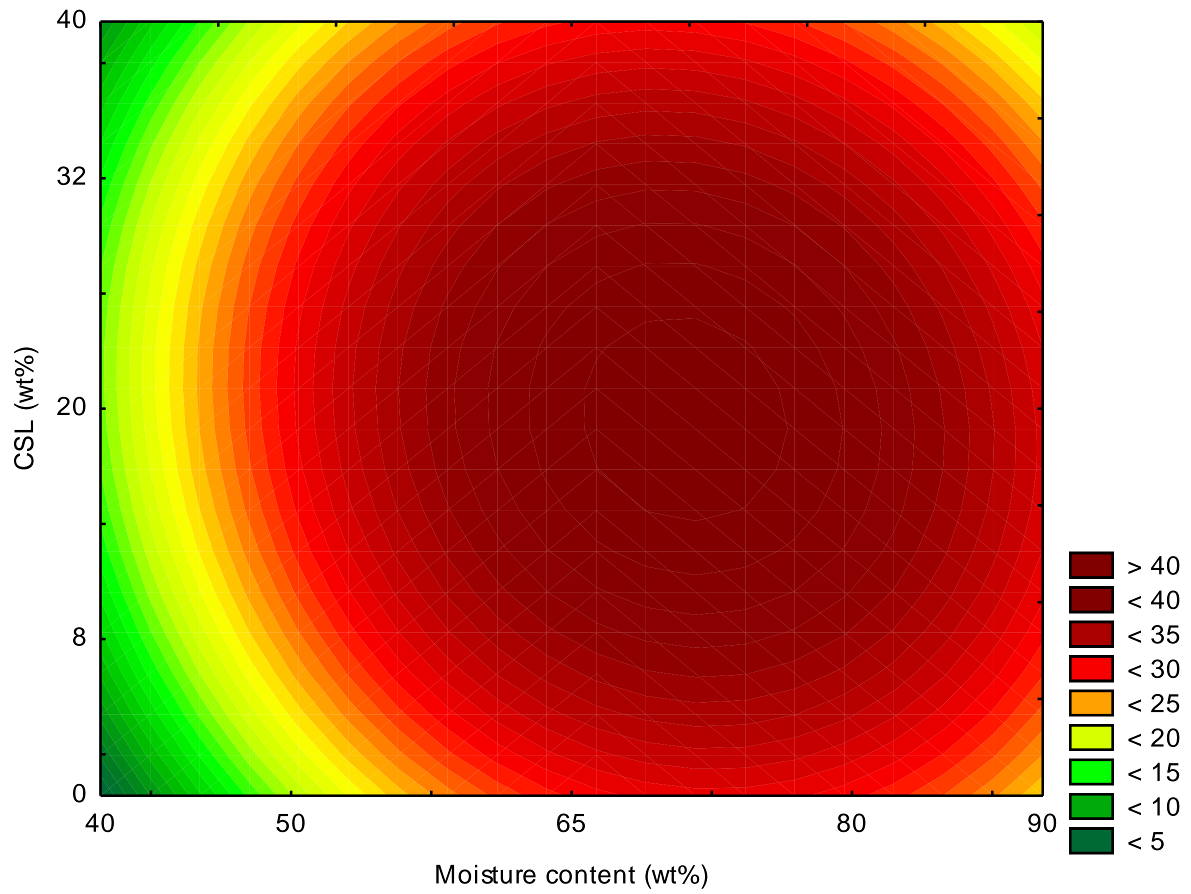

Fig. 1. Contour plot showing the influence of moisture and CSL contents on the phytotoxicity of the bioherbicide produced. Soybean bran content was maintained at the central point of the CCRD.

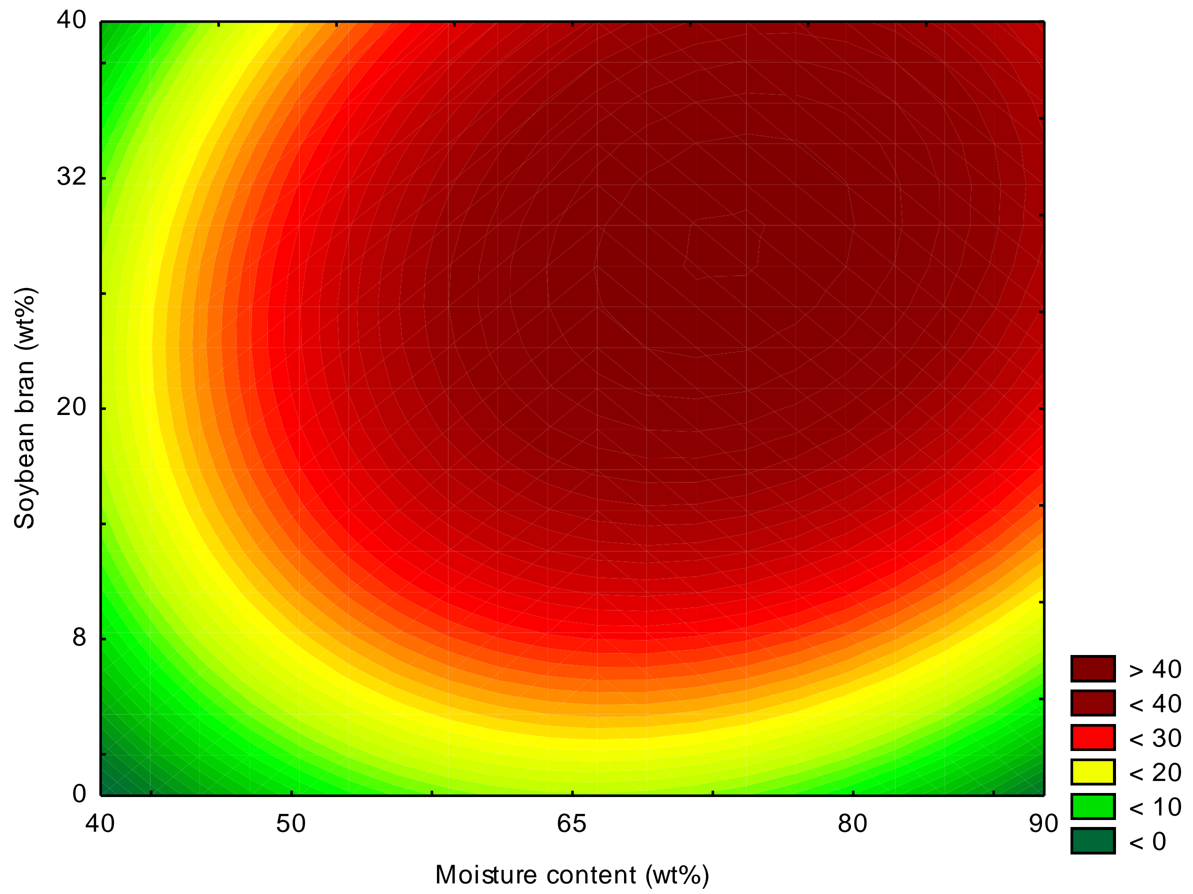

Fig. 2. Contour plot showing the influence of moisture and soybean bran contents on the phytotoxicity of the bioherbicide produced. CSL content was maintained at the central point of the CCRD.

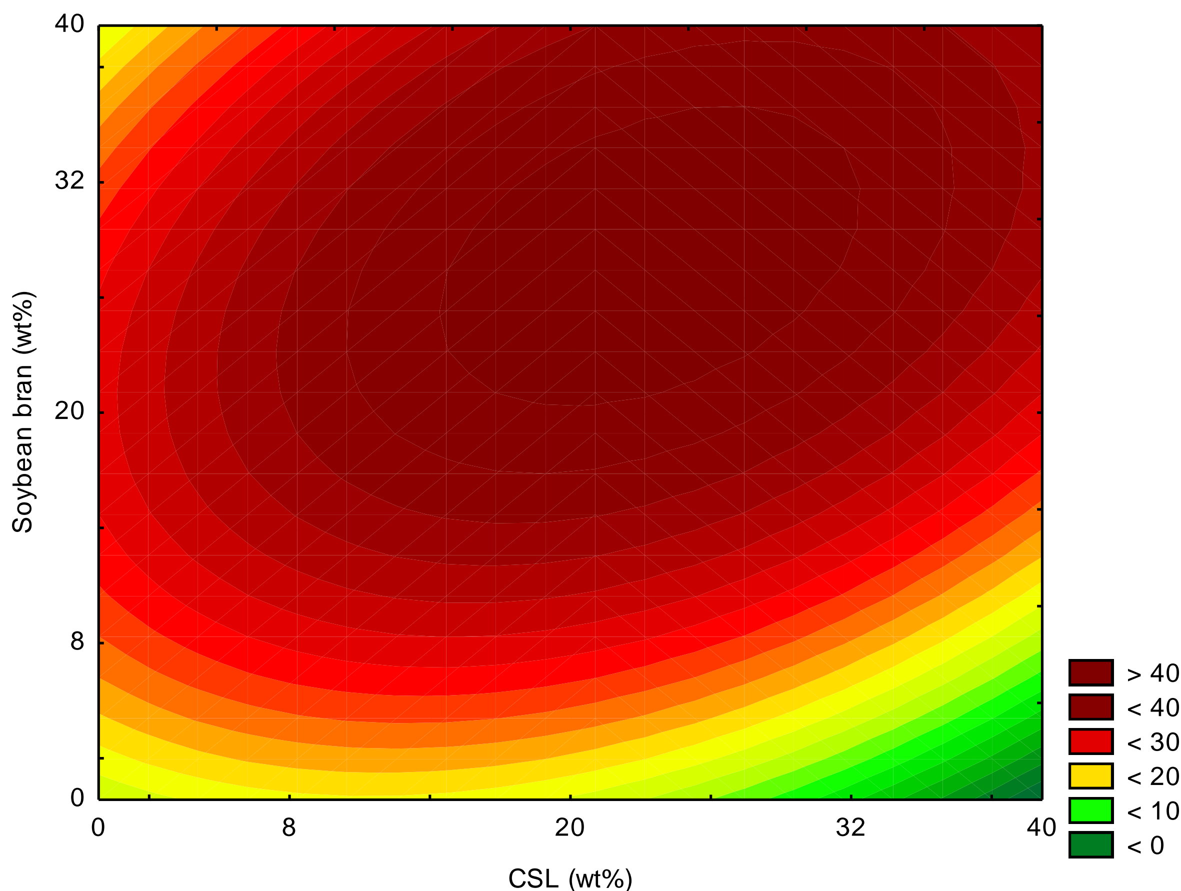

Fig. 3. Contour plot showing the influence of CSL and soybean bran contents on the phytotoxicity of the bioherbicide produced. Moisture content was maintained at the central point of the CCRD.

The model allows to delimit the optimum region in the contour plot. Furthermore, it is possible to affirm that the optimized condition was found in this work that is moisture content of 70%, soybean bran content of 30% and CSL content of 20 wt%. At this condition, the maximum bioherbicide action on the target plant was around 40 in the scale used.

The action of a bioherbicide on the plant depends of absorption, translocation, metabolism and the plant sensitivity to the active compound. It is necessary that the compound penetrates in the plant, translocate and reach the organelle where it will act. The same herbicide may influence various metabolic processes in the plant. However, the first biophysical or biochemical injury that it causes in the plant is characterized by its mechanism of action. The sequence of all reactions to the final action of the product on the plant features its mode of action. The knowledge of the mechanism of action and mode of action is an

important step in the development of a bioherbicide. From analysis of bioassays some common symptoms among the treatments were observed, which allowed to group the possible mechanism of action of bioherbicide produced from *Phoma* sp.

The main symptom observed in the experiments was the bleaching of the leaves of target plants. It is important to point out that only the leaves that came in contact with the bioherbicide presented bleaching. The leaves that were born after the application did not show the symptom. The injury caused on target plants can be seen in Fig. 4, where only the treatments that demonstrate the effect of high injury can be seen. These symptoms are similar to herbicides that have a mechanism of action based on the inhibition of the carotenoids biosynthesis. This mechanism of action is mainly characterized by common symptom of injury, characterized by depigmentation of the leaves caused by photobleaching of chlorophyll that occurs after blockade of the synthesis of carotenoid pigments, resulting in an "albino" appearance [18-19] that is similar to that observed in the bioassays and possibly is the mechanism of action of this bioherbicide.

Fig. 4. Effect of bioherbicide on target plant for some specific runs of the CCRD: a) run 6; b) run 8; c) run 12; d) run 15; e) control test.

3.2.4. Conclusions

In this work the solid-state fermentation for bioherbicide production from *Phoma* sp. was optimized. The culture medium was composed mainly of sugar cane bagasse and complemented with soybean bran and corn steep liquor. The results obtained in the bioassays demonstrate that the bioherbicide presented activity towards the target plant and the intensity of the effect was influenced by the formulation of the fermentation medium. The most severe

injury was seen in the run 6, which showed significant damage in the height, fresh weight of the aerial, dry weight of the aerial part plant and phytotoxicity. The optimized condition for bioherbicide production was at moisture among of 70 %, soybean bran content of 30 wt% and CSL 20 wt%, being possible to obtain an injury level 40. The bioherbicide produced showed a mode of action based on the inhibition of carotenoids biosynthesis.

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References

[1] Boyetchoko SM, Rosskopf EN, Caesar AJ, Charudattan R (2002) Biological Weed Control with Pathogens: Search for Candidates to Applications. Agriculture and Food Production 2:239 – 266.

[2] Charudattan R, Dinoor A (2000) Biological control of weeds using plant pathogens: accomplishments and limitations. Crop Protection 19:691 – 695.

[3] Barton J (2004) How good are we at predicting the Field host-range of fungal pathogens used for classical biological control of weeds? *Biological Control* 31:99–112.

[4] Penariol MC, Monteiro AC, Pitelli RA (2008) Growth and sporulation of *Bipolaris euphorbiae* cultivated under different nutritional conditions. Ciência Rural Santa Maria 38:1907-1913.

[5] Rosskopf EN, Charudattan R, Kadir JB (1999) Use of Plant Pathogens in Weed Control. Handbook of Biological Control.

[6] Zhou L, Bailey KL, Derby J (2004) Plant colonization and environmental fate of the biocontrol fungus *Phoma macrostoma*. Biological Control 30:634–644.

[7] Fravel DR, Deahl KL, Stommel JR (2005) Compatibility of the biocontrol fungus *Fusarium oxysporum* strain CS-20 with selected fungicides. *Biological Control*. 34:165-169.

[8] Ash GJ (2010) The science art business of successful bioherbicides. Biological Control 52:230–240.

[9] Mitchell KJ (2003) Development of a submerged-liquid sporulation medium for the potential smartweed bioherbicide *Septoria polygonorum*, Biological Control 27:293–299.

[10] Pandey A (2003) Solid-state fermentation. Biochemical Engineering Journal 13: 81–84.

[11] Singhania RR, Sukumaran RK, Patel AK, Larroche C, Pandey A. (2010) Advancement and comparative profiles in the production technologies using solid-state and submerged fermentation for microbial cellulases. Enzyme and Microbial Technology 46:541-549.

[12] Hoagland RE, Boyette CD, Abbas, HK (2007) *Myrothecium verrucaria* isolates and formulations as bioherbicide agents for kudzu. Biocontrol Science and Technology 17:721–731.

[13] Bailey KL, Pitt WM, Falk S, Derby J (2011) The effects of *Phoma macrostoma* on nontarget plant and target weed species. Biological Control 58:379–386.

[14] Zhang L, Wang SQ, Li DJ, Zhang AL, Zhang Q, Gao JM (2012) New insight into the stereochemistry of botryosphaeridione from a *Phoma endophyte*. Journal of Molecular Structure 1016:72–75.

[15] Parra R, Aldred D, Magan N (2005) Medium optimization for the production of the secondary metabolite squalestatin S1 by a *Phoma* sp. combining orthogonal design and response surface methodology. Enzyme and Microbial Technology 37:704–711.

[16] Selbmann L, Onofri S, Fenice M, Federici F, Petruccioli M (2002) Production and structural characterization of the exopolysaccharide of the Antarctic fungus *Phoma herbarum* CCFEE 5080. Research in Microbiology 153:585–592.

[17] Frans R, Crowley H (1986) Experimental design and techniques for measuring and analyzing plant responses to weed control practices. In: Southern Weed Science Society. Research methods in weed science, Clemson, 3:29-45.

[18] Oliveira RS Mecanismo de ação de herbicidas. Biologia e manejo de plantas daninhas. 2011.

[19] Roman ES, (2007) Como funcionam os herbicidas. p. 83.

4. DISCUSSÃO

Como visto a principal proposta desta dissertação foi desenvolver um estudo para a produção de um bioherbicida a partir de *Phoma* sp. Entretanto, percebe-se que existem algumas barreiras significativas na produção de bioherbicidas como o desenvolvimento de um processo economicamente viável e a obtenção do ingrediente ativo que está relacionada com a formulação do meio e do tipo de fermentação (MITCHELL, 2003). Estes problemas são compatíveis à proposta de estudo e também foram as principais barreiras enfrentadas neste trabalho na etapa de fermentação submersa e fermentação em estado sólido, logo serão discutidos nesta secção.

Na fermentação submersa foi otimizada a composição do meio de fermentação para crescimento de *Phoma* sp. para maximizar a concentração de biomassa e obter um processo economicamente viável (baixo custo de produção de biomassa). Primeiramente foi defenido um meio sintético de fermentação contendo glicose, peptona e extrato de levedura. Em um segundo momento, a glicose foi substituída por sacarose e, peptona e extrato de levedura por água de maceração de milho caracterizando assim o meio industrial e o crescimento foi otimizado novamente. Após otimizadas as condições em ambos os meios de fermentação a biomassa de fungos foi de 22 gL^{-1} para o meio sintético e 33 gL^{-1} para o meio industrial.

Quando otimizado o meio sintético observou-se o que ponto ótimo para máxima produção de biomassa foi diferente quando analisado o custo de produção, isto mostra que é importante realizar uma análise economica do processo. Para o meio industrial o ponto ótimo foi o mesmo para máxima produção de biomassa e para o custo de produção. Comparando ambos os meios de fermentação é notável que o meio industrial teve maior produção de biomassa e menor custo, isto mostra o beneficio em se utilizar resíduos ou subprodutos agroindustriais em processos fermentativos. Entretanto, é necessário realizar estudos para verificar o substrato ideal a ser utilizado e a referida concentração, pois as condições otimas para um micro-organismo não necessariamente será igual para outro.

Para *Phoma* sp. o meio industrial foi composto basicamente de água de maceração de milho e suplementado com sacarose, este meio de fermentação forneceu melhores condições de cresecimento do que o meio sintético. A melhor adaptação deste micro-organismo ao meio industrial é justificado pelo fato da água de maceração de milho ser um complexo rico em diversos nutrientes e micronutrientes (Xi et al., 2003), logo forneceu melhores condições de crescimento que um meio formulado em laboratório. A condição otimizada para o meio de

cultivo industrial é de 20 gL^{-1} de sacarose, 8% de água de maceração de milho e pH inicial de 6,0, porém para concentrações acima de 15% de água de maceração de milho o crescimento passou a ser inibido.

O meio de fermentação sintético geralmente não consegue fornecer todos os nutrientes que um micro-organismo necessita para maximizar o crescimento. Entretanto, para obter melhores rendimentos utilizando o meio sintético é necessário aumentar a complexidade do meio adicionando uma grande gama de nutrientes e micronutrientes, consequentemene também aumentamos o custo do meio de fermentação invalidando economicamente o processo. Dessa forma, a principal contribuição deste estudo foi demonstrar que o meio de fermentação sintético pode ser substituído por resíduos ou subprodutos agroindustriais, caracterizando um meio industrial com menor custo e os rendimentos de produção de biomassa podem ser iguais ou superiores a o meio sintético.

No estudo da produção de *Phoma* sp. por fermentação sólida também utilizou-se resíduos agroindustriais como substrato para baixar custos de produção e a variável resposta monitorada foi a fitotoxidade, a qual foi avaliada em bioensaios. O meio foi composto basicamente por bagaço de cana e complementado com farelo de soja e água de maceração de milho, além de, ajustar a umidade relativa. O bagaço de cana é a principal fonte de carbono e serve como suporte para crescimento do micro-organismo, o farelo de soja e água de maceração de milho são fontes de nitrogênio, entretanto na água de maceração de milho também podem ser encontrado muitos micronutientes. Dessa forma, observou-se os melhores resultados com 30 % farelo de soja, 20 % água de maceração de milho e 75 % de umidade relativa.

A umidade foi a variável mais significativa e, é citado por muitos autores como a pricipal característica do processo de fermentação em estado sólido (RODRIGUEZ et al. 2013), pois a grande maioria das células vivas apresentam cerca de 70-80% de umidade, assim é necessário uma quantidade específica de água diponivel no meio de fermentação, a qual é característica de cada micro-organismo para necessidades de adaptação e nova sintese celular (RODRIGUEZ et al. 2003), para *Phoma* sp. a umidade foi de 75%. Dessa forma, o meio de fermentação em estado sólido foi otimizado e também forneceu condições favoraveis para o crescimento de *Phoma* sp. e produção do bioherbicida.

Avaliando ambas as fermentações quanto a resposta obtida nos bioensaios a fitotoxidade do bioherbicida, obteve-se resultados ligeiramente diferentes que merecem serem

discutidos. A fermentação em estado sólido tem características bastante diferentes da fermentação submersa e, isto pode fazer com que o micro-organismo passe a comportar-se diferente em cada processo devido as condições extremeas que é submetido principalmente na fermentação em estado sólido. Esse aspecto pode ser bem visualizado quando analisado o modo de ação do bioherbicida. Observou-se que o ingrediente ativo obtido em cada fermentação foi diferente, pois o bioherbicida teve uma ação diferente sobre a planta quando aplicado o extrato da fermentação submersa e quando aplicado o extrato da fermentação sólida. Quando aplicado o extrato da fermentação submersa, observou-se nos bioensaios que na planta de pepino ocorreu uma enrrugamento da folha e redução de estame, como pode-se ver na figura 4 do segundo artigo. Quando aplicado o extrato da fermentação sólida observou-se pricipalmente a clareamento da folha, como pode-se ver na figura 4 do terceiro artigo. Analisado o tipo de fitotoxidade no bioensaios pode-se dizer que o tipo de fermentação e a formulação do meio interferiu no tipo de toxina produzida por *Phoma* sp., entretanto é necessário estudos posteriores para caracterizar cada fitotoxina produzida em cada fermentação.

Dessa forma, avaliando modo geral os resultados obtido em ambos os cultivos por fermentação em estado sólido e fermentação submersa. Observa-se, que a fermentação submersa apresentou um ótimo crescimento celular de *Phoma* sp. quando alterado o meio de fermentação de sintético para industrial, mostrando que o meio industrial proposto pode ser utilizado em um possível *scale-up*. A fermentação em estado sólido se mostrou promissora, pois o fungo *Phoma* sp. se adaptou bem a este tipo de fermentação e os extratos obtidos apresentaram efeitos de injuria moderado sob a planta de pepino. O efeito herbicida de *Phoma* sp. pode ser compravado por bioensaios realizados em casa de vegetação e, a análise de fitotoxidade apresentou um efeito moderado apresentando injúria moderada, mas com recuperação.

5. CONCLUSÃO

Na fermentação submersa foram otimizados os meios de cultivo sintético e industrial, sendo as composições fixadas em 20 gL^{-1} de glicose e peptona, 7,5 gL^{-1} de extrato de levedura e pH inicial de 6,0 para o meio sintético e para o meio industrial, 20 gL^{-1} de sacarose, 8% de AMM e pH inicial de 6,0. Nessas condições a máxima biomassa obtida foi de 22 gL^{-1} para o meio sintético e 33 gL^{-1} para o meio industrial, assim foi possível obter uma produção de biomassa 50% maior e a redução do custo do meio de fermentação de US $41.664 para US $38 para 1000 kg de biomassa produzida. Deste modo, é possível concluir que a utilização de meios industriais é uma alternativa técnica e econômica, uma vez que foi possível reduzir em muitas vezes o custo da fermentação e aumentar a produção de biomassa, o que é importante para possíveis etapas de *scale up* e processo de purificação.

Na fermentação em estado sólido foi avaliada a composição do meio de cultivo para obter a máxima ação do bioherbicida em plantas de pepino. Observou-se que entre as respostas avaliadas que a fitotoxidade foi o efeito mais significativo, outros efeitos vizualizados foram a inibição de crescimento e redução de massa seca e umida da parte aérea da planta. A condição otimizada para produção do bioherbicida foi 70 % de umidade, 30 % farelo de soja e 20 % de AMM.

A avaliação de atividade herbicida do extrato fermentado no controle de plantas de pepino mostrou resultados satisfatórios. O extrato da fermentação submersa apresentou efeito sobre a planta de pepino causando injúria moderada com recuperação. O extrato da fermentação em estado sólido também apresentou atividade em plantas de pepino e a intensidade do efeito foi influenciada pela formulação do meio de fermentação, causando injúria moderada com recuperação de acordo com a escala de Corley (1986). Para o extrado da fermentação sólida foi possível caracterizar o modo de ação em inibição de biossíntese de carotenóides, entretanto não foi possível caracterizar o modo de ação para extratos da fermentação submersa.

Dessa forma foi possível desenvolver e otimizar a composição do meio de fermentação em ambos os processos para produção do bioherbicida, o meio de cultivo se mostrou produtivo e viável economicamente em ambos os processos atingindo os objetivos propostos e o bioherbicida apresentou ação moderada e não duradoura sobre as plantas-teste comprovando o potencial bioherbicida de fungos *Phoma* sp.

5.1. Sugestões Futuras

Como sugestão para trabalhos futuros cita-se o *scale-up* da produção deste bioherbicida em biorreator de fermentação submersa e sólida, visto que, com o crescimento em biorreatores pode-se obter maiores rendimentos em biomassa e produção de metabólitos tóxicos devido ao maior controle das variáveis de processo.

Desenvolver uma metodologia para isolamento e caracterização da estrutura química desta toxina. Por fim, realizar estudos com a utilização de adjuvantes para otimizar o efeito, identificar quais os tipos de ervas daninhas que tem ação e caracterizar o mecanismo e modo de ação deste bioherbicida.

REFERÊNCIAS BIBLIOGRÁFICAS

ASH, G.J. **The science art business of succeful bioherbicides**, Biological Control 52, 230 – 240, 2010.

BAILEY, K.L.; PITT, W.M.; FALK, S.; DERBY, J. T**he effects of *Phoma macrostoma* on nontarget plant and target weed species**. Biological Control 58, 379 – 386, 2011.

BOYETCHOKO, S.M.; ROSSKOPF, E.N.; CAESAR, A.J.; CHARUDATTAN, R. **Biological Weed Control with Pathogens: Search for Candidates to Applications**. Agriculture and Food Production 2, p. 239 – 266, 2002.

CHARUDATTAN, R.; DINOOR, A. **Biological control of weeds using plant pathogens: accomplishments and limitations**. Crop Protection 19, p. 691 – 695, 2000.

GIBBS, P.A.; SEVIOUR, R.J.; SCHMID, F. **Growth of filamentous fungi in submerged culture: problems and possible solutions.** Critical Reviews in Biotechnology 20, p. 17–48, 2000.

HOAGLAND, R.E.; BOYETTE, C.D.; ABBAS, H.K; ***Myrothecium verrucaria* isolates and formulations as bioherbicide agents for kudzu**. Biocontrol Science and Technology 17, p. 721–731, 2007.

JACKSON, M.A.; FRYMIER, J.S.; WILKINSON, B.J.; ZORNER, P.; EVANS, S. **Growth requirements for production of stable cells of the bioherbicidal bacterium *Xanthomonas campestris***. Journal of Industrial Microbiology & Biotechnology 21, 237–241, 1998.

K.J. MITCHELL, Development of a submerged-liquid sporulation medium for the potential smartweed bioherbicide *Septoria polygonorum*, Biological Control, 27 (2003) 293–299.

MITCHELL, J.K. **Development of a submerged-liquid sporulation medium for the potential smartweed bioherbicide *Septoria polygonorum***. Biological Control 27, p. 293–299, 2003.

PENARIOL, M.C.; MONTEIRO, A.C.; PITELLI, R.A. **Growth and sporulation of *Bipolaris euphorbiae* cultivated under different nutritional conditions**. Ciência Rural Santa Maria 38, p. 1907-1913, 2008.

RODRIGUEZ-LEON, J.A.; SOCCOL, C.R.; PANDEY, A.; RODRIGUEZ, D.E. Factors affecting solid-state fermentation. In: Current developments in solid-state fermentation. 2003. Cap. 3, p. 26-46.

ROSSKOPF, E.N.; CHARUDATTAN, R.; KADIR, J.B. **Use of Plant Pathogens in Weed Control**. Handbook of Biological Control, 1999.

SHABANA, Y.; SINGH, D.; ORTIZ-RIBBING, L.M.; HALLETT G.S. **Production and formulation of high quality conidia of *Microsphaeropsis amaranthi* for the biological control of weedy *Amaranthus* species**. Biological Control 55, p. 49-57, 2010.

TEBEEST, D.O.; YANG, X.B.; CISAR, C.R. **The status of biological-control of weeds with fungal pathogens**. Annual Review of Phytopathology 30, p. 637–657, 1992.

WEAVER, M.A.; BOYETTE, D.; HOAGLAND, R.E. **Bioherbicidal activity from spores of Myrothecium verrucaria**. World J. Microbiol Biotechnol 28, p. 1941-1946, 2012.

XI, Y.; CHEN, K.; DAI, W.; MA, J.; ZHANG, M.; JIANG, M.; WEI, P.; OUYANG, K. **Succinic acid production by Actinobacillus succinogenes MJ113 using corn steep liquor powder as nitrogen source**. Bioresource Technology 136, p. 775-779, 2013.

ZHOU, L.; BAILEY, K.L.; DERBY, J. **Plant colonization and environmental fate of the biocontrol fungus *Phoma macrostoma***. Biological Control 30, p. 634–644, 2004.