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ORIGINAL PAPER

Liquid fermentation of *Colletotrichum truncatum* UFU 280, a potential mycoherbicide for beggartick

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Abstract

One isolate of *Colletotrichum truncatum* was found recently causing severe anthracnose symptoms and leading to the death of beggartick (*Bidens pilosa* L. and *Bidens subalternans* DC.), one of the major weeds of Brazilian agriculture. This isolate, namely UFU 280, was selected for development of a mycoherbicide against this weed. Associated of beggartick, one of the, Here, results of a preliminary attempt to develop a protocol for mass production of inoculum (conidia) and fungal biomass of *C. truncatum* was performed aimed at paving the way for greenhouse and field evaluations of this biocontrol candidate. Isolates of *Colletotrichum* spp. have been successfully produced in the past to serve as the active ingredients of mycoherbicides. The method of choice has been the production of propagules through liquid fermentation. We assessed the effect of several options of liquid media recipes, type of seeding of medium, pH levels, incubation lengths, incubation temperatures and agitation speeds on the shaking speed on the concentration of conidia obtained per volume of medium. Additionally, a possible effect of the kind of medium utilized over the virulence of the inoculum was also evaluated through an inoculation study. We found that an adequate amount of conidia of *C. truncatum* (isolate - UFU 280) can be obtained in ME liquid culture medium, adjusted to a pH of 9.0, seeded with a conidial suspension and incubated for 6 days, under a regime of orbital shaking of 150 rpm, at temperatures ranging from 20 to 25 °C. Mortality of beggartick plants using conidia produced in different liquid culture media was of 100%.

Keywords Anthracnose · Bioherbicide · Fermentation · Inoculum · Mass production · Weed control

Introduction

Weeds are major limiting factors for agricultural productivity worldwide. They compete with crops for water, light, and mineral nutrients from the soil, interfere with harvesting, contaminate and increase the moisture content of the harvested product, which reduces the quality of the final product. In addition, weeds can serve as alternative hosts for pests and diseases of cultivated crops (Auld 1998).

Beggartick (*Bidens pilosa* L. and *Bidens subalternans* DC.) are among the worst weeds of tropical agriculture (Holm 1991), including in Brazil (Kissmann and Groth 1999). It is a very prolific species, reproducing three times a year and dense infestations of the plant are often observed in the field. Beggartick control in Brazil is hindered by the increasingly widespread occurrence of herbicide-resistant biotypes (Gelmini 2001; Hrac-BR 2017), especially to acetolactate synthase (ALS) inhibitor herbicides. The extensive and repetitive use of ALS herbicides in Brazil is behind the selection pressure on beggartick populations, which resulted in the predominance of herbicide-resistant biotypes in many important soybean-producing areas of the country (Rizzardi et al. 2002; Baio et al. 2013). In a scenario where herbicide resistant-weeds become widespread, combined with the lack of new herbicide molecules, new approaches to control beggartick are necessary and offer novel opportunities for a much neglected strategy: mycoherbicide development (Charudattan 2001). Mycoherbicides first appeared in the late 1970s and their history has been reviewed by many authors, including, more recently by Hallet 2005; Ghosheh 2005; Yandoc-Ables et al. 2006a, b; Barreto 2009; Barreto et al. 2012).

Results of a recent survey for fungal pathogens occurring on beggartick in Brazil were published recently

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(Guatimosim et al. 2015). This was aimed at subsidizing the classical approach of biocontrol through searches for fungi which might occur in the native range of invasive *Bidens* spp. Mexico and Central America (Guatimosim et al. 2015). The authors did not indicate any of the fungal species found and described in their publication as of having potential for use for mycoherbicide development. Based on this perspective and the current challenge caused by beggartick resistant biotypes in Brazil, we aimed to find potential fungi for biological control of this plant.

To identify fungi that could be developed as mycoherbicides for beggartick, we carried out a short-scale survey for pathogenic fungi associated with *B. pilosa* and *B. subalternans* during 2015 in the Alto Paranaíba region (state of Minas Gerais, Brazil). Of special interest was a group of *B. pilosa* individuals, bearing severe anthracnose symptoms which led to the death of numerous plants (Fig. 1). In the laboratory, we prepared slides under microscope and isolated the fungus in pure culture. The fungus was morphologically and molecularly identified as *Colletotrichum truncatum*. The pathogenicity of the fungus was confirmed after inoculations with conidial suspensions on healthy plants of beggartick (*B. pilosa* and *B. subalternans*).

Isolates belonging to *Colletotrichum* spp. have been exploited to develop mycoherbicides since the early days of mycoherbicide history, including the pioneering products (Collego®, later re-registered as Lockdown®) and Biomal® (Yandoc-Ables et al. 2006a, b). Field observations of extensive damage to beggartick caused by an anthracnose fungus encouraged further investigating *C. truncatum* isolate UFU 280 as a potential novel mycoherbicide active ingredient. This potential was further confirmed in preliminary inoculation assays and by host-specific pathogen of beggartick, being pathogenic only the *B. pilosa* and *B. subalternans* (unpublished data).

To further advance towards a mycoherbicide, it is critical to develop a preliminary method for producing larger amounts of viable and virulent inoculum for greenhouse and field tests. This is a critical issue to be considered for a commercial mycoherbicide to become viable (Bowers 1982; Silman et al. 1991; Shearer and Jackson 2006; Boyette et al. 2014). In general, submerged culture fermentation is considered the most cost-effective production method (Stowell 1991; Silman et al. 1991; Shearer and Jackson 2006; Boyette et al. 2014). During preliminary tests it was observed that virulent isolate of *C. truncatum* UFU 280 obtained from beggartick was capable of sporulating in liquid culture media. Here we report the results of investigation on some factors which are known to be of relevance for the mass production of inocula of biocontrol fungi to establish a preliminary protocol for in vitro production of infective propagules of *C. truncatum* UFU 280 in liquid culture media.

Fig. 1 Beggartick stems (*Bidens pilosa*) naturally infected by *Colletotrichum truncatum* UFU 280

Methods

Culture and maintenance

The *Colletotrichum truncatum* UFU 280 isolate used in this study was obtained from *B. pilosa* individuals, collected near the municipality of Iraí de Minas (Minas Gerais – Brazil) with severe anthracnose symptoms. The isolate was encoded (*C. truncatum* UFU 280) and incorporated into the mycological collection of the Laboratory of Microbiology and Phytopathology (LAMIF) of the Federal University of Uberlândia – Campus Monte Carmelo. The isolate was stored on silica gel according to Dhingra and Sinclair (1995) and when necessary was cultivated on PDA medium.

Biomass and conidial production in selected media

Seven recipes of liquid culture media for cultivation of fungi were arbitrarily chosen and assessed in terms of the resulting amount of biomass and conidia of *C. truncatum* produced. These were: a) JP (Jenkins-Prior) – see recipe in Fargues et al. (2001); b) VB (Vegetable–Broth) – see recipe in Pereira et al. (2003) of VBA, but without agar; c) PD (Potato Dextrose) - Dhingra and Sinclair (1995); d) CD (Czapek Dox) - Dhingra and Sinclair (1995); e) CB (Carrot broth: carrot extract - 20 g/400 mL, 1 L of distilled water); f) ME (Malt Extract) – Bailey and Jeger (1992); g) SYEA (Sucrose yeast extract and asparagine) – see recipe in Zauza et al. (2007).

Three mycelial plugs (ø = 5 mm diam.) taken from the margin of 7-day-old *C. truncatum* cultures growing on PDA were aseptically transferred to each of five separate 250-mL flasks, each containing 100 mL of each of the above liquid culture media. The flasks were kept on an orbital shaker (Nova Instruments® ¯ NT 714) at 180 rpm under room temperature

(25 ± 2 °C) for 15 days. Fungal biomass was ground in a homogeniser (Marconi® – MA 099, Marconi Equipamentos para Laboratório Ltda, Piracicaba, SP, Brazil) for one minute and then were filtered with gauze to remove fungal biomass. 20 μL aliquots were withdrawn from each flask for evaluation of conidia concentration. The conidial concentration in each flask was assessed using a hemocytometer. Dry weight of fungal biomass was evaluated by vacuum filtering the contents of each flask in a Buchner funnel using Whatman No.1 filter, followed by drying the biomass at 70 °C for 24 h in an air circulation oven (SOLAB®). Then the resulting fungal biomass was weighed on a precision balance (accuracy = 0.001 g, Prix AS 310 R2).

Pathogenicity of *C. truncatum* conidia produced in selected liquid culture media

Beggartick plants (*B. pilosa*) at the 2-leaf-pair stage were inoculated with conidial suspension at 1×10^5 conidia/mL. Conidia produced in VB, PD, CB, ME and SYEA were used for inoculations. The JP and CD culture media were excluded from this experiment because there was no sporulation in these media. The conidial suspensions were prepared as described in the previous experiment. As control, plants were sprayed with distilled water +0.05% Tween 20. The conidial suspensions were sprayed onto the foliage of beggartick plants using a hand atomizer until runoff. The inoculated plants were maintained in moist chamber for 48 h in greenhouse with temperatures in the range 25 °C to 30 °C. The moist chamber consisted of wrapping the inoculated plants with a plastic previously moistened internally. The percent control (mortality) was determined 7 days after treatment. The experimental design was completely randomized with five replicates, each replicate consisting of a pot (500 mL) with one plant.

Optimal duration of incubation for biomass and conidial production

This experiment was performed in two rounds aimed at determining the best length of incubation for optimal conidial harvest and the best between two forms of seeding the medium. In the first round 5250 mL flasks containing 100 mL of ME medium each, were seeded with culture disks (three disks per flask) obtained from the margin of 7-day-old actively growing cultures on PDA. The flasks were placed in rotary shakers adjusted to 180 rpm and kept at room temperature (25 ± 2 °C) for 18 days. The second round procedure was identical to that described above except that each flask was seeded with 5 mL aliquots of a conidial suspension of *C. truncatum* UFU 280 (1×10^5 conidia/mL). This suspension was prepared adding 10 mL of sterile water to the surface of 7-day-old actively growing cultures on PDA and gently sweeping the surface with a sterile spreader. The suspension was adjusted to the desired conidial concentration. The flasks were left on the orbital shaker under the conditions mentioned above for periods of incubation of 3, 6, 9, 12, 15 or 18 days, after which the entire content of each flask was removed from them separately. The mycelium was filtered, and the dry biomass retained was determined for each flask content as describe above. The conidial concentration in the filtrate was then evaluated as described above.

Agitation regimes during fermentation and effect on biomass and conidial production

Five 250 mL flasks containing 100 mL of ME medium each were seeded with three mycelial disks of *C. truncatum* UFU 280 as described above. Groups of five flasks were placed separately on an orbital shaker (Nova Instruments® ¯ NT 714) with orbital speeds adjusted respectively to 100, 120, 150 and 180 rpm and temperature adjusted to 25 °C. A group of five flasks was similarly treated, except that flasks were kept stationary, serving as control. After 18 days of incubation, content of all flasks was individually evaluated for dry biomass and conidial concentration of filtrate as described above.

Optimal level of medium pH for biomass and conidial production

25 flasks containing 100 mL of ME divided in groups of five were supplemented either with 1 N lactic acid or with 1 N NaOH to reach a pH level of: 2.3; 3.98; 6.98; 8.78 and 10.08. All flasks were autoclaved (120 °C for 20 min) and the pH of the medium was verified to ensure that no changes in pH level occurred. Seeding of each flask was performed, after the flasks and their content cooled, with culture disks as described above, and the flasks were left on an orbital shaker at 150 rpm under 25 °C for 18 days. Evaluation of fungal biomass and conidial concentration was performed for each flask as described above.

Incubation temperatures and their influence on biomass and conidial production

Groups of five 250 mL flasks, containing 100 mL of ME medium each with their pH adjusted to 9.0, were seeded with *C. truncatum* UFU 280, as described above. The groups of flasks were left separately on orbital shakers at 150 rpm under the following incubation temperatures: 15, 20, 25, 30 or 35 °C. After a period of 18 days, fungal biomass production was evaluated, and conidial concentration measured as described above.

Experimental designs and data analysis

All experiments were a randomised design with five replications for each treatment. The data were subjected to the Shapiro-Wilks and Levene tests for normality and homogeneity of variances. Analysis of variance (ANOVA, F Test, $P = 0.05$) was performed and the treatments were compared by means of the Tukey test (P = 0.05), and the quantitative factors were evaluated after adjusting the regression eqs. (P = 0.05). All analyses were done using the statistical package R version 3.1.1 (R Development Core Team 2011).

Results

Conidial concentrations obtained in the liquid culture media are given in Table 1. They were higher for ME (mean of 4.4×10^6 conidia /mL) followed by SYEA, VB, CB and PD. Concentration ranged between 2.3×10^4 and 1.3×10^6 conidia/mL. *C. truncatum* UFU 280 did not sporulate in CB nor in JP (data not shown). Larger amounts of mycelial mass were produced in JP, with an average of 1.55 g/100 mL of medium (Table 2). Fungal biomass in PD, AS, ME ranged from 0.11 to 0.77 g/100 mL of medium, with no difference among them. Lowest biomass was observed when the fungus was grown in CB and VB (Table 2).

Application of inoculum of *C. truncatum* (UFU 280) obtained from colonies grown in either in ME, SYEA, PD, CB or VB killed 100% of plants 7 days after spraying (Table 2). Conidia were not produced in either JP or CB and hence were unavailable for including in the test.

The longest incubation period of all tested (18 days) yielded the highest conidial concentration either for flasks seeded with culture disks or with conidial suspension (Table 1). The highest final conidial concentration obtained was for flasks seeded with culture disks but seeding with conidial suspension already produced a conidial concentration of 10^5 conidia/mL within a period of incubation of just 6 days of incubation. A much smaller conidial concentration (10^3 conidia/mL) was obtained at the 6th day of incubation for flasks seeded with culture disks. An increase in mycelial biomass was observed with the increase of the incubation period when both mycelial discs and conidial suspension were used as the inoculum source (Table 2).

Different speeds of orbital agitation had a significant effect on conidial yields (Table 1). Conidiation was absent under stationary conditions but reached a maximum of 5.1×10^6 conidia /mL at a regime of 150 rpm (Table 1). Sporulation decreased dramatically when orbital speed was of 180 rpm. Nevertheless, for mycelial biomass the 180 rpm regime resulted in the maximum yield (Table 2). Therefore, incubating *C. truncatum* UFU 280 at shaking speed of 150 rpm ensures adequate conditions for sporulation.

Medium pH of 9.0 was ideal for both conidial production and biomass production (Table 1). Either higher or lower pH levels inhibited mycelial growth and conidiation.

The ideal temperature for incubation for both conidiation and biomass production was between 20 to 25 °C (Table 2). Temperatures above or below this range inhibited conidiation (Table 2) and biomass production (Table 2). No conidia were detected within the media either at 15 or 35 °C (Table 2).

Discussion

Some of the basic conditions for producing inoculum of *C. truncatum* UFU 280 under liquid fermentation were first assessed here and the results allows for a preliminary protocol to be utilized in greenhouse and field experiments and, after improved may be useful for a commercial production of a putative mycoherbicide. An adequate amount of conidia can be obtained in ME liquid culture medium, adjusted to a pH of 9.0, seeded with a conidial suspension and incubated for 6 days, under a regime of orbital shaking of 150 rpm, at temperatures ranging from 20 to 25 °C.

The development of effective and low-cost methods for mass production of inoculum is a critical step both for the first stages of the evaluation of a potential mycoherbicide and for its viability as a commercial product (Boyette et al. 1991; Zhang et al. 2001). Liquid fermentation is the best option for industrial production of mycoherbicides (Churchill 1982; Boyette et al. 1991; Zhang et al. 2001). At this stage of the research, *C. truncatum* isolate UFU 280 can be considered as a good potential biocontroller of beggartick, as indicated by the high mortality levels obtained under controlled conditions (reaching 100%) as observed in this study. Greenhouse experiments in microplots are being conducted to corroborate this potential. Additionally, the fungus appears easily amenable to mass inoculum production under a simple set of conditions. The ME is the adequate liquid culture medium for mass production of *C. truncatum* UFU 280. It allows high conidia yield and did not allow a high production of mycelial mass. According to Dhingra and Sinclair (1995), a suitable culture medium supports high sporulation and low mycelial growth, as the sporulation is usually favored by nutritional exhaustion. The ME (Malt extract) liquid culture medium is also simple to manufacture and cheap. Conversely, mass production of microsclerotia of *C. truncatum* NRRL 18434, a host-specific pathogen of the weed *Sesbania exaltata*, requires more expensive and complex conditions, such as incubation at 28 °C in highly aerated liquid medium with 80 g glucose, 13.2 g casamino acids L^{-1} and salts (Jackson and Schisler 1995).

The shortest time for the suitable production *C. truncatum* UFU 280 conidia is reached after 6 days of incubation when conidial suspension was used as inoculum source. Longer incubation periods make a biological product unfeasible

Table 1 Effect of liquid culture media, incubation period, agitation and pH on the sporulation of *Colletotrichum truncatum* UFU 280

Liquid culture media	Conidia/mL (× 10^5)*	Incubation period	Mycelium disc** Conidia/mL (× 10^5)	Conidia suspension**
SYEA	0.22 (± 1.98) b	3 days	0.08 (± 0.33)	1.10 (± 0.55)
PD	2.25 (± 2.53) b	6 days	0.09 (± 1.45)	2.24 (± 1.18)
CB	10.22 (± 3.55) b	9 days	1.23 (± 2.21)	2.96 (± 3.81)
VB	12.81 (± 1.34) b	12 days	3.45 (± 3.33)	4.15 (± 2.94)
ME	44.42 (± 2.87) a	15 days	7.58 (± 2.98)	6.57 (± 2.22)
		18 days	13.53(± 3.35)	7.16 (± 1.67)
		Regression model	$Y = -4.816 + 0.8752\times$ $R^2 = 0.8525$	$Y = -0.418 + 0.4238\times$ $R^2 = 0.9684$
Agitation	Conidia/mL (× 10^5)	pH	Conidia/mL (× 10^5)	
0	0.23 (± 0.55)	2.3	0.025 (± 0.12)	
100 rpm	4.39 (± 3.55)	3.98	0.19 (± 0.15)	
120 rpm	4.56 (± 4.38)	6.98	1.1 (± 0.05)	
150 rpm	49.75 (± 5.56)	8.78	1.95 (± 2,25)	
180 rpm	13.52 (± 4.78)	10.08	0.17 (± 0.22)	

Liquid culture media = SYEA – Sucrose yeast sucrose yeast extract and asparagine (SYEA), *PD* Potato dextrose, *CB* Carrot broth, *VB* Vegetables broth, *ME* Malt extract. Means of five repetitions (± standard deviation). *Means followed by the same letter did not differ under the Tukey test ($p < 0.05$). **Forms of seeding the medium

commercially. Cost-efficient mass production of fungi requires the maximum yield of propagules within the shortest time possible (Butt et al. 2001). Maximum yield of biomass and chlamydospores of the weed biocontrol agent *Plectosporium alismatis* is also achieved after incubation in submerged culture for 6 days (Cliquet et al. 2004). In its turn, the production of melanized *C. truncatum* NRRL 18434C microesclerotia requires 9–10 days of incubation (Jackson and Schisler 1995).

Intensity of agitation of medium during incubation may be an important factor affecting propagule production. It can dramatically influence the amount of biomass and the kind of structures produced by the fungus in submerged culture (Calam 1986; Cliquet et al. 2004; Jackson and Schisler

Table 2 Effect of different conditions of incubation on mycelial mass and sporulation of *Colletotrichum truncatum* UFU 280 and mortality of beggartick plants using conidia produced in different liquid culture media

Liquid culture media	Dry mycelial mass	Mortality (%)	Incubation period	Mycelial discs**	Conidial suspension**	
				Dry mycelial mass		
JP	1.55 a	–	3	0.11 c	0.15 c	
CD	0.77 b	–	6	0.28 bc	0.29 bc	
ME	0.55 b	100	9	0.35 b	0.39 b	
SYEA	0.31 b	100	12	0.47 ab	0.51 a	
PD	0.11 b	100	15	0.56 a	0.58 a	
CB	0.06 c	100	18	0.61 a	0.59 a	
VB	0.09 c	100				
Agitation	Dry mycelial mass	pH	Dry mycelial mass	Temperature	Dry mycelial mass	Conidia mL^{-1} (× 10^5)
0 rpm	0.13 d	2.0	0.17 c	15 °C	0.42 b	5.5 c
100 rpm	0.34 cd	4.0	0.18 c	20 °C	1.10 a	43.3 a
120 rpm	0.51 bc	6.0	1.27 a	25 °C	1.14 a	13.5 ab
150 rpm	0.56 b	9.0	0.94 b	30 °C	0.62 b	2.1 d
180 rpm	2.18 a	12.0	0.21 c	35 °C	0.76 b	0.03 e

Means of five repetitions. Means followed by the same letter did not differ under Tukey test ($p < 0.05$). **Forms of seeding the medium

2002). It is possible that the combination of the levels of oxygenation of the medium and mechanic disruption of the mycelium influences conidial production. The ideal intensity of agitation (in the case, rotatory speed) found for propagule production of *C. truncatum* UFU 280 was 150 rpm, such as reported for the weed biocontrol fungi *P. alismatis* (Cliquet et al. 2004) and *Lewia chlamidosporiformans* (Vieira and Barreto 2010); however, *C. truncatum* NRRL 18434C required a speed of 300 rpm for microesclerotia production (Jackson and Schisler 1995) and *Pleospora papaveracea* requires a speed of 125 rpm (Bailey et al. 2004).

Most of the studies involving liquid fermentation to produce biocontrol fungi have concluded that the ideal range of pH is between 5 and 7 (Garraway and Evans 1984; Griffin 1994; Deacon 2006; Schisler et al. 1991; Jackson and Schisler 1995; Van Winkelhoff and McCoy 1984; McQuilken et al. 1990; Gardner et al. 2000; Tan et al. 2002). The optimal pH for *C. truncatum* UFU 280 sporulation is 9.0. Higher and lower pH resulted in lower fungal biomass yields and conidia production. Similarly, Zhao and Shamoun (2006) observed that the pH range between 6.0 and 9.0 was ideal for the sporulation of *Phoma exigua*, a potential mycoherbicide for the control of *Gautheria shallon*. For *C. truncatum* NRRL 18434C, the pH 5.5 is more appropriate for conidia production (Jackson and Schisler 1995).

In general, the optimum temperature for vegetative growth and sporulation of a fungus under fermentation process is similar to the predominant temperature conditions where this organism occurs in nature, but different optimum temperatures can be found for different isolates of a single fungal species (Dhingra and Sinclair 1995). We found that maximum mycelial growth and sporulation of *C. truncatum* isolate UFU 280 occurs within the range of 20–25 °C. Other bioherbicide fungi are also known to have specific temperature requirements. *Bipolaris euphorbiae* is a potential biocontrol agent for wild poinsettia (*Euphorbia heterophylla*), and its optimal sporulation temperature is 22 °C (Moraes, 2009). *C. truncatum* NRRL 18434C produces more conidia at 28 °C (Jackson and Schisler 1995), while optimal temperature for *Phytophthora parasitica* is 18 °C (Gardner et al. 2000); for *Fusarium sulphurum* is 37 °C (Barran et al. 1977); and for *Fusarium oxysporum* isolate M12-4A is 32 °C (Diarra et al. 1996). Additionally, the conversion of macroconidia of *Fusarium oxysporum* fsp. *Batatas* and their conversion to chlamydospores occurs at 24–28 °C (French and Nielsen 1966).

The preliminary protocol for mass production of conidia of *C. truncatum* isolate UFU 280 at a laboratory scale presented herein, paves the way towards a series of tests on fungus-host interactions, formulation and application of the potential mycoherbicide and its test in the greenhouse, in mini-plots and in field situations. Some experiments are already being conducted now and others are planned, benefiting from the information generated here. Also, in case the potential of this novel mycoherbicide candidate is further confirmed, scale-up tests will be necessary to verify if the performance of the fungus remain consistent for inoculum produced in large capacity bioreactors as well as the choice of stabilizing and storage methods for the final mycoherbicide product. The challenge of managing beggartick remains a major stimulus for the effort towards the development of a novel mycoherbicide tool.

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