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Original Article

Production of Phytotoxic Metabolite Using Biphasic Fermentation System from Strain C1136 of *Lasiodiplodia pseudotheobromae*, a Potential Bioherbicidal Agent

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Abstract

Formulation of effective and environmental friendly bioherbicides depends on the type of fermentation medium used for the production of phytotoxic metabolites. The effect of biomass, colony forming unit and the phytotoxic metabolite produced from the biphasic fermentation was carried out, while the phytotoxic metabolite was tested *in vivo* and *in-vitro* on *Echinochola crus-galli* and dicotyledonous *Chromolaena odorata*. The mutant strain of *Lasiodiplodia pseudotheobromae* C1136 (Lp90) produced the highest amount of conidia and the largest necrotic area on the two tested weeds when compared to its wild strain in the different biphasic media combinations. The study revealed that the biphasic system containing PDB + rice produced the highest bioherbicidal activities. Therefore, the phytotoxic metabolites from strain C1136 are suggested for large scale production of bioherbicides for the management of weeds in conventional farming to improve yield and enhance food security.

Keywords: biphasic fermentation, bioherbicides, phytotoxic metabolite, biomass, necrotic induction

Introduction

The use of microorganisms as biological control agents has been considered an important tool in integrated pest management (IPM) because the use of synthetic herbicides has posed a serious threat to food safety, the ecosystem, environmental and health hazards, hence there is a need for safe and effective bio-herbicides (Barranco Florido *et al*., 2002; Adetunji and Oloke, 2013; Adetunji *et al*., 2017a, b).

Kayode and Ige (1998) discovered that *C. odorata* (L) and *T. procumbens L*. are one of the major weeds that constitute a major impediment to agricultural and natural ecosystem in Nigeria. *Echinochloa crus galli* is one of the most devastating weeds commonly associated with rice. It is an annual plant similar to rice, which can grow and flower in a photoperiod ranging between 8 and 16 h. Due to its vigorous growth, it competes heavily with crops for water, nutrients, and light (Auld and Kim, 1996).

Biphasic fermentation system combines the benefits of high biomass production in liquid fermentation and production of stable and hydrophobic aerial conidia on a solid substrate (Veloorvalappil *et al*., 2015). The importance of biphasic fermentation, when compared to other fermentation systems, include: prevention of contaminants that might be present from the culture stock, promotion increased the competitiveness of the fungus, ensuring a uniform colonization of the solid substrate, production of conidia is faster, reducing the incubation time and economizing physical space (Vladimir *et al*., 2014).

Phytotoxins are defined as microbial metabolites that are harmful to plants at low concentrations. The phytotoxins contain a variety of classes of natural products with low molecular weight; they are involved in pathogenicity and the induction of disease symptoms while the virulence of the pathogen may depend on its capacity to produce one or more toxins (Evidente and Motta, 2001).

Recently, research has been directed towards isolating phytotoxins produced by some fungi pathogenic to weeds and using the isolated, non-specific compounds as natural

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herbicides either in their native form or as derivatives and analogs (Strobel *et al*., 1991; Boyetchko, 1999; Adetunji *et al*., 2017a,b). With the increasing number of herbicide-resistant weeds, phytotoxins provide a new pool of compounds to control problematic weeds. Therefore, this study intends to evaluate the effectiveness of biphasic fermentation method for the production of phytotoxic metabolites from *Lasiodiplodia pseudotheobromae* for the management of weeds.

Materials and Methods

Source and maintenance of fungal isolates

Fungal strains were isolated from small chlorotic and necrotic lesions on leaves of *Tridax procumben* weeds as previously described by Adetunji and Oloke (2013). The isolated strain was identified as *Lasiodiplodia pseudotheobromae* using 18s rRNA gene sequencing and coded C1136 with an accession number KY432690. 18S rDNA gene sequence was submitted to Gene Bank. The fungal isolates were incubated on potatoes dextrose agar (PDA) for 7 days at 25 ± 1 °C in BOD incubator.

Exposure of the fungal bioherbicidal strain to UV light to induce random mutation

This experiment was performed using the protocol described by Adetunji *et al*. (2017a,b). This was carried out to compare the efficacy of the phytotoxic metabolites produced by the wild strain when compared to that by the mutant strain. The sterile plate containing several mycelia plugs was placed under UV lamp at 300-nm wavelength at a distance of 30 cm to the plates. At different time intervals (30, 60 and 90 min), five mycelia plugs were withdrawn and used as inoculants for potatoes dextrose medium. The wild strain was coded WLp while the mutants were coded Lp 30, Lp 60 and Lp 90. The mycelia plugs from the domesticated type culture serve as the control (Adetunji and Oloke, 2013).

Mass production of Lasiodiplodia pseudotheobromae in a biphasic system

The biphasic system was set following the protocol of Machado *et al*. (2009). The system consists of PDB and YMB as liquid media and whole rice and millet as solid substrates were tested. One hundred milliliter of PDB and YMB was prepared in 250 mL conical flasks and inoculated with 1×10^6 spore's mL^{-1} at 150 rev/sec for 72 hours. Fifteen milliliters of cultures from 3 days old was used to inoculate solid substrates. The solid substrate fermentation was prepared according to the protocol of Penairol *et al.* (2008). The number of the conidia produced from the suspension was determined using a Neubauer chamber, according to the method described by Francisco *et al*. (2006).

Determination of biomass

Dry weights of plant shoot material (biomass) were determined at the end of the experiment after excising stems at the soil line and drying for 48 h at 60-70 °C.

Herbicidal activity and leaf necrosis assay

The leaves of *Chromolaena odorata* and *Echinocholoa crus-galli* were surface sterilized with ethanol and washed with sterile distilled water to remove ethanol from the surface. The leaf bioassay with respective extracts was performed with 2.5µL from different fermentation broth produced after seven days of fermentation was carried out by wounding the sterilized leaves with a sterile needle on the surface of the leaf and transferred to Petri plate containing moistened cotton ball and filter paper. Later plates were incubated at 25 °C for one week. An observation was made for the development of necrotic lesions on the extracts inoculated leaves after inoculation (Amusa, 2005).

Pathogenicity tests

Fungal inocula from liquid culture were applied to of 2-wk-old (2- to 4-leaf stage) of *Echinochola crus-galli* and *Chromolaena odorata* by spraying with an atomizer until run-off occurred. Control plants received distilled water. Plants were placed in a dew chamber for up to 24 h, then transferred to the greenhouse for assessment of infection over a 10-d period. Three replicates were used for each treatment. Each replicate contained 4 *Echinochola crus-galli* and *Chromolaena odorata*. The experiment was repeated twice. Inocula from solid culture fungus-rice extracts, prepared as described above, were applied to leaves and stems of jimsonweed seedlings by spraying to run-off with an atomizer. Control plants received filtrates of autoclaved rice and millet. Plants were placed in the greenhouse immediately after treatment. Treated and control plants were observed daily and symptoms were evaluated using a visual injury rating scale described below in a dew chamber for 10-20 hours before been placed in the greenhouse.

Injury or mortality determination

Injury to and mortality of *Echinochola crus-galli* and *Chromolaena odorata*seedlings by liquid culture inocula, fungal rice extracts and millet extract was visually assessed 2 weeks after treatment, using a scale based on that described by Hoagland and Boyette (1994). The injury was assigned a value of 0-4, where 0 = no injury (0%) and 4 = severe chlorosis, necrosis, growth inhibition, wilt, or mortality 100%). Ratings were combined averages of rating values for two observations of three replicates (composed of 10 seedlings) of each treatment. Percent mortality was determined 2 weeks after treatment by direct counts of collapsed seedlings. Values of the replicates of each treatment were combined and averaged.

Data analysis

The data were analyzed by using SAS software 8.2(2001). Significant means were separated using Duncan's multiple range test.

Results

Effect of biphasic system on biomass production, colony forming unit and necrotic induction from wild and mutant strain of strain C1136

The result obtained from the biomass produced during the biphasic fermentation showed that media containing

potatoes dextrose broth (PDB) plus rice (PDB + rice) had the highest while the lowest was obtained in yeast malt broth plus millet (YMB +Millet) media. The different media used had a significant influence on the amount of biomass produced from the mutant strain of strain C1136 Lp 90 when compared to other strains. The highest amount of biomass was found in (PDB + rice) with 956 mg/L (Fig. 1A), while the lowest yield value of 182 mg/L was obtained in YMB +Millet media from the wild strain of C1136 (Fig. 1B).

The effect of the phytotoxic metabolite produced from the biphasic fermentation tested on weed from leaves of monocotyledon (*Echinochola crus-galli*) and dicotyledonous (*Chromolaena odorata*) showed that the phytotoxic metabolites from the mutant strain C1136 Lp 90 produced the largest necrotic area on the two tested weeds at all the teste concentrations. When the phytotoxic metabolite was inoculated on *Chromolaena odorata* leaves segment the following necrotic areas were obtained from the following media: PDB + rice (3.6 mm^2), PDB + Millet (3.3 mm^2), YMB + rice (3.0 mm^2), YMB + Millet (2.9 mm^2) while the following necrotic area were observed on *Echinochola crus-galli* leaves segments from the following media PDB + rice (3.3mm^2), PDB + Millet (3.1mm^2), YMB + rice (2.7 mm^2), YMB + Millet (2.7 mm^2). Moreover, the wild strain of C1136 produced the lowest amount of phytotoxic metabolite during the biphasic fermentation on the two tested weeds. When the phytotoxic metabolite was inoculated on *Chromolaena odorata* leaves segment the following necrotic areas were obtained from the following media; PDB + rice (1.8 mm^2), PDB + Millet (1.5 mm^2), YMB + rice (1.4 mm^2), YMB + Millet (1.2 mm^2), while the following necrotic area were observed on *Echinochola crus-galli* leaves segments from the following media PDB + rice (1.4 mm^2), PDB + Millet (1.0 mm^2), YMB + rice (1.4 mm^2), YMB + Millet 0.8 mm^2).

The mutant strain of strain C1136 Lp 90 had the highest amount of colony forming unit compared to wild strain WLp C1136 produced in the different biphasic combinations. PDB + Rice produced the highest conidia with 7.9×10^9 CFU/g (Fig. 3A), while YMB + Millet produced the lowest conidia of 1.4×10^9 CFU/g among all the different combinations (Fig. 3B).

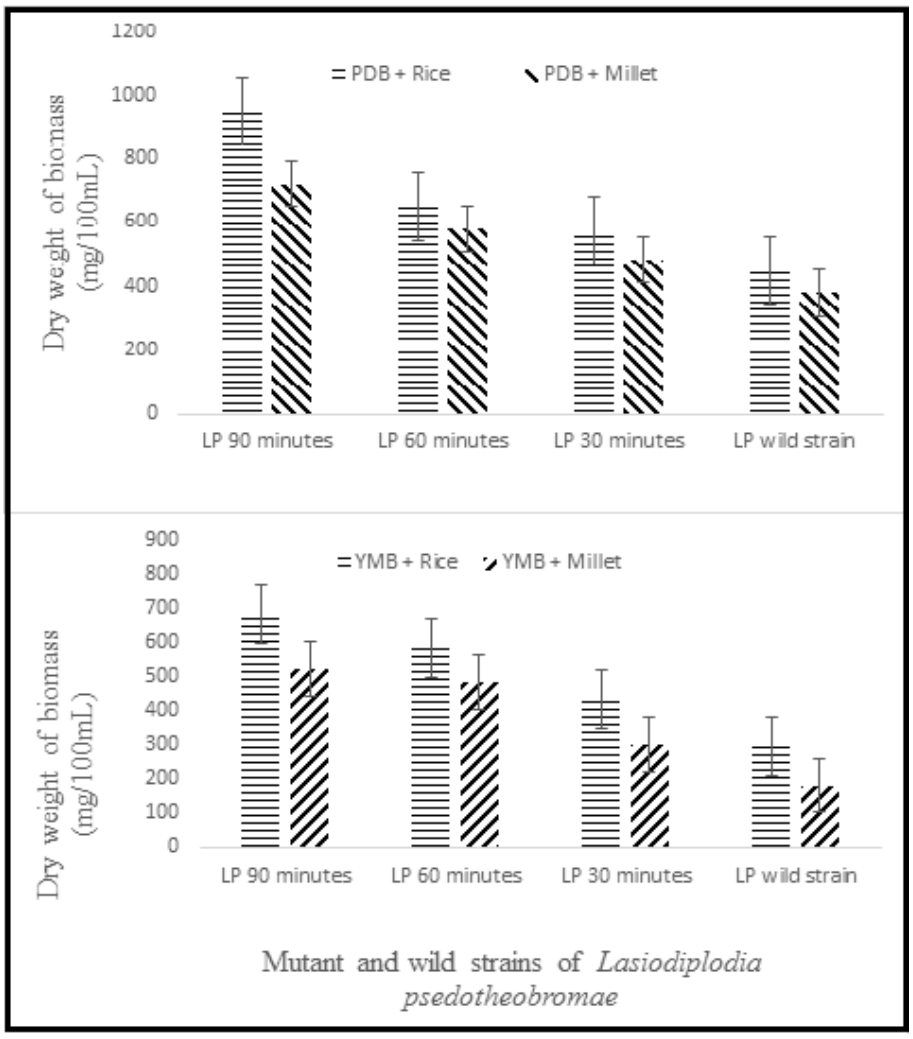

Fig. 1. Effect of biphasic system on biomass production from wild and mutant strains of C1136; (A) The dry weight biomass of strains C1136 produced from PDB + rice and PDB + millet; (B) The dry weight biomass of strains C1136 produced from YMB + rice and YMB + millet

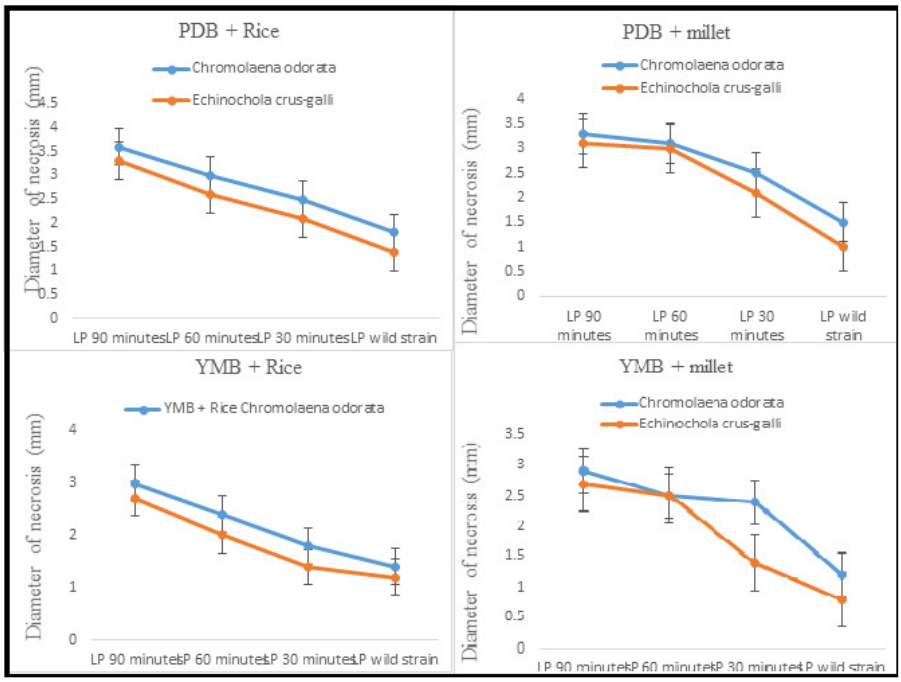

Fig. 2. Effect of biphasic system on necrotic induction from wild and mutant strains of C1136; (A) Diameter of necrosis induced by Lp90; (B) Diameter of necrosis induced by Lp60; (C) Diameter of necrosis induced by Lp30; (D) Diameter of necrosis induced by Lp wild strain

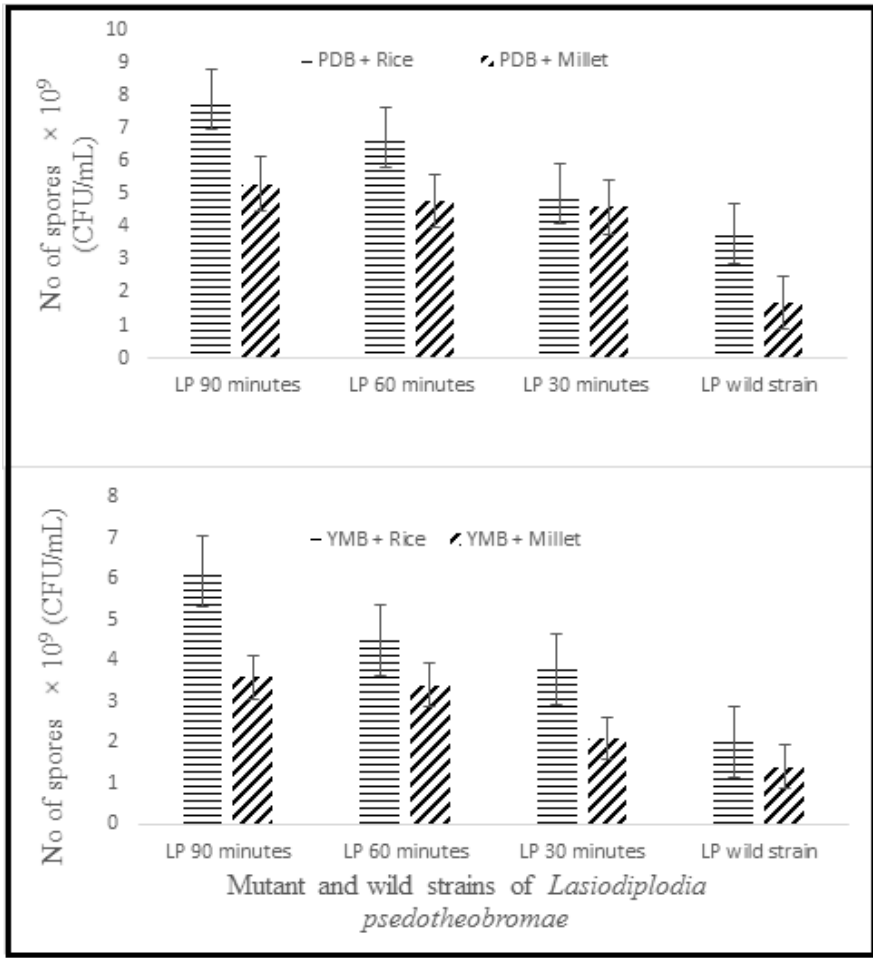

Fig. 3. Effect of biphasic system on the rate of sporulation from wild and mutant strain of C1136; (A) Number of spores produced from PDB + rice and PDB + millet by different strains of C1136; (B) Number of spores produced from YMB + rice and YMB + millet by different strains of C1136

Herbicidal properties of the phytotoxic metabolite from Lasiodiplodia pseudotheobromae grown in a biphasic system

The phytotoxic metabolite produced during biphasic fermentation showed that the combination from PDB + rice, PDB + millet, YMB + rice and YMB + millet had 100% injury and mortality rate on *Chromolaena odorata* weed, while YMB + rice and YMB + millet induced 97% and 95% injury rate and 76% and 65% mortality rate on *Echinocholoa crus-galli* weed respectively. Moreover, PDB + rice and PDB + millet showed a greater antagonism against the tested weeds and this lead to a greater mortality on the two tested weeds. The pytotoxic metabolite from YMB + rice caused a mortality rate of 68% on *Chromolaena odorata* and 59% on *Echinocholoa crus-galli* respectively, while YMB + millet caused a mortality rate of 50% on *Chromolaena odorata* and 54% on *Echinocholoa crus-galli* respectively (Table 1).

Discussion

Optimization of media is generally done by studying the effects of the ingredients and nutrients for growth using fermentation studies, selecting and optimizing a few parameters. Culture medium influenced germination, mycelial growth, and virulence of fungi employed as mycoherbicidesand thus affected their production cost and efficacy (Silman *et al*., 1991; Schisler *et al*., 1993).

Industrial production systems for some biocontrol fungi, use a biphasic method in which the fungal inoculant – mycelia or hyphae – is produced in liquid culture and then is transferred to solid substrates in order to increase conidial production (Guillon, 1997). During this study, the same approach of the biphasic system of fermentation was used by mixing artificial liquid medium serving as the liquid phase with an agricultural solid substrate that produced the best result from the optimization study which serves as solid state phase of the fermentation. The result of this study showed that the combination of potatoes dextrose broth with broken rice (PDA + rice) produced the largest mycelia weight, colony forming unit of fungus and largest amount of phytotoxic metabolite because they were able to induce the largest amount of necrosis on the tested weeds. The reasons for the best result obtained from the combination of PDA + rice might be due to the large surface area of the broken rice to the *Lasiodiplodia pseudotheobromae* strain which enabled it for easily colonize the substrate rice grains.

This is also similar to the findings of Jenkins *et al*. (1997) who discovered that biphasic fermentation process ensures a uniform colonization of the solid substrate which resulted in homogeneous fungal growth. Also, they observed that the colonization and production of conidia were faster which led to a reduction in the incubation time and thereby economizing the physical space.

Another reason for the better outcome of the combinations from PDA + rice in the biphasic fermentation was that there was a slow release of nutrients from the lower solid phase of the medium containing broken rice grains, which supports the growth of the organism when the liquid medium was exhausted just as in the batch culture. This is in line with the findings of Kaur *et al*. (2003). It was also observed that combinations from PDB +Rice produced the largest amount of conidia in the medium with 7.9×10^9 CFU g^{-1}. This is due to the fact that the PDB provided the necessary nutrients while the rice grains provided both nutrients the needed surface area for the colonization of *Lasiodiplodia pseudotheobromae*. Lomer and Lomer (2008) stated that the structure of the substrate may be as important as the available nutrients. According to these authors, an ideal substrate should provide a high ratio between the superficial area and volume where the individual particles remain separated, in order to provide space between particles for aeration and conidia formation.

Derakhshan *et al*. (2008) evaluated different methods for the production of *L. lecanii*, including production using liquid and solid substrates and a combination of them. These authors observed that the production of conidia using the biphasic culture system with a combination of 4% sugar cane molasses and grain rice provided an increase of 2.43 and 1.16 times when compared to production in liquid and solid media, respectively.

Using the biphasic culture system for the production of *Beauveria bassiana*, Santoro *et al*. (2005) obtained the greatest sporulation when rice grain was combined with liquid media made with crysalid flour or with crysalid flour + potato + dextrose, reaching a yield of 2.7 and 2.8×10^{12} CFU/g, respectively. According to these authors, the

Table 1. Effect of herbicidal properties of the phytotoxic metabolite from strains of C1136 grown in a biphasic system on *Chromolaena odorata* and *Echinocholoa crus-galli* weeds

	Chromolaena odorata	*Echinocholoa crus-galli*	*Chromolaena odorata*	*Echinocholoa crus-galli*	*Chromolaena odorata*	*Echinocholoa crus-galli*
Media	Injury (%)		Mortality (%)		Dry weight (% reduction)	
Distilled water	0.0 ± 0.0^b	0.0 ± 0.0^b	0.0 ± 0.0^b	0.0 ± 0.0^d	0.0 ± 0.0^c	0.0 ± 0.0^c
Autoclave rice extract	0.0 ± 0.0^b	0.0 ± 0.0^b	0.0 ± 0.0^b	0.0 ± 0.0^d	0.0 ± 0.0^c	0.0 ± 0.0^c
Autoclave millet extract	0.0 ± 0.0^b	0.0 ± 0.0^b	0.0 ± 0.0^b	0.0 ± 0.0^d	0.0 ± 0.0^c	0.0 ± 0.0^c
PDB broth	0.0 ± 0.0^b	0.0 ± 0.0^b	0.0 ± 0.0^b	0.0 ± 0.0^d	0.0 ± 0.0^c	0.0 ± 0.0^c
YMB broth	0.0 ± 0.0^b	0.0 ± 0.0^b	0.0 ± 0.0^b	0.0 ± 0.0^d	0.0 ± 0.0^c	0.0 ± 0.0^c
PDB + rice	100.0 ± 5.0^a	100.0 ± 6.0^a	100.0 ± 6.0^a	100.0 ± 8.0^a	ND	ND
PDB + millet	100.0 ± 5.2^a	100.0 ± 2.0^a	100.0 ± 3.0^a	100.0 ± 6.0^a	ND	ND
YMB + rice	100.0 ± 4.0^a	97.0 ± 2.0^a	100.0 ± 7.0^a	76.0 ± 4.0^b	68.0 ± 2.0^a	59.0 ± 1.0^a
YMB + millet	100.0 ± 8.0^a	95.0 ± 3.0^a	100.0 ± 2.0^a	65.0 ± 5.0^c	50.0 ± 4.0^b	54.0 ± 4.0^b

Results are the means of three replicates ± one standard error. ND = Not determined due to mortality

available nutritional source in liquid media influenced sporulation on solid media and, compared to the conventional production of this fungus, there was a 100 to 1,000 fold increase in productivity, which also reduced the production time.

The combination of the liquid media made with soy flour and dextrose and solid media with a broken corn, rice, barley, sorghum, oats and coconut fiber base were evaluated for the production of seven *Hirsutella thompsonii* isolates, and one *H. nodulosa* isolates (Rosas Acevedo *et al*., 1995). These authors concluded that the biphasic method increased sporulation of both fungi. The mutant strains of *Lasiodiplodia pseudotheobromae* exposed to 1 hour 30 minutes produced a higher amount of biomass, colony forming unit and necrotic induction compared to its wild strain. Furthermore, the results of this study have shown that genetic improvement carried out using random mutagenesis enhanced the activities of the mutant strains when compared to the wild type strain (Adetunji and Adejumo, 2017).

The strategy for optimizing bioherbicide production is based on developing a medium which maximizes propagule yield and propagule fitness as a bioherbicidal agent. The first step in this optimization strategy is the development of a defined medium which supports good growth and propagule formation by the potential bioherbicide (Jonsbu *et al*., 2002). The propagule of interest will depend on the bioherbicidal agent being evaluated. Once a basal medium is developed, nutritional components of the medium can be varied and the impact of these changes assessed in terms of propagule yield, propagule fitness as a bioherbicidal agent, and propagule stability as a dry preparation. All of these factors must be considered during optimization since all are required for an effective biocontrol agent. On the whole, the enhanced bioherbicidal activities from the phytotoxic metabolites of wild and mutant strains of *Lasiodiplodia pseudotheobromae* during this study is in line with all other authors that have produce various useful metabolite using biphasic fermentation approach (Hafiza *et al*., 2014; Ke *et al*., 2015).

Conclusions

The current study has provided information on the effectiveness of biphasic fermentation for the production of phytotoxic metabolites from local isolates of *Lasiodiplodia pseudotheobromae* as bioherbicidal agent. The phytotoxic metabolite could be a replacement for synthetic herbicide which is more economical in controlling weeds than the synthetic herbicides. Overall, the authors assert that this work will accelerate agriculture production and serve as an alternative to the chemical herbicides because of its safety in the environment.

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References

Adetunji CO, Oloke JK, Prasad GS, Adejumo IO (2017a). Effect of *Lasiodiplodia pseudotheobromae* isolates, a potential bioherbicide for *Amaranthus hybridus* L. in maize culture. Notulae Scientia Biologicae 9(1):131-137.

Adetunji C, Oloke J, Kumar A, Swaranjit S, Akpor B (2017b). Synergetic effect of rhamnolipid from *Pseudomonas aeruginosa* C1501 and phytotoxic metabolite from *Lasiodiplodia pseudotheobromae* C1136 on *Amaranthus hybridus* L. and *Echinochloa crus-galli* weeds. Environmental Science and Pollution Research 15(24):13700-13709.

Adetunji CO, Adejumo IO (2017). Nutritional assessment of mycomeat produced from different agricultural substrates using wild and mutant strains from *Pleurotus sajor-caju* during solid state Fermentation. Animal Feed Science and Technology 224:14-19.

Adetunji CO, Oloke JK (2013). Efficacy of freshly prepared pesta granular formulations from the multi-combination of wild and mutant strain of *Lasiodiplodia pseudotheobromae* and *Pseudomonas aeruginosa*. Albanian Journal of Agricultural Sciences 12(4):555-563.

Auld BA, Kim KU (1996). Weed management in rice (No. 139). Food and Agriculture Org.

Amusa NA (2005). Microbially produced phytotoxins and plant disease management. African Journal of Biotechnology 5:405-414.

Barranco-Florido JE, Alatorre-Rosas R, Gutierrez-Rojas M, Viniegra-Gonzalez G, Saucedo-Castaneda G (2002). Criteria for the selection of strains of entomopathogenic fungi *Verticillium lecanii* for solid state cultivation. Enzyme and Microbial Technology 30(7):910-915.

Boyetchko SM (1999). Innovative application of microbial agents for biological weed control. In: Mukerji KG, Chamola BP, Updahyay RK. (Eds). Biotechnological Approaches in Biocontrol of Plant Pathogens. Kluwer Academic/ Plenum, New York pp 73-97.

Derakhshan A, Rabindra RJ, Ramanujam B, Rahimi M (2008). Evaluation of different media and methods of cultivation on the production and viability of entomopathogenic fungi *Verticillium lecanii* (Zimm.) Viegas. Pakistian Journal of Biological Science 11(11):1506-1509.

Evidente A, Motta A (2001). Phytotoxins from fungi, pathogenic for agrarian, forestal and weedy plants. Bioactive Compound Natural Resources pp 473.

Francisco EA, Mochi DA, Correia ADCB, Monteiro AC (2006). Influence of culture media in viability test of conidia entomonopathogenic fungus. Ciencia Rural 36(4):1309-1312.

Guillon M (1997). Production of biopesticides: scale up and quality assurance. British Crop Production Council Symposium pp 151-162.

Hoagland RE, Boyette CD (1994). Pathogenic interactions of *Alternaria crassa*and phenolic metabolism in jimsonweed *(Datura stramonium* L.) varieties. Weed Science 42:44-49.

Jenkins NE, Goettel MS (1997). Methods for mass-production of microbial control agents for grasshoppers and locusts. The Memoirs of the Entomological Society of Canada 129(S171):37-48.

Jonsbu E, McIntyre M, Nielsen J (2002). The influence of carbon source and morphology on nystatin production by *Streptomyces noursei*. Journal of Biotechnology 95(2):133-144.

Ke W, Wei L, Jianrui S, Tao L (2015). Production, purification, and identification of cholest-4-en-3-one produced by cholesterol oxidase from *Rhodococcus* sp. in aqueous/organic biphasic system. Biochemistry Insights 8(S1):1-8.

Kaur P, Grewal HS, Kocher GS (2003). Production of α-amylase by *Aspergillus niger* using wheat bran in submerged and solid state fermentations. Indian Journal of Microbiology 43:143-145.

Kayode J, Ige OE (1998). A quantitative study of weed flora in three abandoned farmlands in Ekiti State. Biosciences Research Community 3:169-178.

Lomer CH, Lomer CJ (2008). Mass production of fungal pathogens for insect control: insect pathology manual. Retrieved 14 March 2017 from http://www.lubilosa.org.

Machado ACR, Monteiro AC, Mochi DA, Yoshida L (2009). Agroindustrial residues and byproducts and grains as substrates for the production of the entomopathogenic fungus *Lecanicillium lecanii*. Bragantia 68:483-491.

Penariol MC, Monteiro AC, Pitelli RA, Pereira GT (2008). Production of *Bipolaris euphorbiae* in solid and liquid culture media obtained from grains and agroindustrial residues. Bragantia 67:805-814.

Rosas Acevedo JL, Alatorre Rosas R, Sampedro Rosas L, Valdez Carrasco J (1995). Sporulation of entomopathogenic fungi *Hirsutella thompsonii* Fisher and *H. nodulosa* petch in mixed culture. Revista Latinoamericana de Microbiologia 37:59-64.

Santoro PH, Neves PM, de Silva OJ, da Akimi RZ, Zorzetti J (2005). Spore production of *Beauveria bassiana (Bals.) Vuill*. in a two phase process using different liquid media. Semina: Agrarian Sciences 26:313-320.

SAS Institute- Statistical Analysis System (2001). Version 8.2 for Windows. Cary, NY: SAS Institute.

Schisler DA, Jackson MA, Bothast RJ (1991). Influence of nutrition during conidiation of *Colletotrichum truncatum* on conidial germination and efficacy in inciting disease in *Seshania exaltata*. Phytopathology 81(4):458-461.

Silman RW, Bothast RJ, Schisler DA (1993). Production of *Colletotrichum truncatum* for use as a mycoherbicide - effects of culture, drying and storage on recovery and efficacy. Biotechnology Advances 11(3):561-575.

Strobel G, Kenfield D, Bunkers G, Sugawara F, Clardy J (1991). Phytotoxins as potential herbicides. In: Phytotoxins and their involvement in plant disease. Experientia 47:819-826.

Veloorvalappil NJ, Robinson BS, Prakasan P, Sreedharan S,Sailas B (2015). Biphasic fermentation is an efficient strategy for the overproduction of δ-Endotoxin from *Bacillus thuringiensis*. Applied Biochemistry and Biotechnology 175(3):1519-1535.

Vladimir G, Svetlana G, Jae SK (2014). Production of *Beauveria bassiana a*ir conidia by means of optimization of biphasic system technology. Brazilian Archives of Biology and Technology 57(4):571-577.