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Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

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Full Length Research Paper

Effect of inoculum morphology on production of Nigerloxin by solid state fermentation

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Nigerloxin (2-amido-3-hydroxy-6-methoxy-5-methyle-4-(prop-1'enyl) benzoic acid) is a Lipoxygenase and Aldose reductase inhibitor produced by *Aspergillus niger* MTCC 5116. It is produced only under solid state fermentation (SSF) and the inoculum required for the inhibitor production is developed in submerged condition. As this is a newly discovered enzyme inhibitor with potential commercial success against diabetic complications, such as neuropathy and cataract, its complete fermentation process parametric study is not reported. In this study, the role of physical parameters (spore suspension, initial pH, incubation temperature and agitation) in spore germination and pellet size in inoculum development broth in submerged fermentation for the enhanced production of nigerloxin through SSF has been studied. It was concluded that 500 µl of spore suspension in the inoculum development broth at pH 7, incubated at 30°C and 200 rpm gave an ideal pellet size 1.23 mm resulting in 6.0 mg of nigerloxin/g dry weight of wheat bran in SSF.

Key words: Nigerloxin, spore germination, solid state fermentation, *Aspergillus niger*.

INTRODUCTION

Inoculum development is one of the major unit operations in a fermentation process, involving production of required quantity of viable desired microbial biomass in its most productive state (Hockenhull, 1980). There are successful commercial fermentations using pellets and others use dispersed forms. Much information exists on inoculum development for submerged fermentations. In fungal solid state fermentation, the thin line between success and failure of a productive fermentation process is the quality of biomass produced as inoculum. Unlike bacteria, fungal inoculum can be manipulated to required pellet sizes or suspension forms through physical fermentation parameters to subsequently yield large quantities of the product in the main fermentation process. Further, not many reports exist on the influence of physical parameters on the fungal morphology in inoculum development broth on the production of desired metabolites through solid state fermentation.

Nigerloxin, produced by solid state fermentation of *Aspergillus niger* MTCC 5116 on wheat bran medium, is a Lipoxygenase and Aldose reductase inhibitor (Rao et al., 2002). It shows beneficial effects against diabetic

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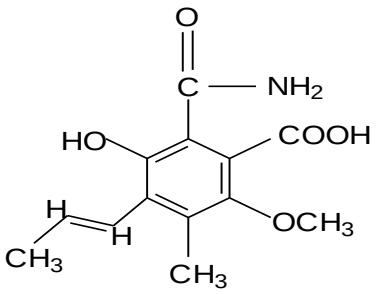

Figure 1. Structure of nigerloxin

complications such as oxidative stress and cataract formation in *vivo* (Suresh et al., 2012; Suresh et al., 2013). Production of nigerloxin is associated with the sporulation of the organism in SSF. The fermentation process is not completely reported for this potential commercially successful inhibitor against diabetic complications. While we have earlier described conditions for its optimum production (Chakradhar et al., 2009), here, we report the effect of physical parameters on inoculum by submerged fermentation and its subsequent production of nigerloxin in SSF.

MATERIALS AND METHODS

Culture

Aspergillus niger MTCC 5116 used in the present study was maintained on potato dextrose agar (Hi Media, Mumbai, India), at 4°C and subcultured once in every three weeks.

Solid state fermentation

Experiments were conducted in 500 ml Erlenmeyer flasks containing 10 g of wheat bran supplemented with 5 % (w/w) trisodium citrate with an initial moisture content of 60%. This medium served as a control wheat bran medium. After a thorough mixing, the flasks were autoclaved at 121°C for 1 h, cooled to room temperature and 2 ml of *A. niger* MTCC 5116 cell suspension was inoculated and incubated at 30°C for 6 days (Rao et al., 2005).

Extraction and determination of nigerloxin

At the end of fermentation, 100 ml of ethyl acetate was added to the fermented bran and kept on a rotary shaker at 200 rpm for 2 h. The bran was then filtered through cheesecloth followed by Whatman No 1 filter paper. The solvent was evaporated to yield a crude extract. 1.36 g of crude was suspended in 25 ml of chloroform and centrifuged at 2000 rpm for 20 min to obtain an orange precipitate. This was resuspended in 50 ml of warm ethanol to which 200 mg of activated charcoal was added. This content was filtered through Whatman No 1 filter paper and concentrated to obtain nigerloxin, which was used as standard. The nigerloxin concentration in samples was determined at 292 nm in UV-VIS spectrophotometer (Shimadzu UV 1601) (Rao et al., 2002)

Spore germination count

The spores from three day old slant were scraped and suspended in 4 ml of 2 % Tween 20 (v/v) solution for several minutes to facilitate wetting, and centrifuged for 1 min at 4000 rpm. The supernatant was then poured off and the spores resuspended in 2 ml distilled water. This suspension was then added to 5 ml of inoculum broth. Germination was determined based on the number of empty spore cases counted at 12^{th} hour of growth using a Spencer Bright-Line Haemocytometer (American Optical Company) under magnifications of 10X and 40X and expressed as percentage spore germination (Braun, 1971).

Fungal pellet measurement

Fungal pellets grown in inoculum broth were collected and the measurements of fungal pellets were done either directly under microscope or a centimetre scale on a digital photograph projection (Pazouki and Panda, 2000).

Optimization of inoculum development parameters

To optimize the inoculum development parameters several media components and cultural conditions were altered in submerged condition. The inoculum produced was inoculated to controlled wheat bran media and observed for the enhanced production of nigerloxin: 1) The effect of various standard media like potato dextrose broth, oatmeal broth, czapeckdox broth, tryptone yeast extract broth, modified egg yolk broth, yeast extract malt extract broth, potassium tellurite broth, and glycerol asperagin broth on the production of inoculum was studied by inoculating loopful (5×10^5 spores) *A. niger* CFR-W-105 to each broth and pellet morphology, media pigmentation, spore germination and biomass production was studied. Nigerloxin production was evaluated by using inoculum developed by each standard medium and inoculating on to controlled wheat bran media; 2) the effect of initial pH of the inoculum development broth on biomass production, pellet size and spore germination was determined by altering the initial pH of the fermentation media with the addition of acid or alkali. Nigerloxin production was evaluated by using inoculum developed by each pH range and inoculating on to controlled wheat bran media; 3) effect of temperature was studied by incubating the organism at various temperatures ranging from 10 to 50°C in inoculum development broth.The nature of pellets, rate of spore germination, biomass production and nigerloxin production was studied; 4) The effect of agitation condition on the development of pellet size, spore germination, biomass production and nigerloxin production was studied by incubating the inoculated flasks at various agitation conditions on a rotary shaker. Nigerloxin production was evaluated by inoculating the inoculum developed under all temperature ranges.

RESULTS AND DISCUSSION

Nigerloxin (2-amino–3hydroxy-6-methoxy-5-methyl-4-(prop-1'-enyl) benzoic acid) (Figure 1) with a molecular weight of 265 and molecular formula $C_{13}H_{15}NO_5$ was discovered in our laboratory as a potent inhibitor of rat eye lens aldose reductase and lipoxygenase with a free radical scavenging property. The inhibitor is produced only by solid state fermentation and not in submerged

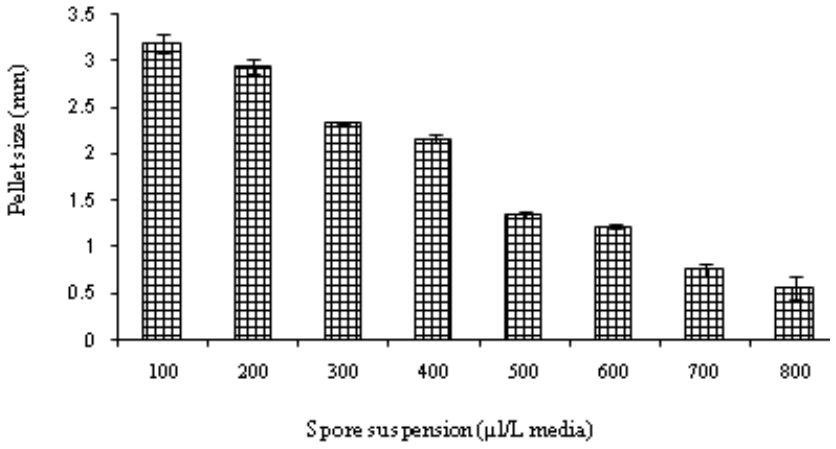

Figure 2a. Effect of spore suspension on pellet size.

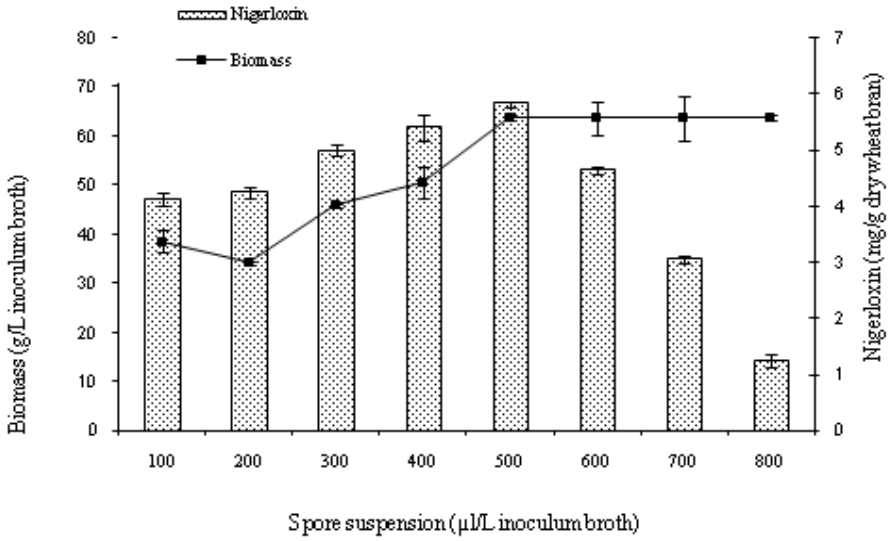

Figure 2b. Effect of spore suspension on biomass of inoculums and nigerloxin production by SSF.

fermentation conditions and the production of the inhibitor is directly related to sporulation of the culture (Chakradhar et al., 2009).

Optimization of physical parameters of inoculum

For these studies, Czapekdox broth with 6 g/L yeast extract was used as the inoculum medium since it showed good production compared to other nutritional media and solid state fermentation using wheat bran medium with 5% trisodium citrate, for the evaluation of nigerloxin production.

Effect of spore suspension

The amount of spores provided to a particular media in submerged fermentation condition has a direct impact on the morphology of pellets developed (Papagianni and Moo-Young, 2002). Increasing spore suspension from 100 µl/L inoculum broth to 800 µl/L inoculum broth showed a steady decrease in pellet size (Figure 2a). Spore suspensions below 400 µl/L media showed pellets sized above 2.1 mm which were not ideal for nigerloxin production in SSF (Figure 2b) perhaps as these yielded a biomass below 50 g/L. Spore suspensions greater than 700 µl/L produced smaller pellets perhaps due to lack of

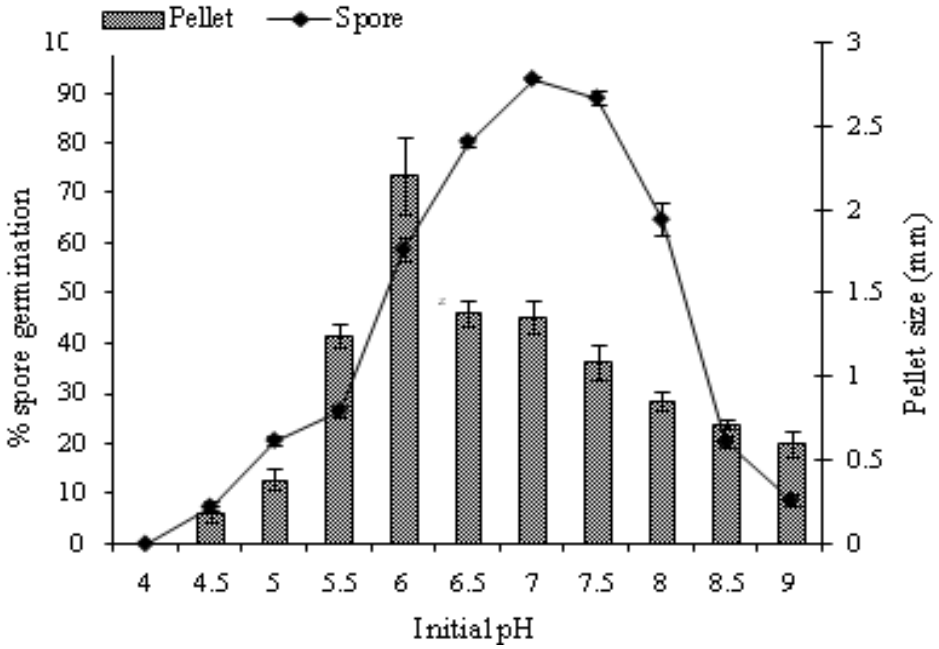

Figure 3a. Effect of initial pH of inoculum development broth on the spore germination, and pellet size.

space and did not translate into more amount of biomass and saw a significant decrease in Nigerloxin production (Figure 2b). In fact, the biomass produced above 500 µl/L, at around 50 g/L, was the same till 800 µl/L, whereas nigerloxin fell below 2 mg/g dry wheat bran. These results are in agreement with that of Van Suijdam et al. (1980) and Calam (1987) where higher concentration of spore suspension failed to produce pellets and suspension form of inoculum was produced. The highest Nigerloxin concentration was produced by using 500 µl/L inoculum broth spore suspension which yielded 5.81±0.04 mg/g dry wheat bran. Hence, 500 µl/L, from the stock of 7 x 10^9 /ml spores concentration was standardized for the rest of the experiments.

Effect of pH

The effect of initial pH of inoculum medium on pellet size, spore germination and biomass production was studied by adjusting the pH of the broth from 2.0 to 9.0 (Figure 3a). It was observed that spore germination was less than 20% below pH 5 and above 8.5 with the highest germination of 92% at pH 7. Further, as pH increased from 4 to 7, there was an increase in pellet size but interestingly, the pellet size over the entire pH spectrum tested was less than 1.5 mm, except for pH 6.0 where it reached 2.4 mm. Production of biomass of 40-53 g/L was seen between pH 5.5 to 7.0 with a complete absence at pH 4 and a slight increase between pH 4.5 to 5. There seems to be a correlation between spore germination and biomass production rather than with pellet size. The phenomenon of pellet formation being strongly influenced by pH is in agreement with the result of Galbraith and Smith and Carlsen et al. (1969, 1995). There was a complete absence of nigerloxin production by SSF when inoculum was developed below pH 5 (Figure 3b). The highest nigerloxin production of 5.7 mg/g dry wheat bran was observed at pH 6.5 after which it fell drastically.

Effect of temperature

Temperature plays an important role in the development of pellets in inoculum medium and spore germination in solid state fermentation (Estrada et al., 2000). It was seen that spore germination below 20 and above 40°C was less than 30% increasing to 91% at 30°C (Figure 4a). A similar pattern was observed with pellet size formation in the range of 1.2 to 1.3 mm between 25 and 35°C and around 0.4 mm at the extreme temperatures tested. Unlike the results seen in initial pH of the medium, biomass produced correlated to both spore germination and pellet sizes. The highest biomass produced was 58 g/L inoculum broth at 30 °C. The absence in nigerloxin production was seen in both extreme ends of temperature and the highest nigerloxin was at 30 °C which produced 5.76±0.07 mg/g dry wheat bran (Figure 4b).

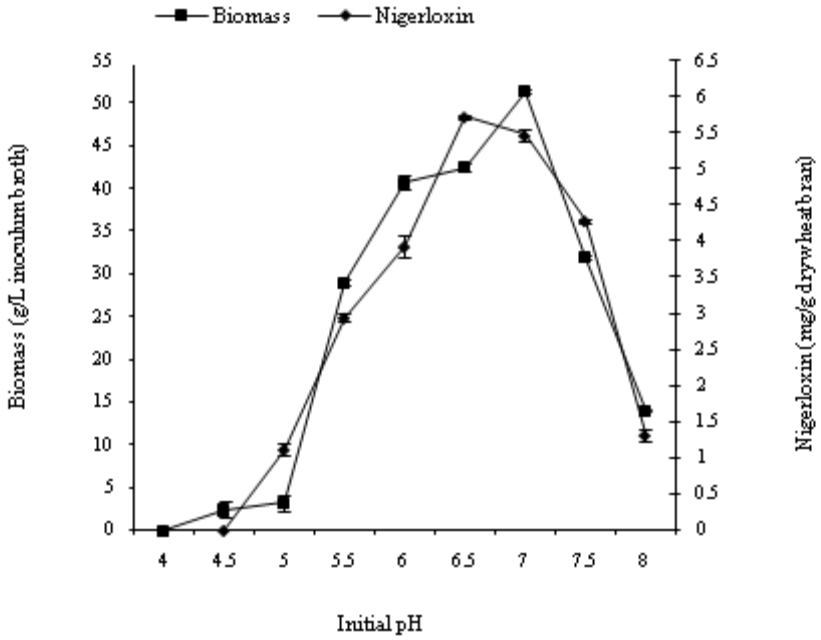

Figure 3b. Effect of initial pH of inoculum development broth on the production of biomass and nigerloxin.

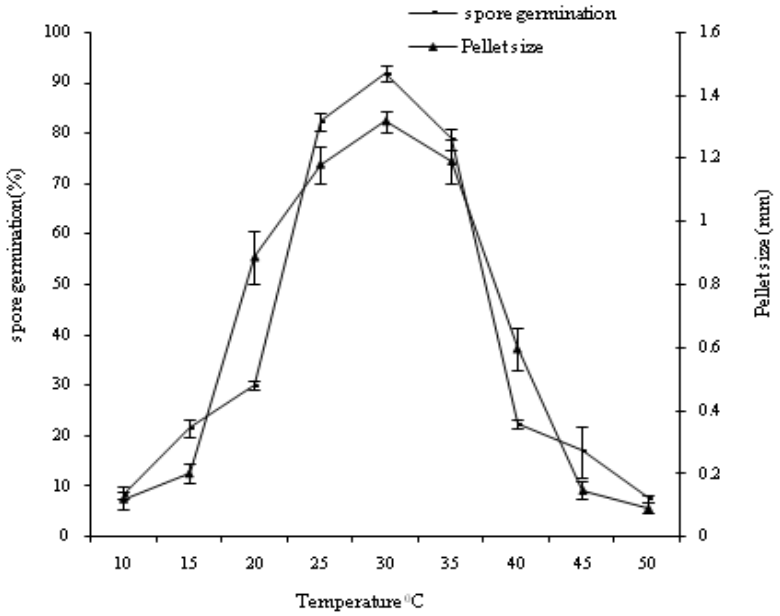

Figure 4a. Effect of incubation temperature of inoculum development broth on spore germination, and pellet size

Effect of agitation

Agitation is the by far the most important physical parameter in inoculum development for SS. Hence the effect of agitation condition on pellet size and spore germination was studied in inoculum broth and nigerloxin production in SSF.

Interestingly, the spore germination remained almost the

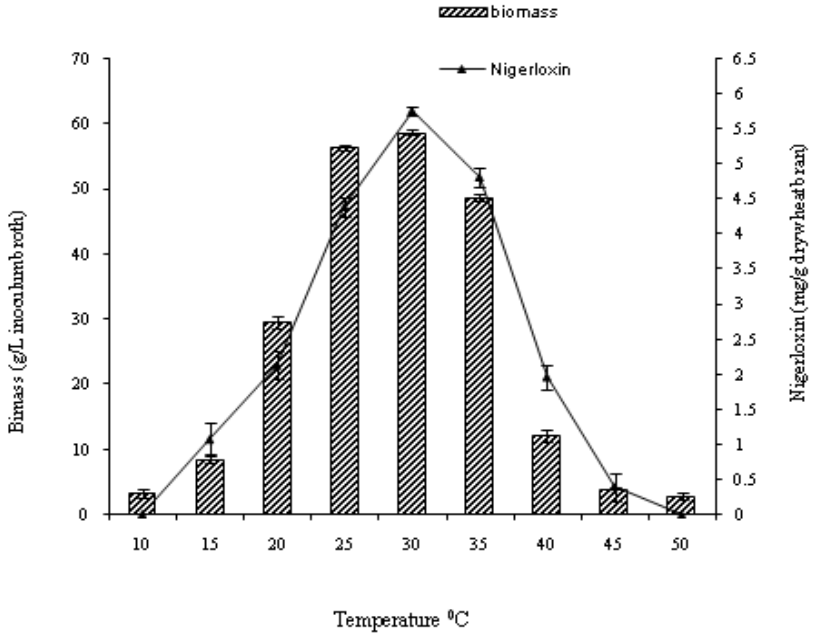

Figure 4b. Effect of incubation temperature of inoculum development broth on biomass and nigerloxin production.

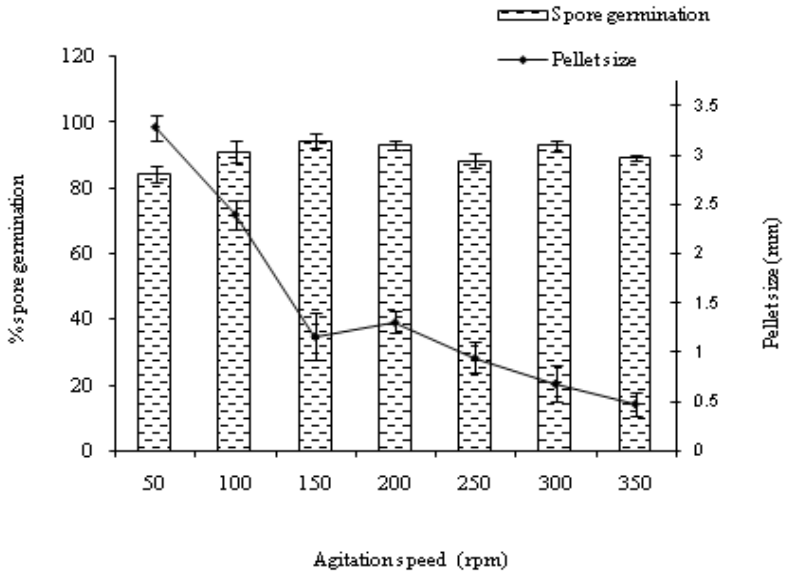

Figure 5a. Effect of agitation condition of inoculum development broth on spore germination, and pellet size

same in all agitation speeds employed with the highest at 93% at 200 rpm, whereas pellet sizes fell steadily from the highest of 3.2 mm at 50 rpm to 0.5 mm at 350 rpm (Figure 5a). Further, biomass produced was more than 40 g/L under all ranges of agitation conditions. The highest production of nigerloxin was observed at 200 rpm which yielded 6 mg nigerloxin/g dry wheat bran (Figure 5b).

The optimized inoculum condition for the better production of nigerloxin was at 500 µl of spore

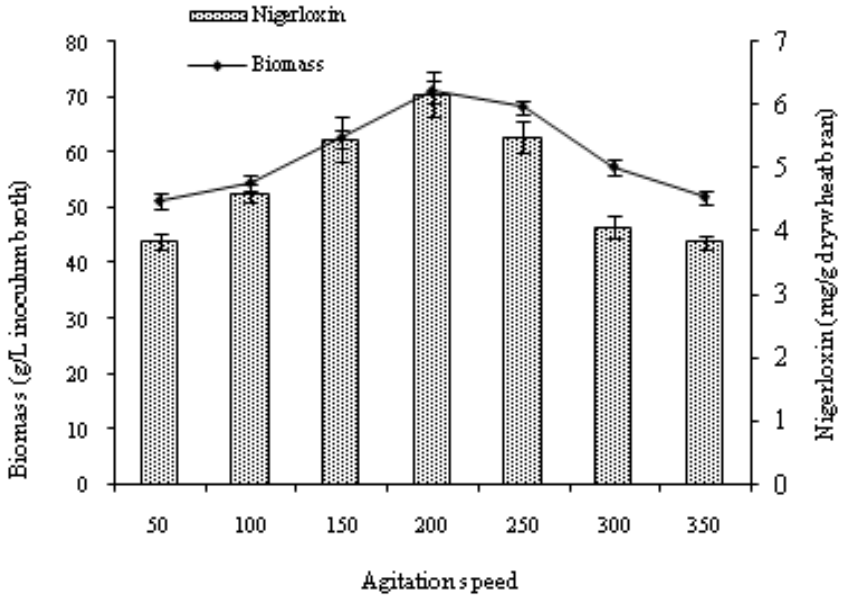

Figure 5b. Effect of agitation condition of inoculum development broth on biomass and nigerloxin production.

suspension in the inoculum development broth at pH 7, incubated at 30 °C and 200 rpm gave an ideal pellet size of 1.23 mm resulting in 6.0 mg of nigerloxin/g dry weight of wheat bran in SSF.

Production of secondary metabolite from micro-organism is a complex task as they require a number of optimal conditions. Inoculum is one of such factors, which if not optimized, may lead to poor or absence of desired product yield. The successful production of a fungal metabolite requires knowledge of the growth characteristics and the physiology of the fungus in question. Thus, for each fermentation, the precise physiological condition and optimised media for inoculum development must be established. The present work was an attempt to understand the type of inoculum conditions required to enhance the production of nigerloxin in solid state fermentation using controlled wheat bran medium by *A. niger* CFTR-W-105. The data generated in this study would be helpful in producing nigerloxin on large scale and has the potential for further developing it into a powerful therapeutic molecule against diabetic complications.

Conflict of Interests

The author(s) have not declared any conflict of interests.

ACKNOWLEDGEMENT

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Full Length Research Paper

Effect of hot-water treatments *in vitro* on conidial germination and mycelial growth of *Colletotrichum musae* isolates

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Anthracnose caused by *Colletotrichum musae* is the most important postharvest disease of banana fruit. The present study was conducted to evaluate the sensitivity of *C. musae* isolates to hot water treatment *in vitro*. The effect of hot water treatment (HWT) *in vitro* was determined by placing conidial suspensions and plugs of agar with mycelia of 12 single spore isolates of *C. musae* in hot water bath at 45, 50 and 55°C for 2, 5, 8, 11, 14 and 17 min. Percent inhibition of conidial germination and colony growth increased with increased temperature and time combinations. HWT at 55°C for 5 min had an equivalent inhibition of conidial germination and reduction of mycelial growth to that of HWT at 50°C for 17 min in the 12 *C. musae* isolates. Remarkable variability in thermo-tolerance was found among 12 isolates of *C. musae* and isolate HC2 and HC3 were found to be the most sensitive, while DL1 and DL2 were comparatively heat tolerant isolates.

Key words: Banana anthracnose, *Colletotrichum musae*, hot water treatment, sensitive, tolerant.

INTRODUCTION

Anthracnose, caused by the fungus *Colletotrichum musae* is one of the most common fungal diseases in banana producing countries (Ranasinghe et al., 2005). Anthracnose is a latent infection where fungal spores infect immature banana in the field but symptoms occur as peel blemishes, as black or brown sunken spots of various sizes on fruit that may bear masses of salmon-colored acervuli with their associated conidia on the fruit peel after ripening (Maqbool, 2010). Chemical methods are widely used for the control of this postharvest disease in banana (De Costa and Kalpage, 2006). However because of the increasing concern about the fungicides, many countries have demanded a fresh product without treatment with any chemicals, particularly fungicides applied after harvest (Maqbool et al., 2010).

Hot water treatment is an effective non-chemical method for management of postharvest banana anthracnose (De Costa and Erabadupitiya, 2005; Li et al., 2006). However, a major practical difficulty in using hot water treatment is to operate at precise temperatures and periods, since the thermal thresholds of pathogens and physiological actions are close to temperatures that can damage the fruits (Arauz, 2000). In addition, there is a lack of information about the *in vitro* effects of hot water

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treatment at different temperature levels and exposure time on *C. musae* isolates.

Thus, there is a need for determining hot water treatment (HWT) *in vitro* that could inactivate the pathogen without detrimental effects to the banana fruit quality. Further, from the result of *in vitro* hot water treatments, information can be extrapolated to develop a possible integrated use of hot water treatments with other postharvest management strategies. Thus, the objective of this research was to evaluate the sensitivity of *C. musae* isolates to hot water treatment *in vitro*.

MATERIALS AND METHODS

Isolation of the pathogen and inoculum preparation

Banana fruits showing anthracnose disease symptoms were collected from local markets in Harar and Dire Dawa, Eastern Ethiopia and were used for isolation of *C. musae*. From each local market, three banana retailers were considered for sample collection, and samples of about three fruits from two different varieties local and cavandish were randomly taken per retailer. Diseased areas of the banana fruits were surface disinfected, cut into small pieces and each piece was sterilized in 1% NaOCl (sodium hypochlorite) for 1 min and subsequently rinsed three times in sterile distilled water. The pieces were then aseptically placed on the surface of potatoes dextrose agar (PDA) medium amended with 40 mg L^{-1} streptomycin sulphate. The petri plates were incubated at 28°C for three days and developing colonies were transferred to fresh PDA medium to obtain pure cultures. Single spore was obtained by dilution method on 2% water agar and cultured on PDA to obtain monoconidial culture. The isolates were transferred to PDA slants and kept at 4°C for preservation and further use.

Hot water treatments versus conidial germination

Conidial suspensions of 12 single spore isolates of *C. musae* were adjusted with sterile distilled water to 10^6 conidia ml^{-1} using a hemacytometer. Screw-cap glass tubes containing 1 ml conidial suspension were placed into a hot water bath and were kept at three constant temperatures, viz., 45, 50 and 55°C. The periods of exposure at each temperature were 2, 5, 8, 11, 14 and 17 min. On removal from the hot water bath, the glass tubes were immediately plunged into a cool water bath to stop the heating process. After HWT, 0.2 ml of each treated conidial suspension was pipetted on 2% water agar and spread with a sterile bent-glass rod. Glass tubes kept into a water bath at room temperature (ca. 22°C) for 17 min served as control. The experiment was laid out in a completely randomized design (CRD) with three replications. After 24 h of incubation, disks of agar were placed on glass microscope slides, germinated conidia were counted out of 100 randomly selected conidia per plate and percent inhibition of conidial germination over the control was calculated as 100 x (*gc*-*gt*/*gc*); where, g*c*=number of germinated conidia in control and g*t* = number of germinated conidia in the treatment.

Hot water treatments versus mycelial growth

Mycelial discs, 6 mm diameter and taken from the edge of 10 days culture of 12 *C. musae* isolates, were dipped into screw-cap test tubes at three constant temperatures, viz. 45, 50 and 55°C. The duration of exposure at each temperature was 2, 5, 8, 11, 14 and 17 min. On removal from the hot water bath, the glass tubes were immediately plunged into a cool water bath. After HWT, agar plugs were removed from heated glass tubes and blotted briefly, agar side down, on sterile filter paper. Treated plugs were placed in the centre of PDA plates and incubated at 28°C. Glass tubes kept in a water bath at room temperature (ca. 22°C) for 17 min served as control. The experiment was laid out in a completely randomized design (CRD) with three replications. Four days after incubation, colony diameter was measured along two axes perpendicular to each other and the average of the two dimensions was recorded as the radial colony diameter. The percent inhibition of mycelial growth over the control was calculated as: 100 x (*dc*-*dt*/*dc*); where, d*c* = colony diameter of control and d*t* = colony diameter of heat treatment.

Statistical data analysis

Percentage data on conidial germination inhibition and mycelial growth inhibition were arcsine transformed before statistical analysis. The data were subjected to analysis of variance (ANOVA) using SAS software version 9.2 (SAS Institute, 2002). Probit analysis was used to calculate the effective time (ET) values that inhibited conidial germination by 50% (ET_{50}) and 95% (ET_{95}). Means were separated using Duncan's multiple range test (DMRT) at ($P<0.05$).

RESULTS AND DISCUSSION

Effect of hot water treatments on conidial germination

Hot water at 55°C for 5 to 17 min durations of exposure resulted in the highest inhibition of conidial germination of 11 *C. musae* isolates (except isolate DL1) (Table 1). In addition, hot water treatment at 50°C for 8 to 15 min for isolate HC1, HC2, HC3, DC3 and HL2, 11 to 17 min for isolate DC2 and DL3, 14 min for isolate DC1, and 17 min for isolate DL2 exhibited the highest inhibition of conidial germination which was on par with the treatments involving 55°C for 5 to 17 min. HWT at 55°C for shorter exposure time (2 min) showed significantly higher inhibition of spore germination than HWT at 45°C, including the longer exposure time, 17 min, for all isolates (Table 1). Moreover, HWT at 55°C for 5 min had an equivalent conidial germination inhibition effect to that of HWT at 55°C for 17 min on the 12 *C. musae* isolates. In this study, conidial germination of isolates DL1 and DL2 was less inhibited (<10%) at 50°C for 5 min, than HC2 and HC3 which were highly inhibited (95.4 and 93.8%, respectively). Moreover, HWT at 45°C for all periods of exposure was less effective in inhibiting conidial germination (<30%) in all isolates tested than HWT at either 50 or 55°C.

Among the twelve *C. musae* isolates, HC2 and HC3 were comparatively the most sensitive to HWT at all levels of experimental temperatures for all periods of exposure (Table 2). Higher inhibition of conidial germination of 90.7 and 95.4% was recorded on isolate HC2 at 50°C for 2 and 5 min, respectively. The time to

Table 1. Effect of temperature and exposure period on inhibition of conidial germination of *Colletotrichum musae* isolates.

Temperature (°C)	Time (min)	Inhibition of conidial germination (%)[1]											
		Isolate											
		HC1	HC2	HC3	DC1	DC2	DC3	HL1	HL2	HL3	DL1	DL2	DL3
45	2	7.2^{c}	7.8^{d}	12.8^{e}	2.5^{fg}	0.9^{ef}	6.6^{e}	1.6^{i}	0.3^{d}	0.3^{hi}	0^{h}	0^{f}	0^{i}
	5	8.0^{c}	9.7^{d}	17.2^{de}	4.0^{fg}	0^{f}	10.9^{de}	4.7^{h}	0.9^{d}	0.4^{hi}	0^{h}	0^{f}	7.8^{h}
	8	8.0^{c}	10.6^{d}	24.8^{de}	4.8^{efg}	0.2^{f}	10.5^{de}	5.6^{h}	1.7^{d}	0.3^{hi}	1.1^{gh}	0^{f}	8.5^{h}
	11	10.8^{c}	11.1^{d}	22.4^{de}	6.6^{efg}	0.3^{ef}	10.5^{de}	5.3^{h}	1.4^{d}	1.2^{h}	4.4^{efg}	0^{f}	11.9^{gh}
	14	15.0^{c}	15.2^{cd}	27.3^{de}	7.9^{ef}	2.9^{de}	12.3^{c}	5.8^{h}	1.7^{d}	3.5^{g}	0.8^{gh}	0^{f}	14.8^{fg}
	17	19.2^{c}	22.9^{c}	29.6^{d}	14.6^{de}	2.7^{def}	26.0^{c}	6.7^{h}	2.6^{d}	10.0^{f}	10.1^{ef}	6.1^{e}	20.3^{ef}
50	2	13.4^{c}	90.7^{b}	64.9^{c}	23.8^{d}	3.4^{d}	28.8^{cd}	20.3^{g}	13.0^{c}	1.7^{h}	0.2^{h}	5.3^{e}	21.9^{e}
	5	88.0^{b}	95.4^{b}	93.8^{b}	66.0^{c}	38.6^{c}	57.5^{b}	28.6^{f}	15.4^{c}	17.9^{e}	2.1^{fgh}	7.6^{e}	45.0^{d}
	8	98.2^{a}	100^{a}	97.1^{ab}	67.4^{c}	82.6^{b}	98.8^{a}	66.5^{f}	99.1^{a}	36.5^{d}	8.9^{e}	32.0^{d}	92.6^{b}
	11	100^{a}	100^{a}	100^{a}	79.4^{bc}	98.3^{a}	100^{a}	94.9^{c}	100^{a}	98.2^{b}	23.9^{d}	33.2^{d}	99.5^{a}
	14	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	96.7^{b}	100^{a}	98.2^{b}	26.1^{d}	94.3^{b}	100^{a}
	17	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	98.7^{b}	74.2^{c}	98.7^{ab}	100^{a}
55	2	77.5^{b}	100^{a}	97.4^{ab}	91.1^{b}	40.4^{c}	98.7^{a}	45.9^{e}	58.9^{b}	63.3^{c}	27.2^{d}	59.8^{c}	86.4^{c}
	5	100^{a}	100^{a}	100^{a}	99.5 ^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	92.7^{b}	98.4^{ab}	100^{a}
	8	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	98.6^{a}	99.6^{ab}	100^{a}
	11	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}
	14	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}
	17	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}
Unheated control		0^{d}	0^{e}	0^{f}	0^{g}	0^{f}	0^{f}	0j	0^{d}	0^{i}	0^{h}	0^{f}	0^{i}
	CV (%)	10.14	7.43	9.04	13.05	7.70	7.41	5.14	12.24	5.28	13.84	13.01	5.28

[1]Percent inhibition of conidial germination after 24 h incubation (mean of three replications); Means within a column followed by the same letter (s) are not significantly different (P<0.05) DMRT; HC = Cavandish at Harar, DC = Cavandish at Dire Dawa, HL= Local at Harar, DL = Local at Dire Dawa.

inhibit spore germination by 50% (ET_{50}) and 95% (ET_{95}) of this isolate at 50°C were much shorter (0.67 min) and shorter (3 min), respectively. In addition, it was totally inhibited at 55°C for short exposure time (2 min). The other sensitive isolate was HC3, with shorter ET_{50} and ET_{95} value at 50°C (1.66 and 5.19 min, respectively). On the other hand, isolate DL1 was comparatively the most tolerant isolate to HWT as the ET_{50} and ET_{95} at 50°C was longer (16.67 and 39.9 min, respectively) than the remaining isolates (Table 2). The other comparatively tolerant isolate was DL2, with longer ET_{50} and ET_{95} value at 50°C (9.98 and 23.1 min, respectively).

Previously, Chen et al. (2006) investigated the inhibition effects of HWT on the spores of *C. musae in vitro*. In this study, HWT at 52°C for 3 min did not make significant difference, while 5 and 10 min significantly reduced the conidial germination. The effects of heat treatments on the same genus *Colletotrichum* was reported by Sopee and Sangchote (2005) that conidial germination of *C. gloeosporioides* was inhibited by application of hot water at 55°C for 5 min. In trails with hot water treatment for another genus, *Fusarium*, the control of *F. oxysporum* required higher temperatures to completely inhibit conidial germination, that is, 55°C for 25 min and 60 or 65° for durations of 5, 15 or 25 min (Sharma and Tripathi, 2008). Although differential response to hot water treatment *in vitro* between *C. musae* isolates had not been reported yet, notable variability in thermo-tolerance was found among conidia of 16 isolates of the insect-pathogenic fungi *Metarhizium anisopliae* var. *anisopliae* (Drauzio et al., 2005). This is due to the fact that heat stress affects different cell properties and requires different repair mechanisms (Drauzio et al., 2005).

Effect of hot water treatments on mycelial growth

The highest mycelial growth inhibition in all isolates was recorded with the treatment combination of 55°C for 17 min (Table 3). At this temperature and exposure time regime, mycelial growth of isolate HC2 was completely inhibited (100%), while DL1 and DL2 were slightly inhibited to 26.7 and 29.5%, respectively. In this study, HWT at 55°C for 5 min had an equivalent effect to 50°C for 17 min in inhibiting mycelial growth of the 12 *C. musae* isolates. In all isolates, the inhibition of mycelial growth increased with increased temperature and time in the hot water bath; however, it is worthwhile to note that

Table 2. The ET_{50} and ET_{95} value of *in vitro* effect of hot water treatment at 50°C for inhibition of conidial germination of *Colletotrichum musae* isolates.

Isolate	ET_{50}	95% CI* for ET_{50}	ET_{95}	95% CI* for ET_{95}
HC1	3.12	2.95-3.29	6.32	5.86-6.90
HC2	0.67	0.38-0.94	3.00	2.56-3.59
HC3	1.66	1.48-1.83	5.19	4.64-5.96
DC1	4.00	3.67- 4.29	18.67	16.88-21.46
DC2	5.39	5.41-5.61	10.43	9.81-11.22
DC3	3.61	3.40-3.83	8.94	8.27-9.77
HL1	5.21	4.90-5.51	16.88	15.40-18.78
HL2	5.24	5.01-5.46	10.31	9.69-11.10
HL3	7.53	7.27-7.79	13.95	13.19-14.89
DL1	16.67	15.97-17.47	39.99	35.61-46.23
DL2	9.98	9.61-10.35	23.10	21.39-25.30
DL3	3.96	3.72-4.20	10.93	10.08-12.01

*CI-confidence interval.

Table 3. Effect of temperature and exposure time on mycelial growth of *Colletotrichum musae.*

Temperature (°C)	Time (min)	Inhibition of mycelial growth (%)[1]											
		Isolates											
		HC1	HC2	HC3	DC1	DC2	DC3	HL1	HL2	HL3	DL1	DL2	DL3
45	2	0.2^{k}	1.8m	13.6^{i}	2.5^{i}	0l	2.1^{j}	0^{j}	10.6^{h}	1.2^{i}	0^{f}	0^{k}	1.7^{j}
	5	9.7^{j}	3.6l	13.2^{i}	5.9^{h}	2.9^{k}	1.5^{ij}	0^{j}	11.5^{gh}	3.7^{h}	0^{f}	0^{k}	9.3^{i}
	8	9.8^{j}	7.1^{k}	19.5^{h}	5.9^{h}	4.8^{j}	4.2^{h}	1.3^{i}	17.6^{fg}	3.7^{h}	0^{f}	3.8^{j}	10.5^{h}
	11	12.0^{j}	11.1^{j}	21.6^{h}	14.5^{g}	11.3^{i}	9.5^{g}	3.2^{h}	20.0^{f}	12.3^{g}	2.0^{ef}	5.3^{ij}	17.0^{g}
	14	21.4^{gh}	10.1^{jk}	20.8^{h}	17.9^{fg}	12.3^{i}	10.1^{g}	6.4^{g}	21.2^{f}	12.8^{g}	3.3^{cd}	7.6^{hi}	18.7^{g}
	17	22.7^{fg}	16.7^{i}	27.6^{g}	20.1^{efg}	16.8^{h}	10.7^{g}	6.4^{g}	24.5^{ef}	19.8^{f}	6.0^{c}	7.6^{hi}	21.2^{fg}
50	2	13.3^{ij}	26.5^{h}	20.7^{h}	21.0^{ef}	22.4^{g}	3.6^{hi}	6.4^{g}	21.2^{f}	8.8^{g}	2.3^{de}	6.8^{hij}	9.3^{hi}
	5	16.8^{hi}	37.8^{fg}	29.9^{g}	26.2^{de}	30.9^{ef}	13.7^{f}	20.5^{f}	25.5^{ef}	20.4^{ef}	16.7^{b}	10.6^{gh}	21.3^{fg}
	8	26.4^{efg}	36.2^{fg}	35.1^{f}	27.5^{de}	30.6^{ef}	17.9^{ef}	33.3^{e}	23.6^{ef}	22.2^{ef}	18.0^{ab}	15.2^{fg}	24.7^{ef}
	11	28.2^{def}	42.6^{ef}	45.4^{e}	27.2^{de}	33.8^{de}	19.0^{def}	37.8^{de}	33.3^{de}	23.1^{ef}	19.3^{ab}	17.4^{ef}	26.7^{de}
	14	29.0^{c-f}	47.3^{e}	51.7^{d}	30.7^{cd}	38.0^{d}	22.6^{cde}	39.7^{de}	33.3^{de}	24.7^{def}	19.3^{ab}	18.9^{def}	29.7^{cde}
	17	31.1^{b-e}	60.7^{d}	55.2^{cd}	37.7^{abc}	48.8^{bc}	28.6^{bc}	43.6^{d}	36.4^{cd}	32.9^{cd}	19.7^{ab}	19.7^{c-f}	33.3^{bc}
55	2	25.9^{efg}	34.5^{g}	38.0^{f}	22.5^{ef}	27.3^{f}	16.1^{f}	41.0^{d}	31.5^{de}	26.9^{def}	16.7^{b}	15.9^{ef}	26.7^{de}
	5	27.1^{d-g}	55.4^{d}	51.8^{d}	34.1^{bcd}	43.9^{c}	23.2^{cde}	53.8^{c}	36.4^{cd}	27.8^{def}	17.3^{b}	22.0^{b-e}	31.3^{bcd}
	8	33.7^{bcd}	73.2^{c}	52.4^{d}	39.5^{ab}	46.9^{c}	24.4^{cd}	57.7^{bc}	40.0^{cd}	28.2^{de}	19.3^{ab}	25.0^{a-d}	34.0^{abc}
	11	35.2^{bc}	82.1^{b}	57.4^{c}	41.4^{ab}	48.8^{bc}	27.4^{bc}	57.7^{bc}	46.7^{bc}	37.5^{bc}	19.3^{ab}	25.8^{abc}	35.7^{ab}
	14	36.3^{ab}	100^{a}	62.7^{b}	44.1^{a}	52.6^{ab}	32.4^{ab}	61.5^{ab}	55.2^{ab}	45.7^{b}	21.7^{ab}	27.3^{ab}	36.3^{ab}
	17	42.2^{a}	100^{a}	70.1^{a}	45.1^{a}	55.9^{a}	37.5^{a}	66.7^{a}	63.0^{a}	58.0^{a}	26.7^{a}	29.5^{a}	39.3^{a}
Unheated control		0^{k}	0^{n}	0^{j}	0^{j}	0^{l}	0^{k}	0^{j}	0^{i}	0^{i}	0^{f}	0^{k}	0^{k}
CV (%)		8.59	6.13	4.67	9.73	5.53	11.31	8.08	11.17	11.58	17.14	12.35	7.03

[1]Percent inhibition of mycelial growth after four days of incubation (mean of three replications); means within a column followed by the same letter (s) are not significantly different (P<0.05), DMRT; HC = Cavandish at Harar, DC = Cavandish at Dire Dawa, HL = Local at Harar, DL = Local at Dire Dawa.

all of the isolates (except isolate HC2) used in the present study were not totally sensitive (that is, 100% colony growth inhibition). Hot water treatment at 45 ^{o}C for all exposure time, including 17 min, was less effective in inhibiting the mycelia growth of all isolates than HWT at 50 and 55°C (Table 3). Among the 12 *C. musae* isolates, HC2 and HC3 were the most sensitive isolates to HWT as they exhibited higher mycelial growth inhibition (>55%)

at 50°C for 17 min, while mycelial growth inhibition of the remaining isolates was less than 50% (Table 3). In addition, isolate HC2 was completely inhibited (100%) at the higher temperature-time combination (55°C for 17 min). On the other hand, isolates DL1 and DL2 were found to be quite tolerant to higher temperature-time combinations, as 50 and 55°C for 17 min inhibited mycelial growth of both isolates to less than 30 and 27%, respectively.

In this investigation, conidial suspensions were generally more sensitive to HWT than mycelia as the highest temperature-time combination (55°C for 17 min) did not completely inhibit the mycelial growth of all isolates except HC2, while 11 min at 55°C was enough to completely kill conidia of all *C. musae* isolates (Table 3). This observation agrees with the finding of Whiting et al. (2001) that hot water treatments were ineffective in reducing the mycelial growth of grapevine trunk pathogens. Gramaje et al. (2010) reported that conidial germination of *Phaeoacremonium* spp. was inhibited at treatments above 53°C for 45 min, while treatments up to 54°C for 60 min were necessary to inhibit the mycelial growth. Neri et al. (2009) also reported that mycelial growth of *Neofabraea alba* was completely inactivated only at higher temperature-time regime of 75°C for 20 min.

Conclusions

Percent inhibition of conidial germination and reduction in colony growth increased with increased temperature and time combinations. Among the level of hot water temperature tested *in vitro*, hot water at 55°C for 5 to 17 min durations of exposure was effective and completely inhibited conidial germination all *C. musae* isolates (except isolate DL1). However, hot water treatment levels tested did not completely inhibit mycelial growth of all *C. musae* isolates (except isolate HC2). Thus, in the present investigation, hot water treatment was less effective in reducing the mycelial growth than the conidial germination. HWT at 55°C for 5 min had an equivalent inhibition of conidial germination and reduction of mycelial growth to that of HWT at 50°C for longer exposure time (17 min) in the 12 *C. musae* isolates. Hot water treatment at 45°C for all periods of exposure was ineffective in inhibiting spore germination against all *C. musae* isolates. In this study, marked variability in thermo tolerance was also observed among 12 isolates of *C. musae* and isolate HC2 and HC3 were found to be sensitive while DL1 and DL2 were comparatively heat tolerant isolates.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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