Evaluation and Optimization of Agro-industrial Wastes for Conidial Production of *Metarhizium anisopliae* isolates under Solid State Fermentation

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ABSTRACT

Metarhizium anisopliae is known to cause high level of epizootics for more than 200 insect species in versatile agro-ecologies. Concerns on environmental pollution and resistance development to chemical insecticides need environmentally safe and economically viable approaches. Therefore, here we investigate a cheap and large scale industrial production of virulent enthomopathogenes on agricultural wastes. Three *Metarhizium anisopliae* isolates were grown on agricultural wastes to evaluate their conidia production potential under Solid state fermentation (SSF) technique. Coffee husk, tea waste, wheat bran and vegetable wastes were used as substrates to determine their capability for maximum conidiation of the isolates. Among these, vegetable wastes were the best media to yield 5.80 ±0.72 (10^7), 4.44±0.55 (10^7) and 5.58±0.66 (10^7) conidia/gram of substrate under quantitative assessment for isolate AUMI1, AUMI2 and AUMI3 respectively, at 60% moisture content. Statistically on two sample t-test vegetable wastes shows significant difference in conidia production when compared to 2 mm and 4 mm sized coffee husk used as substrates. The optimization for temperature indicated that all substrates supported their maximum conidia yield within $27 - 30^0$C range of temperature. The 3.5 pH value used in the present study for optimization was best favored only for coffee husk as substrate. The high conidia yielding substrates were best productive at pH 6.29, 6.63 and 5.4 for vegetable wastes, wheat bran and tea waste, respectively. All isolates incubated on wheat bran was highly productive under sufficient exposure to light. AUMI1 produced high conidia under exposure to light while the higher yield of AUMI2 and AUMI3 was produced under dark condition on vegetable wastes. Therefore, as successful microbial control of insect pests depends on large scale and cheap industrial productivity, cultivation on vegetable wastes and wheat bran under SSF can be a plausible solution.

Keywords: *Metarhizium anisopliae*, Inoculation, Optimization, Substrate, Conidia.

1. INTRODUCTION

The origins of microbial pest control date back to the early 19th century, when the Italian scientist Agostino Bassi studied white muscardine disease on silkworms (*Bombyxmori*). He identified *Beauveria bassiana* as the cause of the disease (Yadav and Neeraj, 2012; Chinnadurai and Ganesh, 2013). His discovery was a major milestone in microbial pest control technology which triggers the use of entomopathogenic fungi, such as *Metarhizium anisopliae* as highly active insect pest under laboratory conditions (Chinnadurai and Ganesh, 2013). The first attempt to control pest with a fungal agent was carried out in Russia in 1888, when the fungus now known

as *M. anisopliae* was mass produced on beer mash and sprayed in the field for control of the beetle weevil *Cleonus Punctiventris* (Lord, 2005).

In the last years entomopathogenic fungi biology and ecology have been studied more and the attention of scientists was focused mainly on the fungi genus *Beauveria* and *Metarhizium* since they attack broad spectrum of insects (Mudrončeková et al., 2013). They are often reported as causing high levels of epizootics in nature and are the most versatile and eco-friendly biological control agents. These fungi contain a heterogeneous group of over 100 genera with approximately 750 species, notified from different insects and many of them are proved to be highly potent to insect pests. Among these *Metarhizium spp., Beauveria spp., Nomuraearileyi, Verticillium lecanii* and *Hirsutellaspp* are highly known (Jitendra et al., 2012).

M. anisopliae is the second most widely exploited entomopathogenic fungi in bio-control trials next to *B. bassiana* (Jitendra et al., 2012) and it is known to attack more than 200 inset species belonging to different orders (Peng et al., 2009; Jitendra et al., 2012; Chen et al., 2014). This has been responsible for a substantial increase in interest for large scale production of good quality inoculums of the fungus for field application sake of the bio control program.

Conidial Biomass production from Agricultural wastes is inexpensive. The annually produced wastes from worldwide Food, Agricultural and Forestry industries are causes of serious environmental, health and disposal management problems (Orzua et al., 2009; Zuriash Mamo and Tesfaye Alemu, 2012). Wastes like sugarcane, fruit bagsse and peels generated in the beverages and juice industries, coffee pulp obtained from coffee industries, sawdust from wood industries and husks from cereals are abundant. Therefore, utilization of these wastes for large scale mycoinsecticide conidia production would be economically viable despite of its role in controlling environmental pollution.

Different studies have been conducted so far, for mass production of conidia using agricultural wastes under solid state and submerged fermentation. Wheat straw, tea waste, vegetable waste, coffee husk and barley bran has been evaluated to determine optimal conditions for conidia production of *Trichoderma* (Zuriash Mamo and Tesfaye Alemu, 2012). Sugarcane bagasse, orange peel, wheat straw, cotton seeds, castor been and rice husk were used alone as carbon source for the production of single cell protein (Khan and Dahot, 2010) Kitchen wastes are used for bio pesticide production under solid state fermentation (Zhang et al., 2013).

According to Wu et al. (2010), different isolates germination, spore production and virulence is associated with the appropriate concentrations of nitrogen and carbon in the culture medium. Nutritional requirements of the entomopathogenic fungi could vary with the fungal species and even the fungal strain under consideration. Generally a fungus requires oxygen, water, source of carbon, and organic or inorganic nitrogen besides minerals that play a major role in growth, pathogenicity and novel metabolite production. Therefore, successful microbial insect pest control also depends on easy and cheap mass production of the virulent microbial agents in laboratory and in industry as well (Sahayaraj and Namasivayam, 2008).

Production of locally isolated entomopathogenic fungi under suitable media for large scale application has not yet been studied. Therefore, in the present study isolates of *M. anisopliae* are screened for mass production of conidia under laboratory scale solid state fermentation using different substrates.

2. MATERIAL AND METHODS

2.1. Source of *Metarhizium* Isolates

Three *M. anisopliae* isolates were obtained from Ababa University (AAU), Department of Microbial, Cellular and Molecular Biology; Mycology Laboratory Culture collection. All *Metarhizium* stains used in this study were isolated from insect cadavers of grasshopper, beetles and locusts from soil samples of south western Ethiopia. The acronyms given to the isolates were derived from initial letters of the university's name and the genus name of the isolate followed by the letter "I" to represent isolate and number to differentiate which isolate number is it.

For laboratory study the fungal isolates was first grown on Sabouraud dextrose agar (SDA) medium. The medium was sterilized at 121^0C for 15 minutes in autoclave, poured to sterilized plates in a hood, cooled and inoculated with pure culture of the fungal isolates on their respective plates under aseptic conditions. Followed the inoculation plates were allowed to incubate at 27^0C for 14 days (Holder and Keyhani, 2005; Masoud et al., 2013). After complete growth the fungal cultures were kept in refrigeration temperature (4^0C) for further study.

2.2. Preparation of conidia suspension:-

Conidia inoculum for solid state media culture were obtained from 14 days old sporulated culture on Sabouraud dextrose agar (SDA) plates at 27^0C. Conidia were harvested using sterile 0.02% (v/v) Tween 80 solution by flooding the plate and scrapping the spores with hockey glass stick to

remove the spores from the mycelial mat of the culture. The suspended spores of *Metarhizium* were collected in to their respective conical flasks and filtered through three layers of cheese cloth. Conidial concentrations were determined by direct counting using Heamocytometer under light microscopy (Wang and Powell, 2002).

2.3. Procurement of agricultural wastes for conidia production

The agricultural wastes used in this study were collected from different areas in Addis Ababa City administration and from college students' cafeteria. All the wastes were initially clean to remove debris and washed with water followed by drying under shade condition. Subsequently, all the wastes were ground in to powder using a Hammer beater mill (Muhammad et al., 2012). The powdered form of each waste was stored in plastic bags for subsequent study.

2.4. Solid state fermentation using agricultural waste substrates for conidia production

Ten gram (10g) of agricultural waste powder was weighed for a single inoculum experiment and autoclaved at 121^0C for 15 minutes in a heat resistant plastic bag (Pham et al., 2010). After sterilization each substrate was transferred in to aseptically sterilized 20x30cm^2 sized plastic bags under microbiologically safe techniques and 1ml of seeding inoculum with conidial concentration of $1.0x10^5$ onidia/ml was sprayed using hand sprayer. The samples were incubated in light transparent incubator at temperature 27^0C for two weeks period of time. Within this form of solid state fermentation technique all high conidial biomass producing wastes were evaluated to optimize conditions such as temperature, pH, moisture content, incubation period, inoculum concentration, inoculum size and the effect of light for maximum conidial productivity. All experiment in this research finding was three times replicated.

2.5. Conidia harvesting from the substrates medium

From each 14 days old conidiated culture 1g was weighed and suspended in 20ml of 0.05% Tween 80 solution containing beaker. To dissociate conidia clamps the beaker was vigorously agitated for 3-5 minutes by hand. The suspensions were then filtered through three layered cheese close and conidia concentration was evaluated by counting under Heamocytometer. Result of the count was calculated arithmetically to know the total spore count in 1g of substrate.

2.6. Conidia Counting

Estimation of the amount of conidia suspension in stock concentration containing bottle was carried out using a standard formula. Serial dilution were prepared from the original suspension by drawing 1ml of spore concentrated solution from the original suspension and transfer it to 9ml

distilled water containing test tube to dilute the spore concentration by a factor of 10. The process was repeated many times as necessary to achieve countable number of spores over the grids. Using a micro pipette 20 microliters of spore suspension were sucked and loaded to a clean cover slip affixed Heamocytometer. Chambers of the Heamocytometer were allowed to fill via capillary action carefully not to overfill or under fill it. There are four big squares in the Heamocytometer with 16 smaller squares in each, all the cells in the four corner squares of each big square were counted by placing the Heamocytometer chamber on microscope stage. Numbers of conidia count on the squares of the grids were recorded. Cell concentration per milliliter was calculated using standard formula. Stock cell concentration per milliliter and conidia concentration per gram of substrate were arithmetically calculated respectively.

2.7. Spore Viability

The viability of conidia was determined by spread plating of 1ml of conidial suspension (titrated to 1×10^3 conidia/ ml) on Sabouraud dextrose agar. Spore was inoculated on eight centimeter (8cm) in diameter plates and examined after 12-18 hours. Spore germination was held by placing 3- separate drops of lactophenol cotton blue, sterile microscopic cover slips were placed over the stained droplets for examination. The proportion of viable conidia was determined by examining 100 spores in each of the three different fields of view at 400X magnification with a compound microscope. Proportion of spores that possessed a distinct germ tube, as defined by germ tube lengths that are two times the diameter of the spore are viable as stated on (Philip et al., 2011 and Fatima et al., 2013).

2.8. Optimization of Moisture Content for Conidia Production

Moisture content of the agricultural wastes was determined by oven drying method as stated on (Rao et al., 2006). Labeled small glass bottles were placed in an oven at approximately 80^0C for about two hours to ensure that they are completely dry. The bottles weighed and recorded after allowed cooling at room temperature with cover lids put on. Ten (10g) gram of substrate from each agricultural waste were added to their respective glass bottles and make a note of the new weight for wet weight of the substrates by replacing the cover lids.

The container bottles with substrates were placed back in to the oven at 60^0C for one to six hours until constant weight of the dry weight is found. Finally moisture content of the substrates was calculated based on wet-weight basis and express it as a percentage to one decimal place, using the following formula (Rao et al., 2006).

$$\text{Moisture content (\%wb)} = \frac{wetweight - dryweight}{wetweight} * 100$$

Based on the formula moisture content of each agricultural waste was adjusted to 35%, 45%, 60% and 80% respectively. All the determined moisture levels were evaluated for maximum conidial production capability of *M.anisopliae* isolates under solid state fermentation (SSF).

2.9. Optimization of Temperature for Conidia Production

Metarhizium isolate inoculated agricultural waste Medias prepared to incubate at 24^0C, 27^0C and 30^0C at high productive moisture level with respect to isolate type. Each of the substrate containing plastic bags were sprayed with 1ml of $1x10^5$ conidia concentration and tie up with rubber band to prevent air blow and contamination in the incubator. Aeration was allowed to the culture via two 1cm sized holes provided at the top of the plastic bag. The culture was incubated for 14 days before spore sample were taken to numerate under heamocytometer (Pham et al., 2010).

2.10. Optimization of PH for Conidia Production

Ten (10g) gram of substrates from each agricultural waste was weighed to examine under different pH levels. The pH was adjusted to 3.5, 4.5 and 5.5 using 1N of HCl (hydrochloric acid) and NaOH (sodium hydroxide). The media was autoclaved at 121^0C for 15 min and transferred to their respective plastic bags under aseptically safe condition. Substrates are allowed to cool and 1ml of ($1x10^5$) conidia concentration of each isolate was sprayed to their respective plastic bags followed by incubation at 27^0C for 14 days. The conidia yield was determined by heamocytometer count (Zuriash Mamo and Tesfaye Alemu, 2012).

2.11. Optimization of Inoculum Concentration

The sterilized substrates in the plastic bags were adjusted at the best moisture level. Conidial concentrations of $1x10^3$, $1x10^4$ and $1x10^5$ were prepared in different flasks from stock concentration of each isolate. The formula used for conidial concentration preparation was as indicated in insect pathology manual.

$$x = \frac{\text{Required concentration} * \text{Final volume Neeed}}{\text{Counted concentration}}$$

Where, X= number of ml of spores from original suspension to be added to the distilled water. The prepared conidial concentrations were sprayed into their respective substrates arranged. Each of the waste substrates was evaluated by all the prepared concentration levels with 1ml of

inoculum size and incubated for 14 days. Conidial harvest and numeration were conducted by taking 1g of conidiated substrate.

2.12. Effect of light on conidia production

10g of agricultural waste substrates for each plastic bag were autoclaved at 121^0C for 15 min. sterilized substrates inoculated with 1ml of (1×10^5 conidia/ ml) were incubated under opaque and light transparent plastic bags both with aeration pores at the top. The cultures were incubated for 14 days. Finally, 1g of conidiated substrate was suspended, filtered and numerated by heamocytometer.

2.13. Data analysis

All the data were statistically analyzed using SPSS (version 20). Substrates potential to support growth of *M. anisopliae* isolates were statistically compared using Analysis of Variance (ANOVA). Conidia production results of each substrate with respect to isolate type were expressed as Mean ± Standard Error of Mean (SEM). Mean difference of two isolates conidia yield concentration on different substrates were computed using two sample t-Test. For all experiments, a probability level of $p \leq 0.05$ was considered as statistically significant.

3. RESULTS

3.1. Evaluation of agricultural wastes for conidia production

Substrates such as three different sizes of coffee husk, tea waste, wheat bran and vegetable wastes were used to evaluate their support to grow entomopathogenic fungi *M. anisopliae* isolates. Among the substrates tested, vegetable wastes support high conidial productivity of AUMI1, AUMI2 and AUMI3 with $4.90 \pm 0.33 \times 10^7$, $3.74 \pm 0.57 \times 10^7$ and $3.62 \pm 0.44 \times 10^7$ conidia/gram of substrate respectively. Tea waste substrate produces the second highest conidial yield for AUMI1 and AUMI2 $1.41 \times 10^7 \pm 0.16 \times 10^7$ and $1.50 \times 10^7 \pm 0.14 \times 10^7$ conidia/gram respectively. The second maximum conidial yield for AUMI3 was harvested from wheat bran.

Vegetable wastes favored maximum conidial productivity of *M. anisopliae* isolates when the fungal isolates were treated at 27^0C for 14 days of incubation under solid state fermentation (Table 1). The lowest conidial productivity was overall recorded on coffee husk, but the least productive substrate for AUMI1 and AUMI3 was record by 2mm size coffee husk which gives $0.20 \times 10^7 \pm 0.02 \times 10^7$ and $0.33 \times 10^7 \pm 0.04 \times 10^7$ of conidia/gram of substrate respectively. Least conidial harvest of AUMI2 $0.36 \times 10^7 \pm 0.07 \times 10^7$ was produced by 4mm size coffee husk.

Table 1. Conidia yields of M. *anisopliae* isolates on different agricultural wastes.

Substrates	Mean±SEM of conidia count of substrates at (x10^7 conidia/gram) after 14 days of incubation		
	Metarhizium anisopliae isolates		
	AUMI1	AUMI2	AUMI3
1mm Coffee husk	0.83±0.08	1.06±0.18	0.60±0.07
2mm Coffee husk	0.20±0.02	0.46±0.05	0.33±0.04
4mm Coffee husk	0.62±0.15	0.36±0.07	0.42±0.08
Tea waste	1.41±0.16	1.50±0.14	0.89±0.10
Wheat bran	1.14±0.13	1.38±0.15	1.09±0.10
Vegetable waste	4.90±0.33	3.74±0.57	3.61±0.44

Note: Mean calculated from three replications, all the isolate cultures on each substrate was incubated at 27°C for 14 days.

AUMI1 gives the maximum conidial yield on vegetable waste from the tested isolates and the lowest conidia yield were recorded by AUMI1 on 2mm size coffee husk. Based on two sample t-Test statistical analysis mean conidia yield of AUMI1 was significantly higher on vegetable waste than on 2mm size coffee husk. The same was true for AUMI2 on vegetable waste than on 4mm size coffee husk and AUMI3 on vegetable waste and on 2mm size coffee husk respectively. The mean conidia yield of AUMI2 and AUMI3 on vegetable wastes were significantly different form conidia yields on 4mm and 2mm particle sized coffee husk, with t(11.35) = 3.38*10^7, p = 0, and t(11.23) = 3.28*10^7, p =0 respectively (Table 1).

Table 2. The effect of moisture on conidia yield of *M. anisopliae* Isolates at 27^{0}C after 14 days.

Substrates	Mean ± SEM of conidia count at (X10^7 Conidia/gram) for moisture content optimization after 14 days of incubation											
	AUMI1				AUMI2				AUMI3			
	35%	45%	60%	80%	35%	45%	60%	80%	35%	45%	60%	80%
4mm size coffee husk	0.37± 0.18[a]	0.37± 0.17[a]	1.01± 0.52[a]	0.46± 0.19[a]	0.37± 0.20[a]	0.40± 0.21[a]	0.30± 0.09[a]	0.38± 0.14[a]	0.33± 0.14[a]	0.46± 0.22[a]	0.33± 0.10[a]	0.57± 0.17[a]
2mm size coffee husk	0.18± 0.04[a]	0.26± 0.02[a]	0.24± 0.02[a]	0.14± 0.02[a]	0.53± 0.10[a]	0.39± 0.09[a]	0.57± 0.10[a]	0.34± 0.03[a]	0.24± 0.06[a]	0.49± 0.10[a]	0.40± 0.02[a]	0.20± 0.05[a]
1mm size coffee husk	0.96± 0.12[a]	0.95± 0.09[b]	0.95± 0.18[a]	0.46± 0.06[a]	1.62± 0.44[a]	1.39± 0.20[a]	0.69± 0.18[a]	0.55± 0.08[a]	0.63± 0.03[a]	0.82± 0.07[a, b]	0.68± 0.15[a]	0.27± 0.04[a]
Tea waste	1.07± 0.07[a]	0.96± 0.18[b]	1.55± 0.37[a]	2.05± 0.10[c]	0.94± 0.07[a]	1.27± 0.14[a]	1.89± 0.12[a]	1.90± 0.23[a, c]	0.78± 0.06[a]	1.32± 0.15[a, b]	0.83± 0.24[a]	0.63± 0.05[a]
Wheat bran	1.25± 0.34[a]	1.46± 0.09[b]	1.01± 0.26[a]	0.82± 0.20[a]	1.56± 0.23[a]	1.59± 0.10[a]	1.43± 0.56[a]	0.95± 0.08[a]	1.19± 0.34[a]	1.07± 0.27[a, b]	1.10± 0.09[a]	0.99± 0.13[a]
Vegetable waste	4.34± 1.08[b]	4.56± 0.09[c]	5.80± 0.72[b]	4.49± 0.08[b]	3.53± 2.29[a]	3.80± 0.59[b]	4.44± 0.55[b]	3.19± 0.95[c]	2.98± 0.46[b]	2.38± 0.78[b]	5.59± 0.66[b]	3.52± 0.34[b]

Note: Means in the same column designated by different letters are significantly different, (a-c), at p≤ 0.05.

3.2. Effect of Moisture Content on Conidia Production of *M.anisopliae* Isolates

The optimum moisture content for conidia production of each isolate varies within and between substrate types (Table 2). The optimum moisture content for all isolates in the substrate of vegetable waste was 60% and the conidia count recorded was significantly high among the rest of the substrates. The result in the present study revealed that the total conidia harvest of each strain with respect to competing moisture contents on substrates of 2mm and 4mm size coffee husks is lower than any other substrate used in this study (Table 2). AUMI2, AUMI1 and AUMI3 gives their maximum conidia productivity ($0.40 \times 10^7 \pm 0.21 \times 10^7$, $1.01 \times 10^7 \pm 0.52 \times 10^7$, and $0.57 \times 10^7 \pm 0.17 \times 10^7$ conidia/gram of substrate) at 45%, 60% and 80% moisture content of 4mm size coffee husk, respectively.

There was a gradual increment in the conidia count of AUMI1 and AUMI2 as the effect of moisture content increases from 35 to 80% using tea waste as substrate. However, conidia count of AUMI3 is higher at 45% moisture. Therefore, tea waste specially provides its highest conidia count at 80% of moisture content for the first two isolates. Wheat bran give the maximum conidia yield of AUMI1 and AUMI2at 45% whereas AUMI3 at 35% of moisture content, respectively.

The mean conidia yield of AUMI1 at vegetable waste ($M = 5.81*10^7$, $SD = 1.25*10^7$, $N = 3$) was significantly increased from 2mm size coffee husk ($M = 2.57*10^6$, $SD = 3.79*10^5$, $N = 3$) $t(4) = 5.55*10^7$, $p = 0.002$. The mean conidia yield of AUMI2 at vegetable waste ($M = 4.45*10^7$, $SD = 9.47*10^6$, $N = 3$) was significantly increased from 4mmsize coffee husk ($M = 4.05*10^6$, $SD = 3.60*10^6$, $N = 3$) $t (2.57) = 4.04*10^7$, $p = 0.01$.

The mean conidia yield of AUMI3 at vegetable waste ($M = 5.59*10^7$, $SD = 1.15*10^7$, $N = 3$) was significantly increased from 2mm size coffee husk ($M = 3.63*10^6$, $SD = 5.80*10^5$, $N = 3$) $t (4) = 5.22*10^7$, $p = 0.001$.

3.3. Effect of Temperature on Conidia Production of *M. anisopliae* Isolates

Incubation temperature markedly affects conidia yield as it can be noticed in table 3. The majority of the isolates in all substrate types the optimal temperature for high conidia productivity was 30^0C after 14 days of incubation. AUMI1 and AUMI2 got their maximum conidia yield of $2.09 \times 10^7 \pm 0.25 \times 10^7$ and $1.28 \times 10^7 \pm 0.27 \times 10^7$ conidia/gram of substrate, respectively when incubated for 14 days at 30^0C using coffee husk as a substrate. Whereas the

optimum conidia yield for AUMI3 on coffee husk were found at 24^0C after 14 days of incubation.

Table 3. Effect of temperature on conidial yield of isolates at different substrates.

Substrates	*Mean±SEM of conidia count ($X10^7$conidia/gram)for temperature optimization after 14 days of incubation*								
	Metarhizium anisopliae Isolates								
	AUMI1			*AUMI2*			*AUMI3*		
	24^0C	27^0C	30^0C	24^0C	27^0C	30^0C	24^0C	27^0C	30^0C
Coffee husk	1.14± 0.03ᵃ	1.15± 0.24ᵃ	2.09± 0.25ᵇ	0.97± 0.13ᶜ	0.81± 0.06ᶜ	1.28± 0.27ᶜ	1.22± 0.06ᵈ	0.96± 0.18ᵈ	1.19± 0.04ᵈ
Wheat bran	2.04± 0.24ᵃ	0.57± 0.33ᵇ	3.23± 0.05ᵃ	1.73± 0.28ᶜ	1.22± 0.13ᶜ	1.79± 0.30ᶜ	2.86± 0.31ᵈ	1.10± 0.33ᵈ	3.04± 0.91ᵈ
Tea waste	0.48± 0.03ᵃ	0.70± 0.12ᵃ	1.10± 0.04ᵇ	0.77± 0.04ᶜ	0.81± 0.07ᶜ	0.81± 0.10ᶜ	0.76± 0.28ᵈ	1.43± 0.28ᵈ	0.82± 0.05ᵈ
Vegetable waste	4.03± 0.40ᵃ	5.05± 0.43ᵃ	3.79± 0.48ᵃ	2.29± 0.11ᶜ	2.67± 0.23ᶜ	2.97± 0.16ᶜ	2.94± 0.39ᵈ	3.69± 0.41ᵈ	4.60± 0.94ᵈ

Note: Means in the same row along the substrate within Isolate type designated by different letters are significantly different, (a-d), at p≤0.05.

Among the substrate types examined vegetable wastes for AUMI2 and AUMI3 2.97 $x10^7±0.16$ $x10^7$ and 4.60 $x10^7±0.94$ $x10^7$ conidia/gram of substrate supported for maximum conidia yield at 30^0C. However, it also supported AUMI1 (5.05 $x10^7±0.43$ $x10^7$ conidia/gram of substrate) for maximum conidia yield at 27^0C. The optimum temperature for AUMI1 and AUMI2 grown on tea waste substrate was 30^0C. Among the isolates tea waste at 27^0C favored higher conidia yield for AUMI3 (1.43 $x10^7±0.28$ $x10^7$ conidia/gram of substrate).

The mean conidia yield of AUMI1 on vegetable waste ($M = 5.05*10^7$, $SD = 7.41*10^6$, $N= 3$) was significantly higher from tea waste ($M = 1.10*10^7$, $SD = 7.01*10^5$, $N = 3$), $t(4) = 3.95*10^7$, $p = 0.001$. The mean conidia yield of AUMI2 on vegetable waste ($M = 2.97*10^7$, $SD = 2.71*10^6$, $N = 3$) was significantly higher from tea waste ($M = 8.07*10^6$, $SD = 1.19*10^6$, $N = 3$), $t (2.74) = 2.17*10^7$, $p = 0.002$. The mean conidia yield of AUMI3 on vegetable waste ($M = 4.60*10^7$, $SD = 1.63*10^7$, $N = 3$) was significantly higher from coffee husk ($M = 1.22*10^7$, $SD = 1.12*10^6$, $N = 3$) $t (4) = 3.39*10^7$, $p = 0.02$.

3.4. Effect of pH Levels on Conidia Production of *M. anisopliae* Isolates

pH values significantly affected conidia yield of the isolates on coffee husk (Table 4). The conidia harvest of AUMI1 and AUMI3 on coffee husk when the PH value adjusted to 4.5 was 2.77 $x10^7±0.23$ $x10^7$, and 3.35 $x10^7±0.23$ $x10^7$ conidia/gram of substrate respectively. AUMI2

give its maximum conidia yield of 3.18 x10^7±0.34 x10^7 conidia/gram of substrate when treated on 3.5 pH. The highest mean of conidial harvest on tea waste was recorded by AUMI1 with 1.14 x10^7±0.11x10^7 conidia/gram of substrate. The overall conidia yield result record on AUMI2 and AUMI3 are not significantly varied among the tested pH values (Table 4).

Table 4. Effect of PH on conidia yield of *M. anisopliae* isolates under different substrates.

Substrate	pH	Mean±SEM of conidia count at (x10^7 conidia/gram)for PH optimization after 14 days of incubation		
		Metarhizium anisopliae Isolates		
		AUMI1	AUMI2	AUMI3
Coffee husk	3.5	2.55±0.41[a]	3.18±0.34[a]	2.59±0.21[a]
	4.5	2.77±0.02[b]	2.90±0.16[e]	3.35±0.23[b]
	5.5	2.41±0.33[f]	2.03±0.20[i]	2.97±0.20[d]
Tea waste	3.5	0.67±0.04[a]	0.59±0.17[b, c]	0.77±0.14[a]
	4.5	1.14±0.11[c, d]	0.88±0.05[f, g]	0.76±0.08[c]
	5.5	0.89±0.10[g, h]	0.92±0.12[J]	0.69±0.11[f, g]
Wheat bran	3.5	1.19±0.39[a]	0.87±0.21[b]	2.26±0.37[a]
	4.5	0.74±0.09[c]	0.98±0.35[f]	1.98±0.13[b, c]
	5.5	0.96±0.05[g]	1.49±0.31[i, J]	1.29±0.32[e, f, g]
Vegetable	3.5	1.31±0.99[a]	0.98±0.23[b, d]	1.49±0.85[a]
	4.5	0.44±0.19[c, e]	1.72±0.31[f, h]	2.87±0.61[b]
	5.5	0.33±0.02[g, i]	0.19±0.02[J, k]	0.62±0.51[g]

Note: Means in the same column within the same PH values designated by different letters are significantly different, (a-k), at p≤0.05.

The pH test on vegetable wastes reduces the total conidial harvest record of all isolates from the records on moisture content and temperature determination. The optimal pH for conidia production of all fungal isolates fall between 3.5 and 4.5 (Table. 4). Isolate AUMI1 produce maximum conidia 1.31 x10^7±0.99 x10^7 conidia/gram of substrate at pH 3.5 whereas isolates AUMI2 and AUMI3 produced 1.72 x10^7±0.31 x10^7 and 2.83 x10^7±0.61 x10^7 conidia/gram of substrate respectively, at pH 4.5. Compared to the conidia yield at the substrates natural pH. Most of the tested pH values used in this study gives rise to the lowest conidia yield record in all substrates except on coffee husk. Coffee husk conidia productivity of all *M. anisopliae* isolates was increased when treated under the adjusted pH tests and the records of all treated pH levels was higher than the results treated on natural pH value of the substrate. The rest substrates were produced high conidial yield at their natural pH concentrations.

The mean conidia yield of AUMI1 on coffee husk ($M = 2.77*10^7$, $SD = 4.32*10^6$, $N = 3$) was significantly higher from mean conidia yield on tea waste ($M = 1.16*10^7$, $SD = 1.93*10^6$, $N = 3$) t(2.77) = $1.62*10^7$, p = 0.01. The mean conidia yield of AUMI2 on coffee husk ($M = 3.18*10^7$, $SD = 5.86*10^6$, $N = 3$) was significantly increased from mean conidia yield on tea waste ($M = 9.23*10^6$, $SD = 2.11*10^6$, $N = 3$) t(2.51) = $2.27*10^7$, p = 0.01. The mean conidia yield of AUMI3 on coffee husk ($M = 3.35*10^7$, $SD = 3.97*10^6$, $N = 3$) was significantly increased from mean conidia yield on tea waste ($M = 7.70*10^6$, $SD = 2.36*10^6$, $N = 3$) t(3.26) = $2.58*10^7$, p = 0.002.

3.5. Effect of Inoculum Concentration on Conidia Production

The highest level of conidia yields of AUMI1 5.42 x107±0.57 x107conidia/gram of substrate on vegetable waste was achieved using 1x105conidia/ml inoculum concentration. The optimum conidia count of AUMI2 and AUMI3 was also recorded at 1x105 inoculum concentration on vegetable waste. The maximum conidia count per inoculum concentration was harvested at 1x105 conidia/ml of inoculum on coffee husk. Conidia count record of the fungal isolates on wheat bran treated in all the inoculum concentrations were higher on 1x104 for AUMI1 and AUMI2 and on 1x105 for AUMI3.

Table 5. Effect of different inoculum concentrations on conidia yield of *M. anisopliae* isolates.

Substrate	Inoculum	Mean ± SEM of conidia count at (x107 conidia/gram) for Inoculum concentration optimization		
		Metarhiziumanisopliae Isolates		
		AUMI1	*AUMI2*	*AUMI3*
Coffee husk	$1*10^3$	1.30±0.17	0.75±0.18	1.14±0.02
	$1*10^4$	0.83±0.15	0.95±0.04	1.06±0.40
	$1*10^5$	1.35±0.06	1.32±0.02	1.50±0.51
Tea waste	$1*10^3$	0.64±0.12	1.19±0.11	0.40±0.18
	$1*10^4$	0.88±0.005	0.91±0.13	0.20±0.03
	$1*10^5$	0.63±0.19	0.74±0.02	0.26±0.06
Wheat bran	$1*10^3$	2.47±0.17	0.89±0.01	1.29±0.42
	$1*10^4$	1.93±0.30	1.47±0.33	2.00±0.45
	$1*10^5$	1.50±0.20	1.14±0.34	3.35±1.74
Vegetable waste	$1*10^3$	0.55±0.20	0.79±0.37	0.82±0.03
	$1*10^4$	0.79±0.07	0.88±0.06	1.07±0.14
	$1*10^5$	5.42±0.57	3.25±0.41	4.63±0.53

The optimum inoculum concentrations for *M. anisopliae* isolates on vegetable waste were recorded at1x105 conidia/ml. Inoculum concentration optimization test conducted on tea waste were also have different optimum concentrations for each isolate. AUMI1 were favored for maximum conidia productivity at 1x104 conidia/ml of inoculum concentration. The conidia

count result $1.19 \times 10^7 \pm 0.11 \times 10^7$ conidia/gram of substrate and $0.40 \times 10^7 \pm 0.18 \times 10^7$ conidia/gram of substrate was recorded for AUMI2 and AUMI3 respectively at 1×10^3 conidia/ml (Table 5).
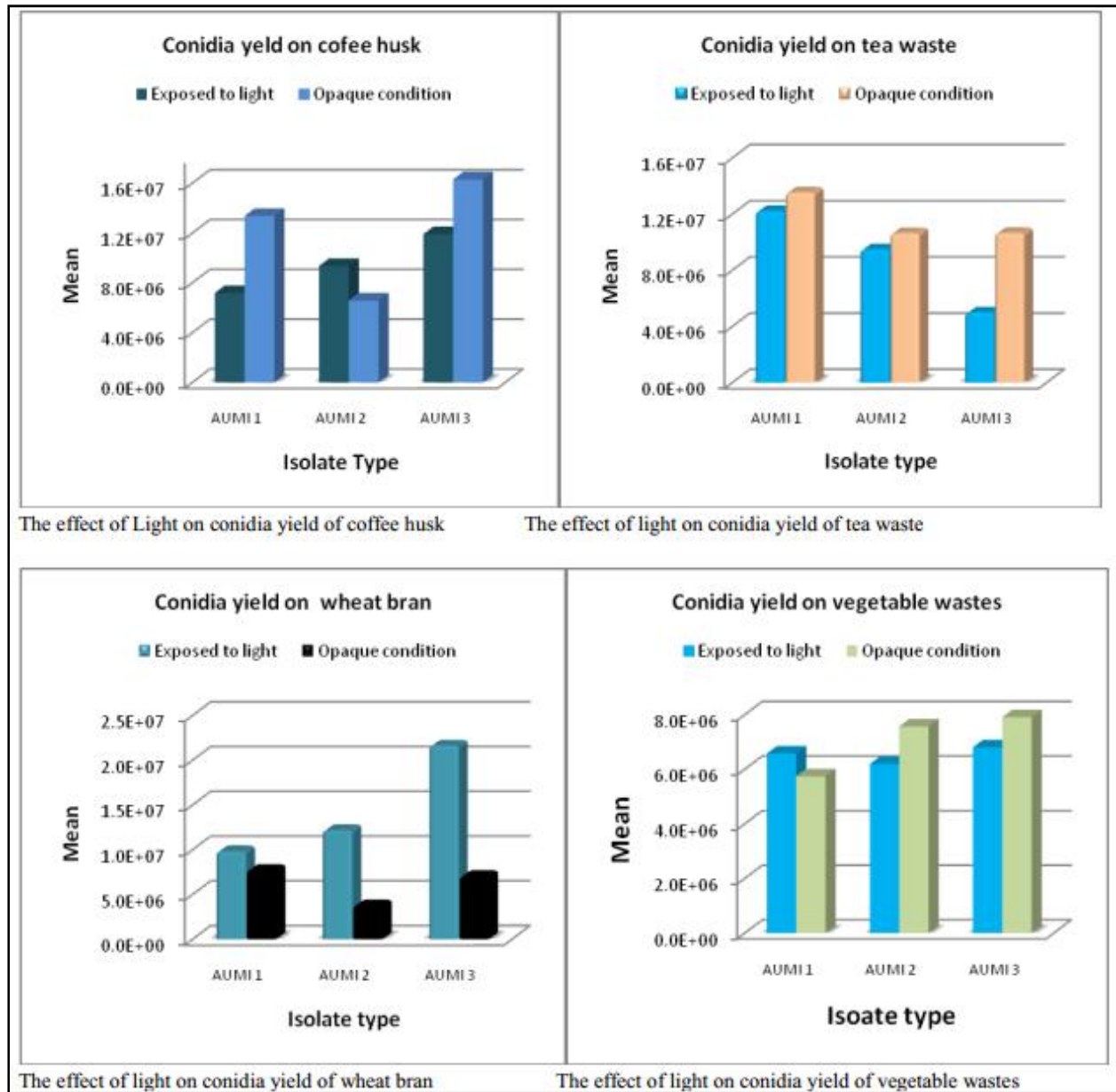

Figure 1. The effect of light on conidia yield of *M. anisopliae* isolates under different substrates.

3.6. Effect of Light on Conidia Production of *M. anisopliae* Isolates

The conidia yields of AUMI1 and AUMI3 in the absence of light were larger than in the presence of light on coffee husk. The substrate afforded comparatively higher conidia growth for AUMI1 and AUMI3 in opaque condition whereas in the presence of light for AUMI2. Excellent

growths of all fungal isolates were afforded by the substrate tea waste at dark condition. It has been observed that the isolates, AUMI1 and AUMI2 was also produced higher conidia in the presence of light but, the maximum conidia yield was observed on cultures in the absence of light (Fig 1). Therefore, conidia production of the fungal isolates on tea waste was light independent when compared with wheat bran in which conidia productivity of all the fungal isolates were light intensive. The conidia production potential of vegetable waste under the presence and absence of light was evaluated as the results shown in figure 1. The conidia production of AUMI1was light dependent whereas Opaque condition was favored for AUMI2 and AUMI3 to produce the highest conidia yield on vegetable wastes. Mean of the conidia yield was calculated from three replicates and each isolate was cultivated at 27^0C for 14 days under sufficient light exposure and complete opaque condition as required.

4. DISCUSSION

Biological pest management using entomopathogenic fungi as microbial agents primarily requires mass production of the candidate on cheap cultivation media (Mohammed, 2006; Masoud et al., 2013). Different agricultural wastes were evaluated in this study as cheap potential substrates for conidia production of *M. anisopliae* isolates. All agricultural substrates were potential producers for high number of conidia even though difference in number was recorded among substrates. *M. anisopliae* isolate AUMI1, AUMI2 and AUMI3 was able to produce significantly high number of conidia (4.90×10^7, 3.74×10^7 and 3.61×10^7 conidia/gram of substrate respectively) on vegetable wastes than their respective less productive substrates. Different vegetables contain different carbon and nitrogen source nutrients and their combination increases nutritional value of the substrate. Therefore, vegetables waste high productivity could be due to high macromolecular composition of lignocellulosic nutrients providing the necessary carbon and nitrogen source. Similarly, Herta et al. (2005); and Gao (2011) have indicated that combination of different carbon and nitrogen source resulted in high spore production.

Initial moisture content and water activity are the key factors in solid state fermentation reaction (Rajan and Nair, 2011). The availability of water strongly affects microbial growth. Therefore, the moisture content of the substrate should be within the suitable range. In the present study, the effects of various initial moisture contents were evaluated for all substrates. The best result for all *M. anisopliae* isolates was obtained at 60% moisture content on vegetable

wastes and a comparable result of conidia count was also obtained at 80% on the same substrate. Therefore, the optimum moisture content 60% was obtained to grow isolates of *M. anisopliae* on vegetable wastes. Despite that, the optimum moisture content of other substrates required to conidiate each isolate varies as shown in Table 2. When 35% of 1mm size coffee husk favored AUMI1 and AUMI2 (0.96 x10^7±0.12 x10^7 conidia/gram and 1.62 x10^7±0.44 x10^7 conidia/gram respectively), 45% of the same substrate supports AUMI3. The results also revealed that, 1mm size coffee husk was efficient in conidiation than the larger used in particle size. This may be due to difficulty to assimilate the larger in particle size by the enzymes secreted from the fungi. Overall coffee husks in the present study at any moisture content were less efficient in conidia production of all fungal isolates when compared to the other substrates. Similarly, (Zuriash Mamo and Tesfaye Alemu, 2012) have observed that the lowest conidia count of *Trichoderma* isolates was recorded on coffee husk under SSF technique.

The optimum moisture content of wheat bran to grow isolate AUMI3 (1.19±0.34 conidia/gram) and AUMI1, AUMI2 (1.46 x10^7±0.09 x10^7, 1.59 x10^7±0.10 x10^7 conidia/gram) are at 35% and 45% respectively. Conidia count reduction in high moisture content may be related to the fact that excess water occupies the space between particles of the substrate and restricts mass oxygen flow across (Pandey, 2003).

There was a gradual increment in conidia count record of AUMI1 and AUMI2 as moisture content increases from 35% to 80% on Tea waste. However, conidia count result of AUMI3 on the same substrate was higher at 45%. Therefore, the highest conidia count record of AUMI1 and AUMI2 at 80% of moisture content was perhaps due to maximum water absorption capacity of the substrate. Pandey and Soccol (2001) have observed that the water absorption potential depends on factors such as solid matrix structure and superficial area, as well as the ability of hydrogen-bond formation sites, among others. As clearly indicated in table 2, the moisture content level of the substrate are not the only factors that affect conidia production, composition and structure of the substrate as well as the type of isolates cultivated are also determinants. Experiment that was conducted by Rosane et al. (2008) for comparison of conidia yield among different strains of *Trichoderma* on different substrate under the same moisture content results with no fungi growth and spore formation this assures that moisture content is not the only factor.

Each isolate was incubated at 24, 27 and 30^0C to evaluate the effect of temperature on conidia production. Among others the substrates examined on vegetable wastes AUMI2 and AUMI3 (2.97±0.16 and 4.60±0.94 conidia/gram of substrate respectively) were supported for maximum conidia yield at 30^0C optimum temperature. However, vegetable waste also supported AUMI1 (5.05±0.43conidia/gram of substrate) for maximum conidia yield at 27^0C as an optimum temperature. The Ethiopian isolates of *Metarhizium spp* were also attained peak conidia production at 28^0C (Seneshaw and Seyum, 2003). The high conidia productivity of wheat bran and vegetable wastes at 30^0C was obtained perhaps due to water stress at a precise growth stage since high temperature causes loss of water via desiccation. Reynaldo and Sevastianos (2013) have clearly stated that water stress under SSF during the maximum growth period or biomass formation helps sporogenesis to start earlier and allows obtaining higher sporulation yield.

pH optimization test completely flipped conidial productivity potential of each substrate. The high conidia productive vegetable waste at natural pH levels of each substrate handovers to coffee husk. AUMI2 was produced the maximum conidia yield (3.18 $x10^7$±0.34 x107 conidia/gram) at pH 3.5 on coffee husk. The optimum pH concentration for AUMI1 and AUMI3 was obtained at pH 4.5 using coffee husk as substrate yielding 2.77 $x10^7$±0.23 $x10^7$, and 3.35 $x10^7$±0.23 $x10^7$ conidia/gram respectively. Similarly, (Zuriash Mamo and Tesfaye Alemu, 2012) have reported that the optimum pH for conidia production of *Trichoderma* isolates was between 4.5 and 5.5. Except for coffee husk the overall conidia count record of the substrates on their natural pH was significantly higher than the initial pH value used for optimization. It is also important to mention that coffee husk high productivity was perhaps due to releasing of its organic matter after highly degraded by the 1N of HCl (1 normality of hydrochloric acid) used to adjust pH at 3.5 into utilizable form by the candidate fungal isolates.

In the present study, conidiation of *Metarhizium* isolates of AUMI1, AUMI3 were best favored in the absence of light on coffee husk. The same was true for excellent conidia production of all isolates on tea waste when compared with wheat bran in which all the isolates were light dependent. Under exposure to light AUMI1 was high productive on vegetable wastes while, AUMI2 and AUMI3 requires opaque condition. Therefore, it is very important to keep in mind that light has essential effect on conidia production of *Metarhizium* isolates under SSF system on different substrates.

5. CONCLUSION

From the study it is clear that all the evaluated substrates are capable to support growth of the Enthomopathogenic fungi *M. anisopliae.* Whereas, vegetable wastes showed to be the best candidate for conidia production. The fungal pathogens are abundantly found in soil and can be isolated using simple techniques and a selective medium. Mass conidial cultivation of these strains on vegetable wastes and applying to control crop loss due to swarm invasion of insect pests like Desert and migratory locust can be efficient and cost effective. Since, the use of chemical pesticides is not financially cheap and environmentally safe. In turn, biological control agents are effective against the target pathogen; they are eco-friendly; and do not affect ecological diversity of the farm. They are considered as excellent bio-control agents.

6. ACKNOWLEDGEMENT

7. CONFICT OF INTEREST

There are no conflicts of interests.

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