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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

Logistic regression applied to the incidence of *Aspergillus* producer of mycotoxin in cocoa beans cultivated in the state of Rondônia, Brazil

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The presence of fungi of the genus *Aspergillus* must be investigated because it is related to the diminishment of quality and consequently to the risk to health of consumers due to the possible production of mycotoxins. In this context, this study was performed with the aim to evaluate the presence of *Aspergillus* species in cocoa beans produced in the state of Rondônia, Brazil, as well as verify the toxigenic potential of these species and the effect of the geographical origin on the incidence of toxigenic and non-toxigenic species of fungi. The technique of logistic regression was used to estimate the probability of occurrence of the genus *Aspergillus*. A total of 185 *Aspergillus* were identified. Among the tested *Aspergillus carbonarius,* 79.41% produced ochratoxin on levels from 0.08 to 44.09 µg/g in the culture medium. For the other *Aspergillus* species, the production potential was only assessed, and 3.13% of the *A. niger* presented potential to produce OTA and 83.33% of *A. flavus* which was able to produce aflatoxins. The presence of these toxigenic species indicates a potential risk of mycotoxin in cocoa beans and in their derived products in the studied region. Besides, the occurrence of *Aspergillus* species differed in function of geographical coordinates and temperature, presenting higher probability of occurrence on cocoa beans of municipalities located in Southern Rondônia, Brazil.

Key words: Cocoa beans, *Aspergillus,* mycotoxins, ochratoxin A, aflatoxins, logistic regression, thin layer chromatography, high performance liquid chromatography.

INTRODUCTION

The importance of cocoa to the industry is attributed to its beans, from which the chocolate and other by products are obtained. Brazil is the sixth largest cocoa producer of the world (International Cocoa Organization, 2013),

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highlighting the state of Rondônia between the three largest national producers (Brazilian Institute of Geography and Statistics, 2013). The state of Rondônia is located in the Western Amazon in northern Brazil and has suitable conditions for the production of this Brazilian commodity. It is located at 11°30'20'' south latitude and 63°34'20'' west longitude. The average annual temperature is 24-26°C, precipitation of 1400-2600 mm/year and has hot and humid climate.

The primary cocoa processing occurs on farms and involves the fruit opening stages, fermentation, drying and storage. The microbial succession that naturally occurs during the fermentation of cocoa was already established and observed on studies developed on Brazil. According to Schwan and Wheals (2004), the cocoa seeds are contaminated with microorganisms when the fruit is opened by the cutting tool, when they are transported on baskets and also through insects that may be present on the local. These microorganisms contribute to the process of spontaneous fermentation that occurs thereafter (Schwan and Wheals, 2004).

On the steps of fermentation, drying and storage, the contamination by filamentous fungi on cocoa beans may occur (Mounjouenpou et al., 2008; Sánchez-Hervás et al., 2008) and the presence of these fungi may cause the cellulose hydrolysis, production of acids and undesirable flavors on the beans (Schwan and Wheals, 2004). Furthermore, some species of these fungi may produce mycotoxins, which may endanger its consumption (Copetti et al., 2010, 2011a, b; Mounjouenpou et al., 2008; Sánchez-Hervás et al., 2008).

One of the mycotoxins of greatest interest on cocoa beans, which is produced by species of the genus *Aspergillus,* is the ochratoxin A (OTA), a potent nephrotoxin with teratogenic and carcinogenic action (International Agency for Research on Cancer, 1993; O'brien and Dietrich, 2005).

Therefore, the knowledge regarding the incidence of fungi of the genus *Aspergillus* and its relation with the processing practices are important for the creation of control strategies during the primary processing of cocoa beans in order to minimize the exposition of the consumer to these toxic compounds.

In this sense, the present study aimed to investigate the presence of fungi of the genus *Aspergillus* on cocoa beans cultivated on municipalities of the state of Rondônia, Brazil, besides evaluating the toxigenic potential of these species, as well as the effect of the geographical origin over the incidence of toxigenic and non-toxigenic fungi.

MATERIALS AND METHODS

Samples

Forty samples of cocoa beans were collected on properties located in the state of Rondônia, the third largest producer of cocoa on Brazil. The samples were collected in 2012 in 12 municipalities: Ariquemes (5), Buritis (3), Cacoal (3), Cacaulândia (3), Colorado do Oeste (4), Governador Jorge Teixeira (2), Jaru (3), Machadinho d'Oeste (5), Ouro Preto do Oeste (5), São Felipe d'Oeste (3), Urupá (3) and Vilhena (1).

Identification of species of the genus *Aspergillus*

The technique of direct plating was used for the isolation of fungi from cocoa beans, according to Samson et al. (2000). One hundred randomly collected beans of each sample were submitted to surface disinfection with sodium hypochlorite (1%) during 30 s and then washed with sterile distilled water three times consecutively. Samples were put on Petri dishes containing the culture medium Dichloran Rose Bengal Chloranphenicol (MERCK). Plates were incubated at 25°C during seven days (Pitt and Hocking, 1997). After seven days, beans were evaluated in relation to the growth of fungi and representative colonies of the genus *Aspergillus* were isolated in medium Malt Agar (MA). Results were expressed as the percentage of contaminated beans according to the methodology of Pitt and Hocking (1997). After obtaining pure colonies, the isolates were transferred to two standardized media: Czapec yest agar (CYA) and malt extract agar (MEA) at 25°C during seven days for posterior identification. The species identification was made according to Klich and Pitt (1988), Klich (2002), Samson et al. (2002) and Samson et al. (2004).

Evaluation of the toxigenic through the thin layer chromatography

The ochratoxigenic and aflatoxigenic potential was evaluated by thin layer chromatography, according to Filtenborg et al. (1983). Species of the Section *Nigri* were cultivated in medium CYA (K_2HPO_4:1.0 g; Czapec Concentrate: 10.0 mL; yeast extract: 5.0 g; Agar: 15.0 g; $NaNO_3$:30.0g, KCl: 5.0 g, $MgSO_4.7H_2O$: 5.0 g, $FeSO_4.7H_2O$: 0.1 g, $ZnSO_4.7H_2O$: 0.1 g, $CuSO_4.5H_2O$: 0.05 g; Distilled Water: 1000 mL) at 25°C for seven days. The *Aspergillus* Section *Flavi* were cultivated in médium – yeast extract sacarose (YES) Agar (yeast extract: 20.0g; sacarose: 150 g; Agar: 20.0 g; metalic solution $ZnSO_4.7H_2O$: 0.1 g, $CuSO_4.5H_2O$: 0.05 g; distilled water: 1000 mL), at 25°C for seven days.

Standard solution of OTA, AFB_1, AFB_2, AFG_1 E AFG_2, (SIGMA-ALDRICH), plates of thin layer chromatography (MERK-SILICA GEL 60, 20x20) and as mobile phase TEF- toluene ethyl acetate and formic acid 90% (60:30:10 v/v/v) were used. Confirmation of toxin production was performed in ultraviolet light (λ 366nm) in Cromatovisor CAMAG (UF-BETRACHTER). The isolates considered producers had a retention factor (Rf), and a fluorescent spot pattern similar to that of the toxin evaluated.

Quantification of ochratoxin A produced by *Aspergillus carbonarius* by HPLC method

The *A. carbonarius* were inoculated into a Petri dish containing the medium Czapek yeast agar (CYA) and incubated at 25°C for 10 days. After this, OTA was extracted according to the modified method of Bragulat et al. (2001). Three plugs of culture were removed from the centre, middle and edge of each colony on the tenth day of the incubation period. The plugs were weighed in test tubes, and then 1 mL of methanol was added. The tubes were homogenised vigorously for five seconds and kept at 25°C for 60 min. The extracts were filtered through polytetrafluoroethylene (PTFE) membranes (0.22 μm) (Millipore) and then analysed by high-performance liquid chromatography (HPLC). The equipment used was an HPLC Shimadzu coupled with two high-pressure

pumps (model SPD-M20A), degasser DGU $20A_3$, interface CBM-20A, auto injector SIL-10AF and RF-10 A_{XL} fluorescence detector. The Agilent ZORBAX Eclipse XDB-C18 (4.6 x 250 mm, 5 µm) column was used and it was connected to a pre-column Agilent ZORBAX Eclipse XDB-C18 4-Pack (4.6 x 12.5 mm, 5 µm). The chromatographic conditions for wavelength were 332 nm for excitation and 476 nm for emission. The flow used throughout the analysis was equal to 0.8 mL min^{-1}, and the injected volume of the samples and standard was 20 µl. The elution was performed using an isocratic system of 35:35:29:1 (methanol : acetonitrile : water : acetic acid). The average retention time for OTA determination was 11 ± 0.1 min. The amount of OTA in the samples was determined using an analytical curve obtained by linear regression ($y = 1.11756 \times 10^7x - 2592.1485$, where y = peak area and x = OTA concentration). The calculation defined the peak area versus the concentration of the respective standard solution obtained by the setting of coefficient ($R^2 = 0.9999$). The detection limit (DL) and quantification limit (QL) were estimated through parameters obtained by the analytical curve and were calculated according to the following: DL = 3 SD/m and QL = 10 SD/m (where SD = standard deviation and m = angular coefficient of the linear regression). The values obtained for the DL and QL were 0.0004 and 0.0016 µg/g, respectively. All samples were analysed in duplicate, and the standard OTA solutions were assessed in triplicates.

Statistical analysis

In order to determine the probability of occurrence of total and toxigenic species of the genus *Aspergillus* in the state of Rondônia, Brazil, the technique of logistic regression was used in function of the following independent variable: geographical coordinates (latitude and longitude) and temperature. The response variable was characterized by the presence and absence of total and toxigenic fungi of the genus *Aspergillus*.

Thereunto the models of logistic regression were fitted, adjusted using the software R Core Team (2013), considering the covariates represented by temperature (T), by geographical coordinates that identify a city given by longitude (Long) and latitude (Lat). Thus, it was assumed that the Y = 1 encoding, featuring the presence of total species and Y = 0, indicating the absence of these species (Equation 1). Similarly, considering the same covariates, but assuming Y = 1 coding related to the presence of toxigenic species and Y = 0, the absence, the adjusted logistic model is given in Equation 2.

$$P(Y=1|\text{Long, Lat, T}) = \frac{\exp(36.047 - 0.8294\text{Long} + 0.428\text{Lat} - 0.690\text{T})}{1+\exp(36.047 - 0.8294\text{Long} + 0.428\text{Lat} - 0.690\text{T})} \quad (1)$$

Where, P (.) is set equal to the probability for the occurrence of species of total fungi of the genus *Aspergillus*.

$$P(Y=1|\text{Long, Lat, T}) = \frac{\exp(-20.5807 - 0.4682\text{Long} - 0.2002\text{Lat} - 0.0805\text{T})}{1+\exp(-20.5807 - 0.4682\text{Long} - 0.2002\text{Lat} - 0.0805\text{T})} \quad (2)$$

Where, P (.) is set equal to the probability for the occurrence of species of toxigenic fungi of the genus *Aspergillus*.

After the adjustment of models through the Hosmer-Lemeshow test by means of adjusted probabilities obtained from Equations 1 and 2, the graphs of response surface and contours were built in order to ease the identification of the potential regions for the incidence of total and toxigenic fungi of the genus *Aspergillus*.

Table 1. Species of *Aspergillus* isolated from cocoa beans processed and stored on cocoa properties of Rondônia, Brazil.

Genus/species	Nº of genus/species	(%)
Aspergillus* Section *Nigri	**166**	**89.73**
A. aculeatus	12	7.23
A. carbonarius	34	20.48
A. foetidus	17	10.24
A. japonicus	5	3.02
A. niger	64	38.55
A. niger aggregate	20	12.05
A. tubingensis	14	8.43
Aspergillus* Section *Flavi	**19**	**10.27**
A. flavus	18	94.74
A. oryzae	1	5.26

RESULTS AND DISCUSSION

Identification of *Aspergillus* species

Only 2.5% of all the analyzed samples (40) did not present contamination by fungi species, 97.5% were contaminated by filamentous fungi and nine of these samples presented 100% of contamination.

The fungi were identified to the species level due to the high incidence in cocoa beans and the risk for the production of ochratoxin A in tropical regions. The species identified in this study are presented on Table 1.

The species were isolated and identified as members of the Sections *Nigri* (*Aspergillus niger*, *Aspergillus carbonarius*, *Aspergillus niger* aggregate, *Aspergillus foetidus*, *Aspergillus japonicus, Aspergillus tubingensis* and *Aspergillus aculeatus*) and *Flavi* (*Aspergillus flavus* and *Aspergillus oryzae*), and the species of Section *Nigri* were the most frequent (89.73%).

The following species were isolated and identified: *A. niger*, *A. carbonarius*, *A. niger* aggregate, *A. foetidus, A. japonicus*, *A. tubingensis* and *A. aculeatus*, belonging to Section *Nigri*, and *A. flavus* and *A. oryzae* of the Section *Flavi*. The species of Section *Nigri* were the most frequent (89.73%). A similar result was found by Sánchez-Hervás et al. (2008) on cocoa beans produced on Sierra Leone, Equatorial Guinea and Ecuador. It is known that some species of these two Sections of Aspergillus are considered the most important contaminating and toxin producer fungi, which has increased the interest on the isolation and identification of these species (Moss, 1996; Samson et al., 2004).

Among the species of Section *Nigri*, *A. niger* predominated both in this study and other studies regarding cocoa (Copetti et al., 2011b; Mounjouenpou et al., 2008). *A. niger* is a species of frequent occurrence of the Section *Nigri* and it is known by its wide distribution,

both in the tropics and in temperate regions (Pitt and Hocking, 1997).

Mounjouenpou et al. (2008) isolated the species *A. carbonarius, A. flavus, A. fumigatus, A. niger, A. tamari* and *A. versicolor* from cocoa beans cultivated on Cameroon. Sánchez-Hervás et al. (2008) obtained *A. niger* aggregate as main species of the Section *Nigri* and also related the presence of *A. carbonarius* in lower amounts, but did not find any uniseriate species (*A. aculeatus* and *A. japonicus*). Copetti et al. (2011b) made a similar study on Brazil and also isolated some species mentioned above, like *A. versicolor* and *A. fumigatus*, as well as *A. clavatus, A. niger, A. ochraceus* and *A. parasiticus*, confirming the existence of a toxigenic microbiota associated with the culture of cocoa, as well as the risk of producing ochratoxin A and aflatoxins on cocoa beans.

In relation to Section *Flavi*, *A. flavus* predominated in this section. In the study performed with cocoa beans on Sierra Leone, Equatorial Guinea and Ecuador by Sánchez-Hervás et al. (2008), this species also predominated on the Section *Flavi.* Other main species member of the Section *Flavi* responsible for the production of aflatoxins, *A. parasiticus* (Horn, 2007), was not isolated in this study.

No species of the Section *Circumdati* considered ochratoxigenic, like *A. ochraceus* (Pitt and Hocking, 1997) was identified in this study. Sánchez-Hervás et al. (2008) related a low incidence of species of the Section *Circumdati.* The results shows that probably *A. ochraceus* is not an important source of OTA on cocoa cultivated in these regions.

Potential toxigenic species of genera *Aspergillus*

A total of 166 species of Section *Nigri* were registered in relation to the capacity to produce ochratoxin A by the method of thin layer chromatography. From all the isolates, 29 (17.47%) were capable of producing toxins, it means, they presented retention factor and fluorescence spot similar to the standard solution of OTA. From the 29 isolates with toxigenic potential, 27 were identified as *A. carbonarius* and 2 as *A. niger*.

However, 7 *A. carbonarius* did not present potential to produce OTA. This means that from the total of *A. carbonarius* isolates only 79.41% produced OTA. Some studies showed that the percentage of OTA production by this species may range between 70 and 100% *A. carbonarius.* Only two isolates identified as *A. niger* were able to produce OTA. Differently from the observed in the present work, Mounjouenpou et al. (2008) found 70% of the species of *A. niger* producing OTA in cocoa beans. However, the same percentage (3%) of *A. niger* was recorded as OTA producer in coffee (Taniwaki et al., 2003) and grape (Lasram et al., 2007). The high percentage found by Mounjouenpou et al. (2008) was not confirmed by other studies conducted with this species in different producer regions (Bellí et al., 2004a; Iamanaka et al., 2005; Leong et al., 2007; Magnoli et al., 2007; Taniwaki et al., 2003).

Sánchez-Hervás et al. (2008) did not identify any *A. niger* producing OTA in cocoa, however 44.7% of the fungi identified as *A. niger* produced OTA. In the study developed with cocoa beans in Brazil by Copetti et al. (2010), *A. niger* was most commonly isolated species, but only 2.5% were able to produce the toxin.

A total of 19 species of the Section *Flavi* (18 *A. flavus* and 1 *A. oryzae*) isolated in this study were tested in relation to their capacity to produce aflatoxins. From the 18 isolates identifies as *A. flavus,* 15 (83.33%) were able to produce aflatoxin B_1 and B_2. The isolate of *A. oryzae* tested in the present study did not produce aflatoxins, thus confirming the non-toxigenicity of this species as already related by Magan and Olsen (2004). This indicates that the contamination of cocoa beans by aflatoxins does not seem to be worrying in cocoa beans cultivated in Brazil.

Production of ochratoxin A by *A. carbonarius* isolated from cocoa beans

All isolates identified as *A. carbonarius* were tested in relation to the production of OTA in medium CYA by HPLC (Table 2). Results obtained show that these isolates produced amounts of OTA ranging from 0.08 to 44.09 μg/g of culture medium.

Sánchez-Hervás et al. (2008) related the OTA production by *A. carbonarius* isolated from cocoa ranging from 0.2 to 8.0 μg/g of culture medium. In other studies with their substrates, like grape and coffee, the amount of OTA produced ranged from 1 to 10 μg/g (Bellí et al., 2004a; Lasram et al., 2007; Martínez-Culebras and Ramón, 2007). This difference of production may be related to the interference of the cultivation conditions and the origin of substrate, besides the region from where these fungi were isolated. Few studies were published on non-ochratoxigenic *A. carbonarius.*

Cabañes et al. (2013) identified 3 wild isolated of non-ochratoxigenic *A. carbonarius* from grape of different vineyards of Southern Spain through genetic sequencing. The three isolates did not produce OTA in any medium culture in all tested temperatures and in the different time of incubation, thus proving the existence of non-ochratoxigenic *A. carbonarius.*

Probability of occurrence of total and toxigenic fungi of the genus *Aspergillus* in function of geographic coordinates and temperature

Geographic coordinates and temperatures of the state of Rondônia, Brazil, were analyzed in order to relate it to the

Table 2. Effect of OCHRATOXIN A (OTA) production by *A. carbonarius*[1] isolate from cocoa beans processed and stored on cocoa properties of Rondônia, Brazil.

Isolate	Mean of OTA (μg/g)	Isolate	Mean of OTA (μg/g)
A. carbonarius 1	44.09	*A. carbonarius* 18	0.18
A. carbonarius 2	0.25	*A. carbonarius* 19	30.21
A. carbonarius 3	0.09	*A. carbonarius* 20	0.18
A. carbonarius 4	0.11	*A. carbonarius* 21	20.98
A. carbonarius 5	ND	*A. carbonarius* 22	0.19
A. carbonarius 6	0.13	*A. carbonarius* 23	0.20
A. carbonarius 7	ND	*A. carbonarius* 24	0.12
A. carbonarius 8	0.12	*A. carbonarius* 25	ND
A. carbonarius 9	ND	*A. carbonarius* 26	0.29
A. carbonarius 10	0.08	*A. carbonarius* 27	0.17
A. carbonarius 11	4.82	*A. carbonarius* 28	0.22
A. carbonarius 12	ND	*A. carbonarius* 29	0.37
A. carbonarius 13	0.39	*A. carbonarius* 30	0.29
A. carbonarius 14	ND	*A. carbonarius* 31	14.07
A. carbonarius 15	14.10	*A. carbonarius* 32	17.32
A. carbonarius 16	30.68	*A. carbonarius* 33	11.82
A. carbonarius 17	0.33	*A. carbonarius* 34	ND

[1]Analysis made by HPLC in CYA medium /10days; [ND]Non detected; LOD = 0.0004 μg/g; LOQ = 0.0016 μg/g.

levels of contamination by species of total and toxigenic fungi of the genus *Aspergillus* in cocoa beans, according to what is described in Equations 1 and 2.

The validation of the logistic model (Equations 1 and 2) was made by the probability of significance obtained in relation to the Hosmer-Lemeshow test. When considering the total and toxigenic species of fungi, the estimated probabilities were respectively, 0.907 and 0.992. Thus, confronting with the significance level fixed at 5%, since they presented a non-significant result, both models are adequate to identify the municipalities with higher probability of fungi incidence.

The logistic regression model adjusted for the identification of the occurrence of total fungi (Equation 1) allows interpreting that, considering fixed values for the geographic coordinates and temperature, the resulting probability of the model refers to the probability of occurrence of total fungi of the genus *Aspergillus*, characteristic of a determined region of the state of Rondônia (Figures 1 and 2).

In relation to the presence of toxigenic species, the surface of response with probabilities of occurrence of species in function of geographic coordinates (Equation 2), as well as the contours plot to help in interpretation are presented in Figures 3 and 4, respectively.

In relation to the geographical position of the cultivation areas, several studies with grapes had already demonstrated that the geographic area and meteorological conditions may contribute to the incidence of fungi and production of OTA (Battilani et al., 2006a, b; Bellí et al., 2006; Chiotta et al., 2009; Serra et al., 2006), however no study was done with cocoa regarding this aspect until the present one. In this context, when considering latitude, longitude and temperature, a higher probability of occurrence of total fungi of the genus *Aspergillus* was estimated for municipalities of the state of Rondônia, Brazil, located between the parallels of 11° and 13° of South latitude and meridians of 60° and 62° of West longitude, which correspond to Vilhena, Colorado do Oeste, Cacoal, São Felipe d'Oeste and Urupá.

However, we may verify that for toxigenic species of fungi, the probabilities of occurrence were similar in all the studied regions in the state of Rondônia, Brazil.

The effect of geographical area on the fungi was confirmed by Battilani et al. (2006b), showing a significant effect of localization when the incidence of species belonging to the genus *Aspergillus* Section *Nigri* in grapes was analyzed. The incidence was significantly correlated with geographical coordinates showing the positive correlation with longitude, indicating that the incidence increased from West to East, while the correlation with latitude was negative, showing a positive gradient for Southern Europe. The incidence of *A. carbonarius* also was positively correlated with latitude. These authors also verified that meteorological conditions play an important role in the fungi colonization and on the presence of OTA in grapes in Italy.

Bellí et al. (2006) studied the correlation between meteorological parameters and fungi of the Section *Nigri* in grapes of wine regions of Spain and revealed a positive correlation between the colonization by *Aspergillus* Section *Nigri* and temperature. These authors attributed the higher percentage of infection of grapes by *Aspergillus* Section *Nigri* in the year of 2003, where meteorological conditions are extremely hot during the whole year. Studies with species of the Section *Nigri* suggest that the higher frequency in grapes of warmer climates may be associated with the survival capacity of these species in the soil and the capacity of adaptation to high temperatures and low water activity (Battilani et al., 2006c; Leong et al., 2004), as well as to the fact that the black spores of Section *Nigri* species offer protection to sunlight providing a competitive advantage on warmer climates (Hocking, 2006; Leong et al., 2006; Pitt and Hocking, 1997).

A positive correlation of *A. carbonarius* on grapes with temperature was already related, and the higher occurrence of this species occurred on hot areas (Battilani et al., 2006a; Chiotta et al., 2009). *A. carbonarius* grows on temperatures between 10 and 40°C (Pitt, 2000), with optimum between 25-35°C (Bellí et al., 2004b; Mitchell et al., 2004). The mean temperatures of the municipalities which samples were collected range between 24.33 and 26.13°C, which is propitious to the growth of *A. carbonarius* in the whole state. In relation to the

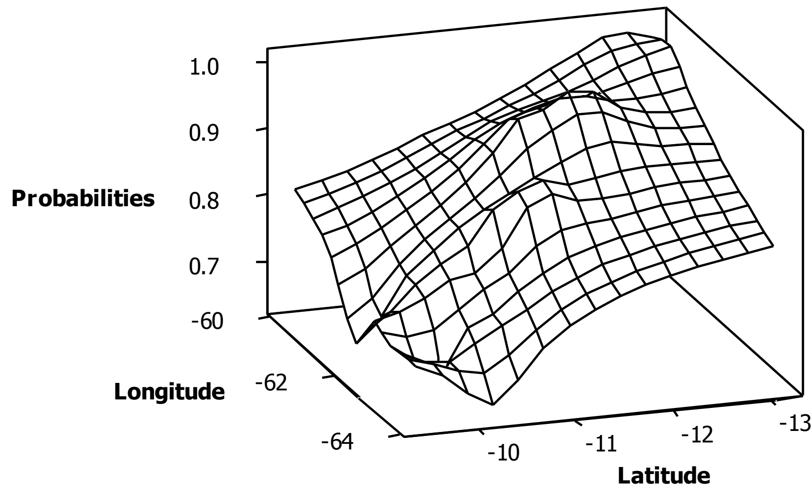

Figure 1. Response surface for the probability of occurrence of total fungi on localities characterized by different ranges of latitude and longitude in Rondônia, Brazil.

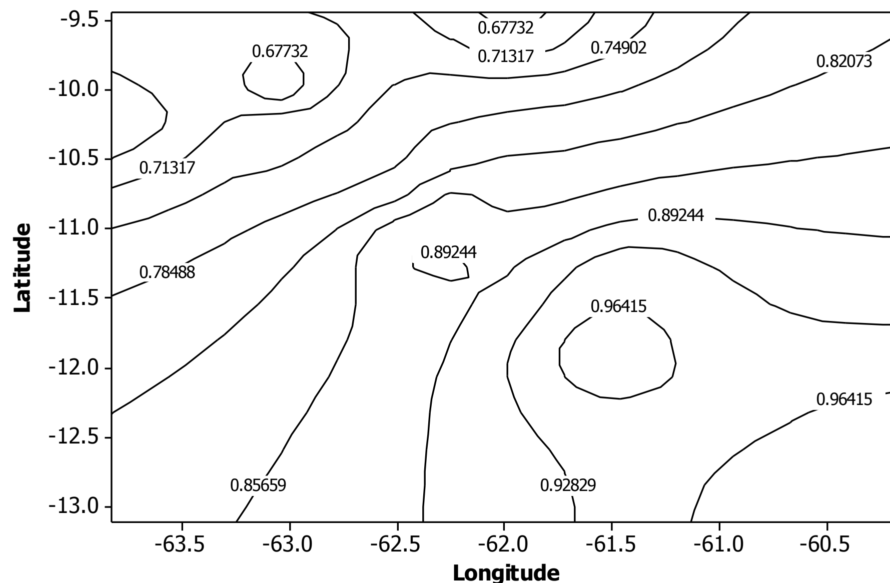

Figure 2. Contour lines obtained from the response surface with percentages of probability of occurrence of total fungi in Rondônia, Brazil.

production of OTA, *A. carbonarius* produced the most elevated levels at 15 or 20°C (Esteban et al., 2004; Passamani et al., 2014), this temperature range below that found in Rondônia which suggests a reduced risk of contamination of the cocoa beans OTA produced by this species.

The uniformity among probabilities predicted by the model adjustment in relation to the geographical coordinates and temperatures may be related to the limited number of collected samples of cocoa beans, thus decreasing the possibility of isolating toxigenic fungi. We highlight that results were obtained in function of geographical coordinates and temperature, and then the inclusion of new variables may identify other municipallities with high index of toxigenic fungi.

Results obtained in the present study suggest a risk of contamination by toxigenic fungi for cocoa beans, once *A. carbonarius, A. niger* and *A. flavus* were isolated and identified. The higher probability of fungi occurrence was estimated for municipalities located between the parallels 11 and 23° S and 60 and 62° W, thus confirming the

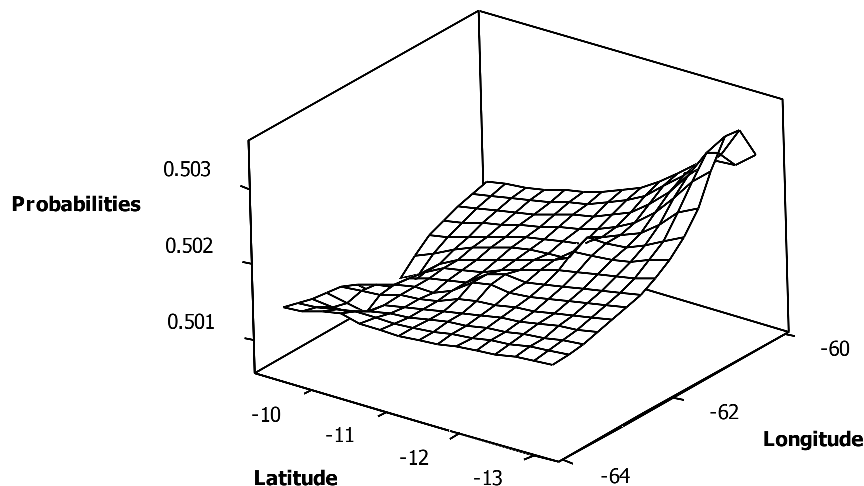

Figure 3. Response surface for the probabilities of occurrence of toxigenic fungi on localities characterized by different ranges of longitude and latitude in Rondônia, Brazil.

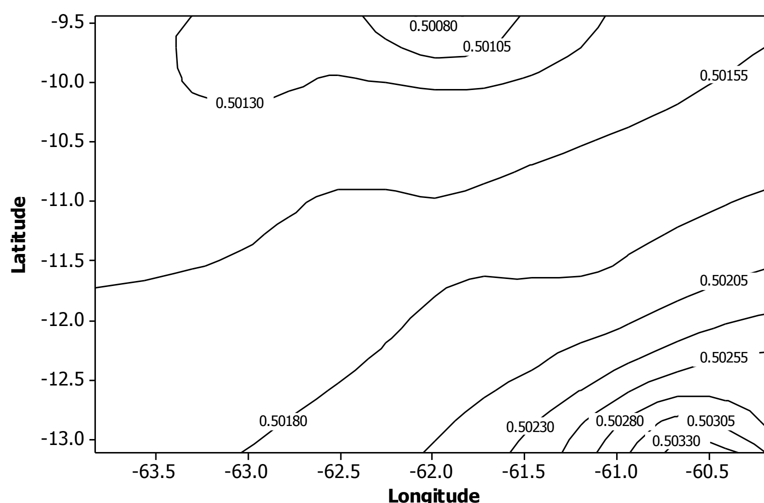

Figure 4. Contour lines for the probabilities of occurrence of toxigenic fungi on localities characterized by different ranges of longitude and latitude, in Rondônia, Brazil.

effect of geographic localization and temperature on the incidence of fungi of the genus *Aspergillus.*

However, some key questions still must be answered to minimize the contamination by toxigenic fungi in cocoa beans processed in these municipalities. We suggest: (a) identify which step of processing represents a greater risk of contamination by these toxigenic fungi; (b) characterize which step of processing provide optimum conditions for the production of toxins by fungi; (c) optimize methods of field control, for example, use of fungicides; (d) create systems of geographic information and maps of risk as support material to producers and researchers.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Soil microbial, chemical properties and crop productivity as affected by organic manure application in popcorn (*Zea mays* L. var. *everta*)

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Soil microbial population, dehydrogenase activity and chemical properties of soil under different doses of farmyard manure (FYM), leaf compost and vericopmpost were examined at the research farm of the Indian Agriculture Research Institute (IARI), New Delhi, India during 2008-09 and 2009-10. Results indicated the higher value of microbial population, dehydrogenase activity, organic carbon, available nitrogen (N), phosphorus (P), potassium (K) and lower bulk density were observed in farmyard manure applied equivalent to 120 kg N ha^{-1} followed by vermicompost equivalent to 120 kg N ha^{-1}. Grain yield of popcorn was significantly higher in the treatments of recommended dose of fertilizers and vermicompost equivalent to 120 kg N ha^{-1}.

Key words: Dehydrogenase activity, farmyard manure (FYM), leaf compost, microbial population, vermicompost.

INTRODUCTION

Popcorn (*Zea mays* L. *var. everta*.) is the most important crop under irrigated conditions of north India. Due to its importance as food, feed and specialty corn, it is a versatile cereal crop for both domestic consumption and for export. popcorn requires huge amounts of nutrients for high productivity. The continued application of chemical fertilizer leads to deterioration of soil health with reduced organic carbon and increased multi-nutrients deficiencies (Swarup, 2002). Maintenance of soil quality is of utmost importance for enhancing productivity which leads to food and nutritional security. High aboveground biomass yield is accompanied by an active root system, which releases an array of organic compounds into the rhizosphere (Fließbach et al., 2007). For maintaining good soil health, nutrient schedules should be correctly followed, including organic manures viz., farmyard

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Table 1. Nutrient concentration in applied organic manures.

Source	N (%)	P (%)	K (%)
Vermicompost	1.60	0.50	1.52
Farm yard manure	0.48	0.28	0.51
Leaf compost	0.67	0.29	0.27

manure (FYM),vermicompost and leaf compost, as these sources have the potential to supply nutrients to crops as well as prevent soil deterioration (Kumar et al., 2005). Application of these organic manures not only helps in enhancing the productivity but also have the beneficial effect on soil properties (Pathak et al., 2005). Nutrients available in organic manures are released slowly, remain in the soil for longer time and are available to plants, thereby maintaining soil fertility and enhance the soil microbial population (Belay et al., 2001). Researchers reported that application of various organic manures stimulated the plant growth, activity of soil micro-organism, resulted in higher population of fungi, bacteria and actinomycetes and higher activity of soil enzymes (Krishnakumar et al., 2005; Sidhu et al., 2007; Alam et al., 2007; Knapp et al., 2010). Application of organic manures increased microbial respiration and resulted in increased carbon and plant nutrient mineralization rates in soil (Powon et al., 2005).

Microbial processes are dynamic, and therefore patterns of temporal fluctuation during crop growth are of great importance in relation to the nutrient supplying capacity of the ecosystem and the crop demand. Consequently, microbial association needs to be studied along with available plant nutrients in soil and crop growth. The information regarding microbial population, soil enzymes and chemical properties of soil in relation to organic manures is limited in crops like popcorn (Meena et al., 2013). Hence, the present study was carried out to evaluate the effect of organic manures on soil chemical, and biological properties as well as productivity in popcorn.

MATERIALS AND METHODS

Experimental location and climate

Field experiments were conducted during 2008-09 and 2009-10 at the research farm of Indian Agricultural Research Institute (IARI), New Delhi (latitude 28.40° N, longitude 77.12°E, 228.6 m above mean sea level). The climate of New Delhi is sub-tropical and semi-arid type with hot and dry summer and cold winter and falls under the Agro-climatic Zone 'Trans-Gangetic Plains'. The mean annual rainfall is 650 mm and more than 80% generally occurs during the south-west monsoon (July-September) with mean annual evaporation of 850 mm. Minimum and maximum temperature ranged between 11.0 and 35.0°C and 10.9 and 37.9°C, with the rainfall of 582.7 and 502.7 mm during growth period of popcorn in 2008 and 2009, respectively.

Soil characteristics, layout and treatments

The soil of the experimental field was sandy loam in texture, slightly alkaline in reaction (pH 7.7) with bulk density of 1.54 Mg m^{-3}, low in soil organic carbon (0.38%) and available nitrogen (159.4 kg ha_{-1}), medium in available phosphorus (12.6 kg ha_{-1}) and potassium (162.2 kg ha_{-1}), as analysed by Singh et al. (2005). The eight treatments (T1 = Control, T2 = RDF ($N_{120}P_{25}K_{35}$ kg ha_{-1}), T3 = Farmyard manure equivalent to 120 kg N ha_{-1}(FYM120), T4 = Leaf compost equivalent to 120 kg N ha_{-1} (LC 120), T5 = Vermicompost equivalent to 120 kg N ha_{-1} (VC 120), T6 = FYM equivalent to 90 kg N ha_{-1} (FYM 90), T7 = Leaf compost equivalent to 90 kg N ha_{-1} (LC 90), T8 = Vermicompost equivalent to 90 kg N ha_{-1} (VC 90)) were applied. The experiment was laid out in randomized block design (RBD) with three replicates.

Application of organic manures/fertilizers and crop management

The required quantity of organic manures was applied 10 days before sowing of the crop as per the treatments. The FYM, vermicompost and leaf compost samples were analyzed for total N using a Kjeldahl digestion method (Jackson, 1967), while total P and K were determined using a wet digestion (Di-acid digestion) method as described earlier (Prasad et al., 2006). Concentrations of different nutrients in organic manures are given in Table 1. Recommended dose of fertilizer ($N_{120}P_{25}K_{35}$ kg ha^{-1}) was applied through urea, single super phosphate and muriate of potash, respectively. Full dose of P and K, $^{1}/_{3}$ of N and 25 kg ha^{-1} zinc sulphate were applied as basal. The remaining N was applied in two equal splits at tasseling and grain formation stage. To ensure the optimum moisture for germination, pre-sowing irrigation was applied and afterwards irrigation was given to the crop at frequent intervals for better crop establishment. The field was prepared by two cross ploughings with disc plough and one ploughing with cultivator followed by planking. Thereafter, a ridge with a spacing of 60 cm for popcorn sowing was made with the help of tractor drawn ridger. The popcorn seed was dibbled at 3-4 cm seed depth on the side of the ridges at a spacing of 15-20 cm. The optimum plant population (83,000 plant ha^{-1}) was maintained by thinning of plants after one week of germination. For weed control, two manual weddings were done at 30 and 50 days after sowing and during second weeding the earthing up operation was also carried out.

Soil sampling, microbial population and dehydrogenase activity

Soil samples (0-15cm) were collected after harvest of the crop from each treatment for analysis. The samples were stored in plastic bags and taken to the laboratory, where soil was sieved (2 mm mesh size), homogenized and stored at 4°C. Organic carbon was determined by using wet digestion method (Walkley and Black, 1934), available N by alkaline permanganate (Subbiah and Asija, 1956), available P by Olsen's et al. (1954) method, available K by Flame photometer method (Jackson, 1967), and bulk density was estimated by using the core sampler method (Bodman, 1942). The fungal, bacterial and actinomycetes population were estimated by standard plate count method using Marten's for fungi (Martin, 1950), and nutrient agar medium for bacteria and actinomycetes (Allen, 1959). Microbial population was calculated and expressed as number of cells, $x10^{n}$/g soil. Dehydrogenase activity was determined by reduction of 2, 3, 5-triphenyltetrazolium chloride (TTC) (Casida et al., 1964). Individual character datasets were subjected to analysis of variance and means were separated by Duncan's multiple range test (DMRT) at the 5% level of probability using SAS version 9.3.

Table 2. Effect of various treatments on microbial population in popcorn cultivated soil (pooled data).

Treatment	Bacteria ($cfu10^5$ g^{-1} soil)	Fungi ($cfu10^2$ g^{-1} soil)	Actinomycetes ($cfu10^2$ g^{-1} soil)
T1=Control	20.37^d	14.77^g	8.34^f
T2=RDF ($N_{120}P_{25}K_{35}$ kg ha^{-1})	23.18^c	16.58^f	9.58^e
T3=Farmyard manure equivalent to 120 kg N ha^{-1}	28.60^a	23.96^a	14.16^a
T4= Leaf compost equivalent to 120 kg N ha^{-1}	26.11^{ab}	21.13^d	11.61^{cd}
T5= Vermicompost equivalent to 120 kg N ha^{-1}	28.37^a	23.03^{ab}	13.49^{ab}
T6= FYM equivalent to 90 kg N ha^{-1}	27.12^{ab}	22.17^{bc}	12.92^b
T7=Leaf compost equivalent to 90 kg N ha^{-1}	24.74^{bc}	19.32^e	10.63^{de}
T8=Vermicompost equivalent to 90 kg N ha^{-1}	26.95^{ab}	21.21^{cd}	12.58^{bc}

Same letters (a, b and c) between the two treatments indicate a non-significant difference at $P \leq 0.05$ (DMRT-test).

RESULTS AND DISCUSSION

Microbial population of bacteria, fungi and actinomycetes

The pooled data of both the years are presented, as there was no significant difference observed among the years (Table 2). The microbial population viz., bacteria, fungi and actinomycetes significantly were affected with application of different organic N sources as compared to control. The application of 120 kg N ha^{-1} either through FYM (T3) or vermicompost (T5) resulted in maximum microbial population of bacteria (28.60 and 27.12 $cfu10^5$ g^{-1} soil), fungi (23.96 and 22.17 $cfu10^2$ g^{-1} soil) and actinomycetes (14.16 and 12.92 $cfu10^2$ g^{-1} soil), respectively (Table 2). The application of leaf compost equivalent to120 kg N ha^{-1} (T4) resulted in higher amount of microbial populations as compared to control and RDF (T2), although it was statistically at par with the application of 90 kg N ha^{-1} either through FYM (T6), vermicompost (T8) and leaf compost (T7). The increase in microbial population with the application of organic manure might be due to stimulated growth and activities of soil microorganism (Upadhyay et al., 2011). The crop plant secreted various types of organic acids from roots, which is an easily available source of food for soil microorganism (Dotaniya et al., 2013). The addition of organic inputs enhanced the microbial counts in soil, which might be due to carbon addition and changes in physico-chemical properties of soil. The recommended dose of fertilizer (RDF) resulted in lower values of microbial populations than organic manure treatments, but significantly higher than the control treatment. Microbial populations were more numerous in the application of 120 kg N ha^{-1} either through FYM (T3) or vermicompost (T5) probably due to the bioavailability of growth-promoting substances. Microbial population composition and density is an important attribute of soil organic matter quality, as it provides an indication of a soil's ability to store and recycle nutrients and energy. It also serves as a sensitive indicator of change and future trends in organic matter level. The increase in microbial biomass was proportional to the addition of nutrients and accelerated microbial activity (Masto et al., 2006).

Dehydrogenease activity in soil

Dehydrogenease activity increased significantly with application of organic manures (FYM, leaf compost and vermicompost) as compared to inorganic fertilizers and control (Figure 1). Farmyard manure equivalent to 120 kg N ha^{-1} (T3) resulted in significantly higher activity of dehydrogenase in soil (87 µg TPF g^{-1} soil 24 h^{-1}). Application of vermicompost equivalent to 120 kg N ha^{-1} (T5) also recorded higher values of dehydrogenase activity in soil, although it was statistically at par with the application of 90 kg N ha^{-1} either through FYM (T6) or vermicompost (T8). From these results, it can be inferred that dehydrogenase activity is influenced more by the quality than by the quantity of organic matter incorporated into soil. Thus, the stronger effects of FYM on dehydrogenase activity might be due to more easy decomposition of FYM on the metabolism of soil microorganisms (Pancholy and Rice, 1973). It has been shown before that dehydrogenase activity was positively influenced by the total porosity of soil and higher dehydrogenase activity was obtained in soil treated with organic manures (Marinari et al., 2000).

Available N, P and K contents in soil

Available N, P and K increased significantly with application of organic manures (FYM, leaf compost and vermicompost) as compared to RDF and control (Table 3). The application of FYM equivalent to 120 kg ha^{-1} (T3) resulted in the highest concentration of available N (175.8 kg ha^{-1}), P (14.8 kg ha^{-1}), and K (184.3 kg ha^{-1}) followed by vermicompost equivalent to 120 kg N ha^{-1} although it was statistically at par with 90 kg N ha^{-1} added as vermicompost or FYM or leaf compost at the equivalent

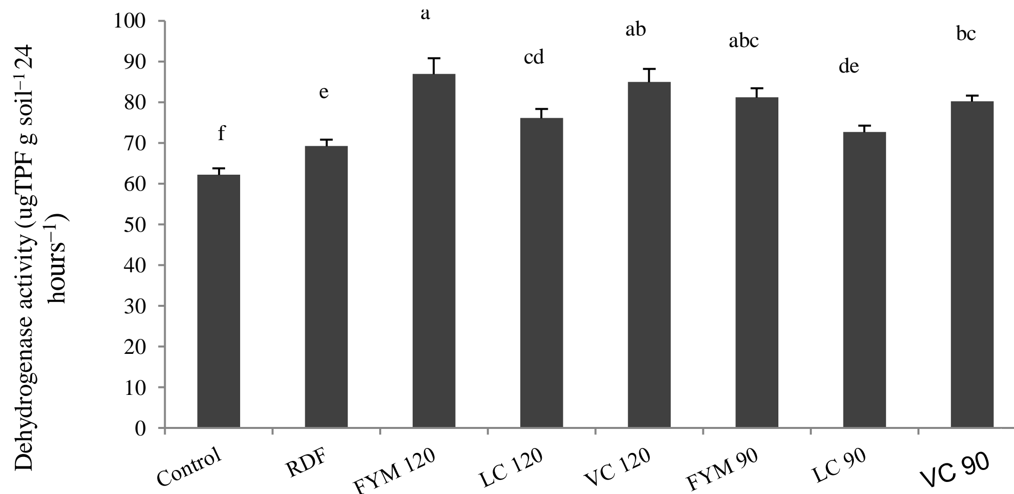

Figure 1. Effect of various treatments on dehydrogenase activity. Same letters (a, b and c) between the two treatments indicate a non-significant difference at P ≤ 0.05 (DMRT-test).

Table 3. Effect of various treatments on available N, P, K, organic carbon and bulk density in popcorn cultivated soil (pooled data).

Treatment	Available N ($kg\ ha^{-1}$)	Available P ($kg\ ha^{-1}$)	Available K ($kg\ ha^{-1}$)	Organic carbon (%)	Bulk density ($Mg\ m^{-3}$)
T1=Control	159.72^{e}	11.65^{f}	161.68^{d}	0.40^{f}	1.53^{a}
T2=RDF ($N_{120}P_{25}K_{35}$ kg ha^{-1})	167.28^{d}	12.78^{e}	170.65^{c}	0.44^{e}	1.51^{ab}
T3=Farmyard manure equivalent to 120 kg N ha^{-1}	175.83^{a}	14.80^{a}	184.31^{a}	0.52^{a}	1.42^{d}
T4= Leaf compost equivalent to 120 kg N ha^{-1}	172.65^{abc}	13.81^{cd}	181.08^{ab}	0.48^{cd}	1.45^{cd}
T5= Vermicompost equivalent to 120 kg N ha^{-1}	174.45^{ab}	14.60^{ab}	183.75^{a}	0.51^{ab}	1.43^{cd}
T6= FYM equivalent to 90 kg N ha^{-1}	172.15^{abc}	14.21^{bc}	178.00^{b}	0.50^{bc}	1.44^{cd}
T7=Leaf compost equivalent to 90 kg N ha^{-1}	169.84^{cd}	13.21^{e}	175.82^{bc}	0.47^{d}	1.46^{bc}
T8=Vermicompost equivalent to 90 kg N ha^{-1}	171.43^{bc}	13.72^{d}	177.15^{b}	0.49^{c}	1.44^{cd}

Same letters (a, b and c) between the two treatments indicate a non-significant difference at P ≤ 0.05 (DMRT-test).

120 kg N ha^{-1}. The available N, P and K with farmyard manures equivalent to 120 kg N ha^{-1} (T3) was higher by 10, 22 and 14%, respectively, over control. All the organic sources of nutrients improved the available N, P and K status of the soil as compared to the inorganic sources of nutrients and control. Higher N, P, K under organic treatments may be due to continuous application of FYM and organic sources. These sources may enhance organic matter status in soil, which further improves soil physical as well as microbiological activities and increases the availability of plant nutrients (Kumar and Dhar, 2010; Meena et al., 2014). Singh et al. (2008) confirmed the role of organic manures in releasing N and improving N availability in soil. During decomposition of organic manures, various phenolic and aliphatic acids are produced which solubilize phosphatase and other phosphate bearing minerals and thereby lowers the phosphate fixation and increase its availability (Dotaniya et al., 2014a). Available K in soil increased with the application of organic manures which is due to solubilising action of organic acids produced during FYM decomposition and its higher capacity to hold K in available form (Vidyavathi et al., 2011).

Soil organic carbon

Soil organic carbon (SOC) concentration in soil is significantly influenced by different sources of nutrients whether organic or inorganic (Figure 2). In general, post-harvest soils of both years had more concentration of SOC (0.48%) than the samples taken before initiation of experiment (0.38%). The SOC contents of soil changes rapidly with addition of organic manures. The highest SOC concentration (0.52%) was observed with the application of FYM equivalent 120 kg N ha^{-1} (T3). The SOC was also higher with the application of 120 kg N ha^{-1} through FYM (T3) followed by application of vermicompost (T5, T6) during both years. The higher value of SOC content in soil with organic manures might be due to biological immobilization and continuous mineralization of FYM on surface soil layer (Ramesh et

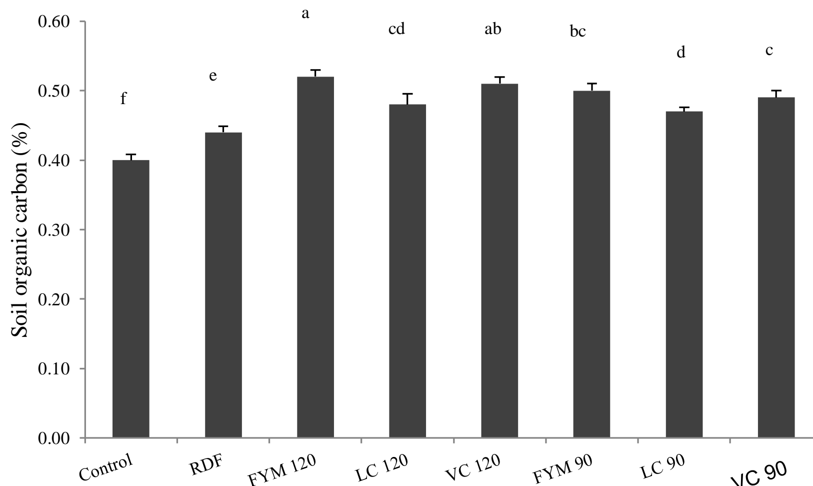

Figure 2. Effect of various treatments on soil organic carbon. Same letters (a, b and c) between the two treatments indicate a non-significant difference at P ≤ 0.05 (DMRT-test).

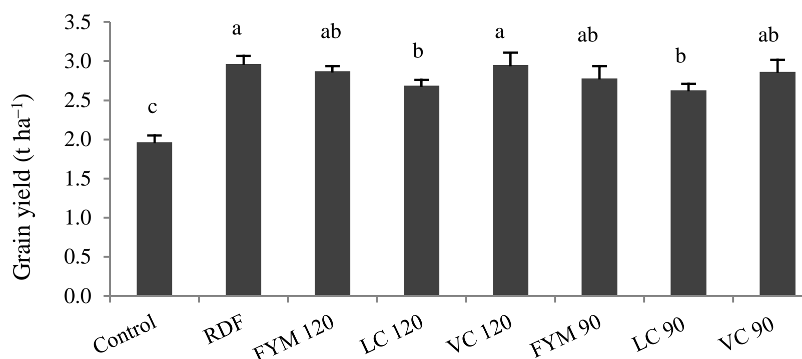

Figure 3. Effect of various treatments on grain yield of popcorn. Same letters (a, b and c) between the two treatments indicate a non-significant difference at P ≤ 0.05 (DMRT-test).

al., 2008). The SOC was also improved with the application of the RDF ($N_{120}P_{25}K_{35}$ kg ha^{-1}) as compared to control plots. The increase in SOC due to inorganic fertilization could be due to higher root biomass accumulation in fertilized treatment than in control as described previously (Masto et al., 2006).

Bulk density

Bulk density (0-15 cm layer) of the popcorn cultivated soil differed significantly due to various levels and sources of nutrients (Table 3). The values of bulk density were significantly lower (1.42-1.46 Mg m^{-3}) with the treatments consisting of organic sources of nutrients than the treatments with inorganic fertilizer (1.51 Mg m^{-3}) and control (1.53 Mg m^{-3}). This could be attributed to higher organic carbon content in these treatments which had better soil aggregate and larger macro pore space (Bellaki et al., 1998). Bulk density was significantly reduced with application of FYM equivalent to 120 kg N ha^{-1} (T3) as compared to RDF (T2) and control (T1), but it remained statistically at par with other organic sources of nutrients during both years. Organic manures (FYM, vermicompost and leaf compost) decreased the bulk density and improved the soil physical properties due to reduced mass per unit of soil (Das et al., 2002).

Popcorn grain yield

The highest grain yield of popcorn was recorded with the application of recommended dose of fertilizers and 120 kg N ha^{-1} applied through vermicompost (T5) during both years (Figure 3). The popcorn yield was 51.3 and 46.4% higher with the application of recommended dose of fertilizers and vermicompost, respectively. The higher yield under inorganic fertilizer is attributed to the increased

nutrient availability, which significantly increase the yield parameters such as plant height, stem girth, number of leaves, leaf area, leaf area index and dry matter accumulation (Kolawole and Joyce, 2009).

Conclusions

Integrated use of organic manure and inorganic fertilizers improved the enzymatic activities as well as microbial population of bacterial, fungal and actinomycetes of soil used for growth of popcorn. Based on a two years study, it can be concluded that FYM and vermicompost equivalent to 120 kg N ha^{-1} have almost equally important effects with respect to soil chemical, and biological properties. Decomposition of organic matter and recycling of carbon have substantial effect on the activity of soil enzymes evolved in mineralization of crop plant nutrients. It improved the soil health and microbial population in soil. While, grain yield of popcorn was significantly associated with either recommended dose of fertilizers or vermicompost equivalent to 120 kg N ha^{-1}.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Arbuscular mycorrhizal fungi (AMF) communities associated with cowpea in two ecological site conditions in Senegal

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The objective of this study was to characterize the diversity of arbuscular mycorrhizal fungal (AMF) communities colonizing the roots of *Vigna unguiculata* (L.) plants cultivated in two different sites in Senegal. Roots of cowpea plants and soil samples were collected from two fields (Ngothie and Diokoul) in the rural community of Dya (Senegal). Microscopic observations of the stained roots indicated a high colonization rate in roots from Ngothie site as compared to those from Diokoul site. The partial small subunit of ribosomal DNA genes was amplified from the genomic DNA extracted from these roots by polymerase chain reaction (PCR) with the universal primer NS31 and a fungal-specific primer AML2. Nucleotide sequence analysis revealed that 22 sequences from Ngothie site and only four sequences from Diokoul site were close to those of known arbuscular mycorrhizal fungi. Also, 47.6% of the clones from Ngothie site and 89.47% from Diokoul site were not close to known AMF. A total of 15 operational taxonomic units (OUT) were identified. Phylogenetic analyses showed that these clones belonged to the genera *Glomus*, *Sclerocystis*, *Rhizophagus*, *Scutellospora*, *Gigaspora*, *Racocetra*, *Acaulospora* and *Redeckera*. The genus *Glomus* is the most represented with six OTU, representing 40% of all OTU.

Key words: Agriculture, Glomeromycota, *Vigna unguiculata*, diversity, soil origin.

INTRODUCTION

Cowpea (*Vigna unguiculata* L.) is a legume that plays an important role in the nutritional balance of the rural population in Sub Saharan Africa, particularly in western and eastern regions where it is mostly cultivated. This

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legume as source of protein, occupies an important place in diet and population food security (Ndema et al., 2010). Its leaves are also highly appreciated by livestock (Cissé and Hall, 2003). Dry grains and fresh leaves contain a high level of protein (23-25%), vitamins B and micronutrients such as iron, calcium and zinc (Cissé and Hall, 2003). In addition, another important beneficial role of this legume is its contribution to soil nitrogen status through symbiotic nitrogen fixation, thereby enhancing soil fertility and reducing the need of N-fertilizer application (Bado, 2002).

The average yield per hectare of cowpea is variable. It ranges from 50 to 550 Kg/ha in Africa and depends on climatic conditions, varieties, cropping system and the level of fertilizer used and pesticides (Cissé and Hall, 2003). These yields are considered low as compared to the potential yield of up to 1000 and 3000 Kg/ha for the Senegalese varieties Melakh and Murid, respectively (Cissé and Hall, 2003). The low yield observed is mainly due to low rainfall and soil nutrient (P and N) depletion.

In all legume plants, cowpea is able to form symbiotic association with microorganisms such as rhizobia and arbuscular mycorrhizal fungi (AMF). This last can improve plant growth under low soil fertility condition, confer tolerance to some pathogens, improve the water balance of plants and can influence soil aggregation (Dalpé, 2005; Marulanda et al., 2006; Rillig and Mummey, 2006). Currently, about 250 species of AMF are described in the world (Jansa et al., 2014). These symbiotic microorganisms have been formerly identified by the morphological characteristics of their spores although sporulation is under the control of several parameters such as environment, seasonal variation, host plant, genotype and AMF species (Sanders, 2004; Smith and Read, 2008). Thus, spore counting and identification based on morphological characterization do not adequately reflect the diversity of active AMF communities in plant roots (Schüßler et al., 2001). Nowadays, several DNA-based methods have been used successfully in studies of diversity and could help to overcome these constraints. Among them, there might be mentioned PCR, quantitative PCR, Sanger sequencing and 454 pyrosequencing methods (Chifflot et al., 2009; Buée et al., 2009 ; Stockinger et al., 2010; Rousk et al., 2010; Tedersoo et al., 2010; Orgiazzi et al., 2013).

Furthermore, many AMF species are ubiquitous, occurring worldwide in quite different terrestrial ecosystems (Öpik et al., 2006), while others appear to be restricted to specific ecosystems, land uses types, vegetations or climates (Oehl and Sieverding, 2004; Castillo et al., 2006).

In Africa, several studies have shown that native AM fungi mainly belong to Glomeraceae, Gigasporaceae and Acaulosporaceae families (Bâ et al., 1996; Kachkouch et al., 2012). Most of them showed the predominance of the genus *Glomus* in several ecosystems, particularly in Senegal (Diop et al., 1994; Manga et al., 2007; Ndoye et al., 2012), Burkina Faso (Bâ et al., 1996) and Morocco (Abbas et al., 2006; Bouamri et al., 2006). However, a study conducted in Benin showed an equal distribution between *Glomus* and *Scutellospora* species in a woodland of *Isoberlina doka* (Houngnandan et al., 2009). This AMF diversity described in Benin is low as compared to that found in the reserve of Bandia in Senegal (Sene et al., 2012) and those in the arid and semi-arid areas of Africa and elsewhere (Bâ et al., 1996; Mohammad et al., 2003; Bouamri et al., 2006; Ndoye et al., 2012; Kachkouch et al., 2012). In Senegal, molecular diversity studies of AMF performed on acacia trees showed only the presence of *Glomus* in their roots, suggesting a preferential mycorrhization of these trees because other genera have been isolated in their rhizosphere (Manga et al., 2007; Ndoye et al., 2012).

Some studies have shown that different ecological conditions can influence the AMF community in cultivated soil (Oehl et al., 2010). However, in Sub-Saharan African particularly in Senegal, little is known about the influence of ecological site conditions on AMF community composition.

The aim of this study was to analyze the AMF communities composition in cowpea cultivated under two different ecological site conditions in Senegal.

MATERIALS AND METHODS

Study sites

The study was conducted in the rural community of Dya, located in the region of Kaolack, in the center of Senegal. This zone belongs to the eco-geographical area of the "South groundnut basin" and is characterized mostly by flat terrain dotted with depressions, representing the old dry valleys. The climate is Soudano-Sahelian with 3 to 4 months of rainy season and 8-9 months of dry season during which the temperature varies between 25°C in January and 45°C in May. The annual rainfall varies between 500 to 900 mm. There are four types of soil in this zone: "Dior" soil or arenosoil (75% of area), "Dek-dior" soil or lixisoil (16% of area), "Dek" soil or vertisoil (8% of area), and saline soil (1% of area) (Merlier, 1972).

The study was conducted at two sites. The first site is located in the village of Ngothie (14°N 14' W, 16°N 12' W) and the second in the village of Diokoul (14°N 15'W, 16°N 11' W). In Ngothie site, the field has been installed on a Dek soil whereas in Diokoul site, the field is on a Dior-soil.

On both sites, woody stratum is composed of acacia trees dominated by *Faidherbia albida* whose density is greater on Ngothie site than on Diokoul site. The herbaceous stratum is the same except *Adropogon gayanus* encountered only on Ngothie site (in zone of depression).

Plant material and experimental design

V. unguiculata (L.) seeds used in this study belonged to the "Melakh" variety. This choice was justified by its fast growing cycle (early variety: 60 days) allowing escape of the drought and it's appreciation by farmers. Seeds provided by ISRA (Institut Sénégalais

de Recherches Agricoles, Dakar, Senegal) were directly used in fields without any previous treatment as used in traditional farming system.

The two sites are separated by about 2 km. At Ngothie, the field is installed on a Dek soil. Each field was divided into five plots of 16 m^2 each. In each plot, there are seven lines of seven seed holes each, representing a total of 49 seed holes. The distance between the lines and between the seed holes is 60 cm. At sowing, two seeds were placed into each hole.

Soil sampling and characterization

Before sowing, in each plot, soil was sampled at three points on the horizon 0-40 cm and mixed to get a composite soil sample. Soil samples from the five plots were pooled to one composite soil sample of approximately 2 kg per field. The composite soil sample from each field was passed through a coarse sieve (2 mm mesh) to remove stones and fine roots.

Physicochemical characterization of composite soil samples was performed at LAMA (Laboratoire des Moyens Analytiques de l'Institut de Recherche pour le Développement, Dakar, Senegal). The total amount of carbon and nitrogen was quantified by using the combustion system Thermo-Finnigan Flash EA 1112 (Thermo-Finnigan, France). The colorimetric determination of total and available phosphorus was performed according to the method of Dabin (1965). Soil pH values were measured in 2 M KCl suspensions at a solid liquid ratio of 1:2, 5. Soil physical characteristics were determined according to the method of Gee and Bauder (1986) and exchangeable cations following the method of Thomas (1982).

Plant harvested and analyses

Root sampling and assessment of AM colonization

After pod maturity (55 days after sowing), 10 cowpea plants were randomly chosen in each experimental plot, representing a total of 50 plants per field. Fine roots of 10 pooled plants (5 replicates per field) were collected, gently washed and stored in alcohol 70°C. Before assessment of AM colonization, roots were washed, cleared in KOH (10%) and stained with 0.05% Trypan blue (Philips and Hayman, 1970). Roots were then cut into pieces of 1 cm and placed on slides for microscopic observation at 400x magnification (Abbas et al., 2006). The frequency and intensity of mycorrhization were assessed following the method of Trouvelot et al. (1986) on 50 pieces per sample (250 root pieces per field).

AM fungal spore extraction, enumeration and morpho-anatomical identification

AMF communities were studied by spore extraction from soil samples. Hence 100 g soil (dry weight) of each subsample (with three replicates) was wet-sieving, following the methodology proposed by Gerdemann and Nicholson (1963). Samples were centrifuged in a sucrose gradient (Walker et al., 1982). Depending on the color, size, solitary character or grouped spores, mode of attachment of the hyphae and specific attributes, the spores were grouped into morphotypes. The relative abundance of each morphotype and AMF spore density (total number of spores in 100 g of dry soil) were determined with a normal appearance under a compound microscope (40x).

For taxonomic identification, AMF spores were mounted onto slides in polyvinyl alcohol (PVA) (Omar et al., 1979) with or without Melzer reagent (Morton, 1988). Specimens were identified with the original description and reference isolates described by the International Culture Collection of Arbuscular and Vesicular-Mycorrhizal Fungi (http://invam.wvu.edu/the-fungi/species-descriptions). In most cases, identification of the samples was assessed by the observation of morphological features of spores obtained after sieving and decanting.

Molecular analyses of AMF colonizing cowpea roots

For molecular analysis, roots were stored in alcohol at 70°C prior to DNA extraction. Fine roots from each field were washed, cut into pieces and pooled to get a composite root sample. DNA was extracted from 80 mg of the composite root sample (per field) using Fast DNA SPIN Kit following the manufacturer's recommendations. The DNA extraction (from 80 mg of roots) was repeated three times for each composite sample. The DNAs extracted from the three replicates were mixed in equimolar quantities to get a composite sample by taking into account the concentration.

The partial small subunit (SSU) region of the nuclear rDNA was used as a target region for the PCR experiment. DNA was amplified in a 25 µl reaction volume containing 5 µl of reaction buffer 5X ($MgCl_2$ 1.5 mM), 1 U of GoTaq polymerase, 200 µM dNTPs, 0.5 µl of BSA (200 ng/µl), 0.5 µl of each primer (20 µM), 14.05 µl of sterile water and 2 µl DNA template. The DNA amplification was performed using the universal eukaryotic primer NS31 (5'-TTG GAG GGC AAG TCT GGT GGC-3', Simon et al., 1992) and a fungal primer AML2 (5'-GAA CCC AAA CAC TTT GGT TTC C-3', Lee et al., 2008), designed to target as specifically as possible the *Glomeromycota*. The PCR was carried out as follows: an initial denaturation at 94°C for 3 min followed by 30 cycles at 94°C for 30 s, 58°C for 1 min, 72°C for 1 min 20 s, followed by a final extension period at 72°C for 10 min. PCR products were analyzed by electrophoresis using a 1% w/v agarose gel (Sigma, France) in a Tris-Acetate-EDTA buffer with a size standard (Eurogenic Smart Ladder). PCR products were then purified with the PCR purification kit (Invitrogen) according to the manufacturer's instructions. The purified PCR products were cloned into ultra-competent cells XL2 blue using PGEM-T Easy Vector (Promega/Catalys, Wallisellen, Switzerland) following the manufacturer's instructions. Twenty four Putative positive clones were selected and amplified using M13 forward and M13 reverse primers. Purified PCR products were then sequenced with the universal primers M13 F/M13 R (Genoscreen, France).

The sequences of clones were corrected using the Chromas software and compared by BLAST (Basic Local Alignment Search Tool) with those deposited in the NCBI (National Center for Biotechnology Information) data bank. All sequences obtained from this study were aligned using BioEdit software along with reference sequences from GenBank. Corrected sequences were submitted to GenBank and were allocated with accession numbers (KC588975 to KC589000). The clones from supprimer site of Ngothie site were called DEKRA and those from Diokoul site, DIORRA. The maximum likelihood (ML) tree was constructed using PHYML. The Kimura 2-parameter model with 100 bootstraps was used. The phylogenetic tree was computed using *Mortierella polycephala* (accession number X89436) and *Endogone pisiformis* (accession X58724) sequences as outgroups. Sequence phylotypes (called OUT in the text) were defined as separated monophyletic groups in the phylogenetic tree (Hijri et al., 2006).

Statistical analysis

Species richness, Shannon and Wiener diversity index (H'),

Table 1. Physicochemical characteristics of soils from the two study sites.

Soil characteristics	Ngothie Site (Dek soil)	Diokoul Site (Dior soil)
Clay (%)	4.4	1.3
Silt (%)	19.8	13
Fine sand (%)	50.6	29.2
Coarse sand (%)	14.1	55.7
Nitrogen Kjeldahl (%)	574	114
N(NO_3) mg/kg	9.9	1.4
N(NH_4) mg/kg	14.74	6.77
Total N (%)	0.055	0.022
Total C (%)	0.55	0.22
C/N	10	10
Total P (mg/kg)	139	51
Soluble P (mg/kg)	15	8
pH(H_2O)	6.1	5.8
pH (KCl)	5.5	4.8

(E) and Simpson dominance index (D) were calculated for each field in order to assess the diversity of AMF spores. The Shannon and Wiener diversity index was calculated using the following equation: $H' = -\sum p_i (\ln p_i)$ where *pi* represents the proportion of individuals found in the *i*th species, estimated as *ni /N*, *ni,* the number of individuals in the *i*th species and *N,* the total number of individuals. For a better interpretation of the results, the inverse of the Simpson dominance index was calculated as follows: $1\text{-}D = 1-\sum [ni (ni-1)/N (N-1)]$, where ni represents the number of the ith type and N the number of individuals in the population. The species distribution was determined by calculating the evenness using the following formula: $E = H'/H_{max}$ where *Hmax* is the maximum diversity and is calculated by ln *S*, where *S* is the number of species recorded.

Percentage of root colonization rate was arcsine transformed prior to analysis. Data were subjected to a one-way analysis of variance (ANOVA) using the XLSTAT software version 2010. Mean values were compared using the Student-Newman-Keuls range test ($P<0.05$) in order to determine the effects of site on these parameters.

Data on AMF spore density and relative abundance without any prior processing were also subjected to a one way analysis of variance using the same software. Mean values were compared using the Student-Newman-Keuls range test ($P < 0.05$). This analysis was performed to determine the effect of site in the spore density.

RESULTS

Physicochemical characteristics of soils

Results of soil physicochemical analyses are presented in Table 1. Data showed that both soils were acidic. The soil from Diokoul site (Dior) was slightly more acidic than those from Ngothie site (Dek). The highest percentage of organic and all mineral nutrient contents were observed in soil from Ngothie site. The percentages of fine sand and clay were higher in soil from Ngothie site whereas that of coarse sand was greater in soil from Diokoul site.

AMF spore density, species richness and diversity measurement of AMF spores communities

The AMF spore density varied between the two sites (Figure 1). The AMF spore number was significantly ($P=0.02$) greater in soil from Ngothie (636 spores/100 g of soil) than in soil from Diokoul (293 spores/100 g of soil).

In both sites, the highest spore density was recorded for *Entrophospora* sp. with 254 and 197 spores per 100 of dry soil for Ngothie and Diokoul sites, respectively. However, the lowest spore number was obtained with *Glomus* (7 spores/100 g of dry soil) in soil from Ngothie, and with *Scutellospora* in soil from Diokoul site (2.67 spores/100 g of dry soil).

In both sites, a total of ten AMF morphotypes belonging to seven genera were recorded and *Glomus* represented the most diverse genus with three morphotypes (Table 2). The number of AMF morphotypes (Table 2) showed that the soil from Ngothie site displayed 9 AMF morphotypes and that from Diokoul site, 8 AMF morphotypes belonging to seven genera (*Glomus*, *Scutellospora*, *Gigaspora, Entrophospora*, *Acaulospora, Sclerocystis* and *Rhizophagus*). From the ten AMF morphotypes, seven were common to the two fields whereas *Glomus* sp1 and *Rhizophagus* sp. were isolated only in Ngothie site and *Sclerocystis* sp. only in Diokoul site.

Three diversity indexes were calculated to characterize the communities of AMF in both soils. Shannon-Weiner (H') and Simpson's indices showed variation between the two sites and were higher in Ngothie site than in Diokoul site. However, the species evenness was greater in Diokoul site than in Ngothie site (Table 3).

Intensity and frequency of mycorhization

Before exploring the diversity of AMF in the roots, mycorrhizal parameters of cowpea roots (intensity and frequency of mycorrhization) were first determined. Results showed that the cowpea root mycorrhizal colonization was significantly different between the two sites ($P<0.05$). The higher cowpea root mycorrhizal colonization was recorded in the field installed in Ngothie site (100 and 25% for frequency and intensity, respectively) compared to that of the field located in Diokoul site (60% and 5% for frequency and intensity, respectively) (Figure 2).

Community composition of AMF colonized roots of cowpea grown in both soils

A total of 42 clones obtained from root samples from Ngothie site and 38 clones for samples from the Diokoul

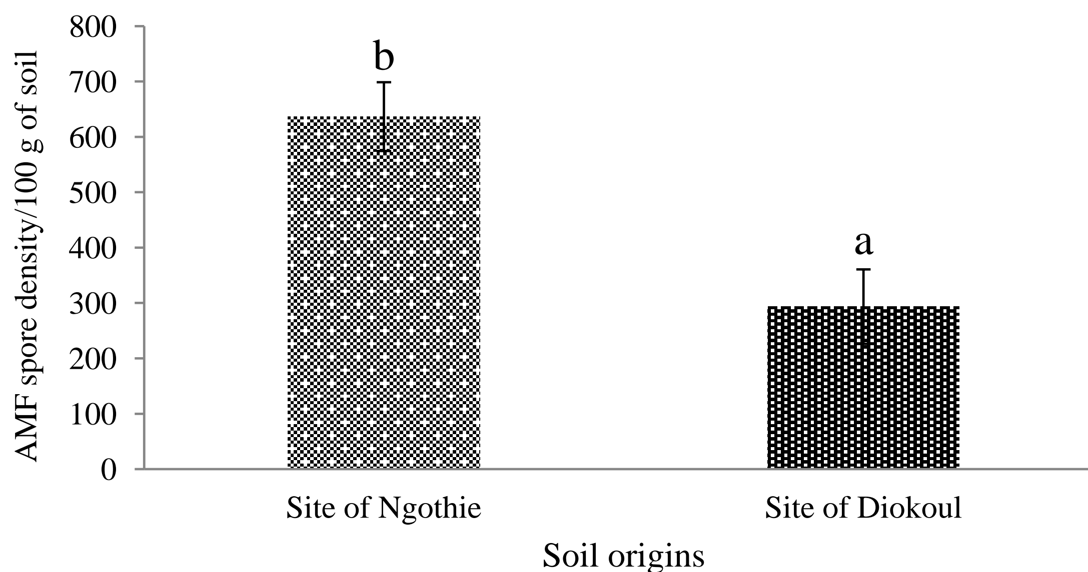

Figure 1. AMF spore density in rhizospheric soils of two cowpea growing fields. Bars with the same letter are not significantly different according to the Student-Newman-Keuls test ($P < 0.05$).

Table 2. Average density of each arbuscular mycorrhizal fungi (AMF) species or morphotype in 100 g of dry soil from the two study sites.

Parameter	Ngothie site (Dek soil)	Diokoul site (Dior soil)
Scutellospora gregaria	8.33	6.67
Scutellospora heterogama	51.33	2.67
Gigaspora spp.	46.00	31.66
Acaulospora longula	72	28.67
Entrosphospora spp.	254.33	198.00
Sclerocystis sp.	0.00	6.00
Glomus coronatum	13.67	4.3
Glomus sp1	7.00	0.00
Glomus spp.	167.00	15.33
Rhizophagus sp.	17.00	0.00
Number of AMF morphotypes	9	8

Gigaspora spp., *Entrophospora* spp. and *Glomus* spp. are considered as morphotypes because it would include more species. *Rhizophagus* sp, *Sclerocystis* sp and *Glomus* sp1 have not been identified and we gave them respectively the names *Rhizophagus* sp. and *Glomus* sp. *Sclerocystis* sp1.

Table 3. Diversity measurements of AMF communities from spores in soils from the two fields.

Parameter	Equations	Ngothie site (Dek soil)	Diokoul site (Dior soil)
Shannon Weiner index	$H' = -\sum p_i (\ln p_i)$	0.713	0.503
Simpson index of dominance (D)	1-D =1-∑ [ni (ni-1)/N (N-1)]	0.746	0.719
Evenness (E)	H'/H_{max}	0.748	0.757

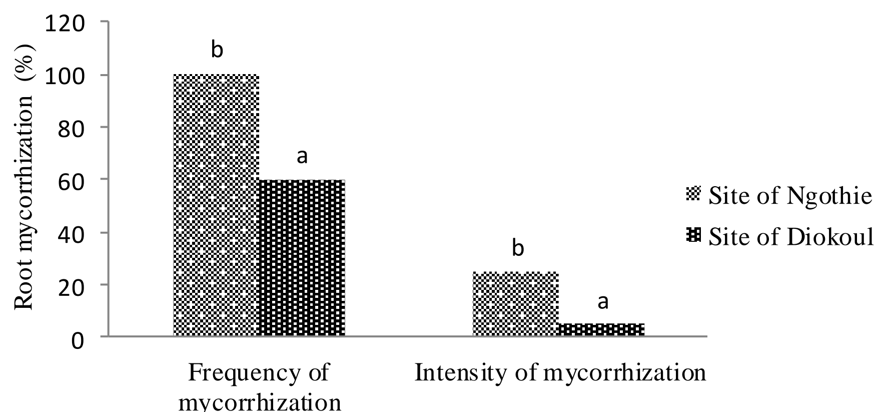

Figure 2. Mycorrhizal frequency and intensity of cowpea roots in the two fields. For each parameter, bars with the same letter are not significantly different according to the Student-Newman-Keuls test ($P < 0.05$).

site were sequenced. The number of clones identified as sequences of arbuscular mycorrhizal fungi in root samples were 22 for the soil from Ngothie site and only 4 for the soil from Diokoul site with a similarity percent of 52.38 and 10.52%, respectively (Figure 3). The number of clones identified as *Mucuna pruriens*, a *Fabaceae* close to the studied plant (*V. unguiculata L.*) is 18 and 30, respectively for the roots from Ngothie site and Diokoul site, respectively with a percentage of similarity comprised between 99 and 100%. Two and four clones were identified as others, respectively for soil from Ngothie site and that from Diokoul site.

The phylogenetic tree was built from alignment of the partial SSU fragments of about 560 nucleotides generated by NS31 and AML2. Different clades of the tree have allowed distinguishing 15 AMF OTU (operational taxonomic unit) belonging to seven known genera: *Scutellospora*, *Gigaspora*, *Redeckera*, *Acaulospora*, *Glomus*, *Sclerocystis* and *Rhizophagus* (Figure 3). The OUT *Gigaspora* (KC588982 and KC588994) formed a separate group in the family *Gigasporaceae* but their position was not well-defined. The different genera identified belonged to four families: *Gigasporaceae* (*Scutellospora*, *Gigaspora*, and *Racocetra*), *Diversisporaceae* (*Redeckera*), *Acaulosporaceae* (*Acaulopora*) and *Glomeraceae* (*Glomus*, *Sclerocystis, Rhizophagus*). These four families belonged to two orders: Diversisporales (*Gigasporaceae*, *Acaulosporaceae* and *Diversisporaceae*) and Glomerales (*Glomeraceae*). The *Glomeraceae* and the *Gigasporaceae* are the most diverse families as compared to *Acaulosporaceae* and *Diversisporaceae,* each represented by a single genus. Also, the results indicated that the sequences of the AMF found in the roots of cowpea were different from known AMF species listed in GenBank database.

Shannon-Weiner index (H') based on DNA sequences of AMF revealed differences between both root origins. This index was relatively higher in samples from Ngothie site (1.06) compared to those from Diokoul site (0.45).

DISCUSSION

The root DNA amplification and sequencing with specific primers revealed the presence of several AMF species in the roots of cowpea. These AMF species belonged to eight genera, which are *Gigaspora*, *Scutellospora*, *Acaulospora*, *Glomus*, *Sclerocystis*, *Redeckera*, *Rhizophagus* (Schüßler and Walker, 2010) and *Racocetra* (Oehl et al., 2008). These results confirmed the ability of the primer AML2 to amplify several groups of AMF and indicated a wide diversity of AMF in roots of cowpea (Lee et al., 2008). However, these findings were opposed to those of Manga et al. (2007) and Ndoye et al. (2012) who found only species of the genus *Glomus* in rhizosphere of *Acacia senegal* in the semi-arid zone of Bambey and Dahra (Senegal), respectively. Nevertheless, Ngonkeu (2009) using the same fungal primers as the previous authors (LR1, FLR4), had already found some Gigasporaceae and Acaulosporaceae species in the roots of corn in Cameroon. These results might be explained by a selective mycorrhization (Helgason et al., 2002) in acacia trees because other species of fungi were found in their root zone particularly in *Acacia senegal* (Ndoye et al., 2012). The genus *Glomus* is the most diverse in our study with six species, representing 40% of all the species found in roots of cowpea. The predominance of this genus in most ecosystems like Senegal (Manga et al., 2007; Sene et al., 2012 ; Ndoye et al., 2012), China (Zhao and Zhao, 2007; Wang et al., 2008), Burkina Faso

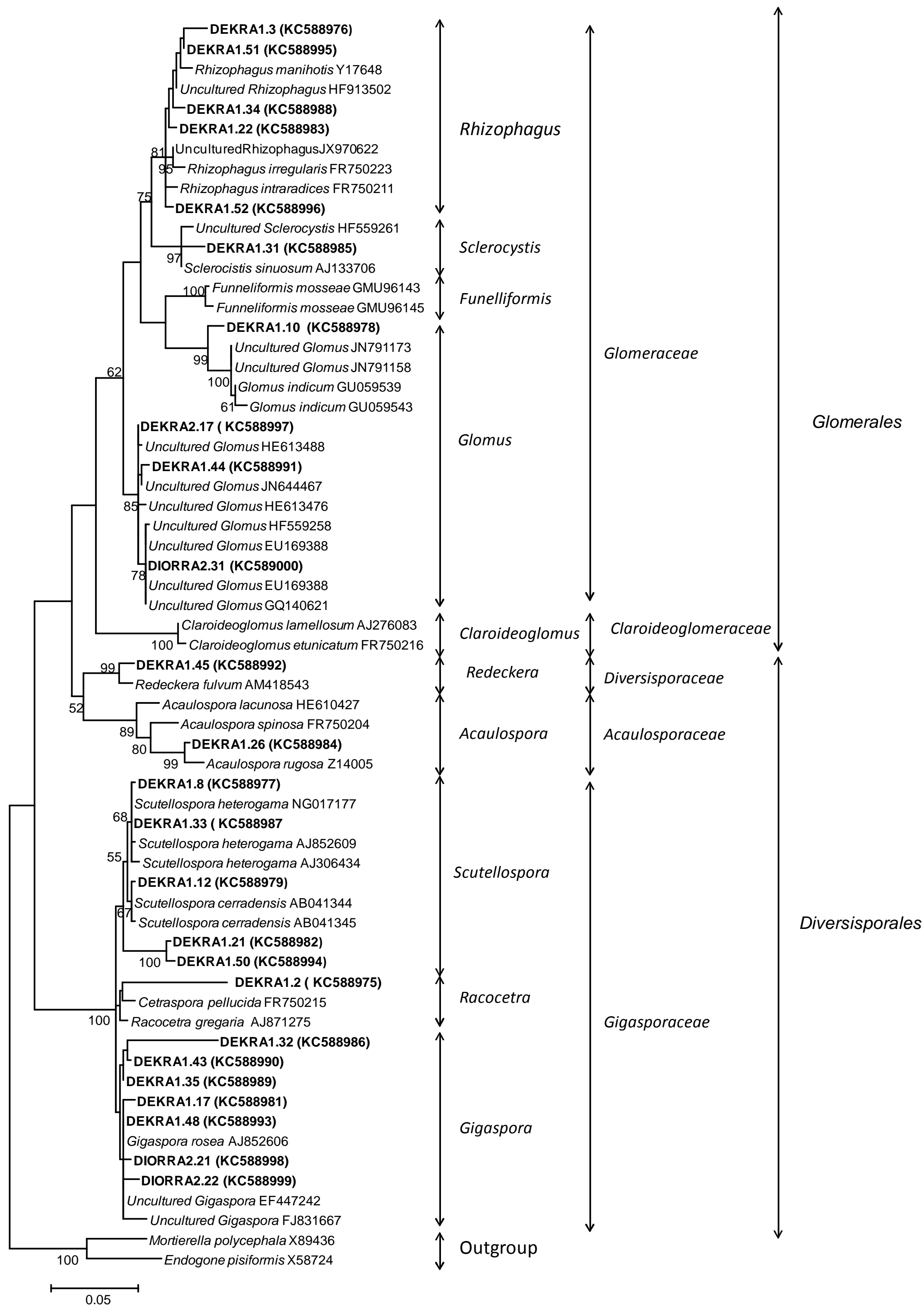

Figure 3. Maximum likelihood tree showing all sequences identified in cowpea roots. Sequences obtained in the present study are shown in bold. Bootstrap support values for branches were estimated from 1.00 replicates. The corresponding accession numbers ranging from KC588975 to KC599000 were given by Genbank. DEKRA means clones from soil DEK (Ngothie site) and DIORRA clones from DIOR soil (Diokoul site).

(Bâ et al., 1996) and central Europe (Oehl et al., 2003) suggests a better adaptation of the genus to adverse conditions such as warm, cold, drought, salinity and other environmental stress (Blaszkowski et al., 2002). The dominance of *Glomus* in these agro-ecosystems might also be related to their ability to form anastomoses after hyphae break. Indeed, as compared to Gigasporaceae, the Glomeraceae form more easily anastomoses between hyphae of the mycelium and may have the ability to restore an interconnected network after mechanical disruption of the hyphae (de la Providencia et al., 2005).

Furthermore, our results indicated that the clones close to uncultured *Gigaspora* EF447242 is the most frequent species encountered in the roots of cowpea with eight sequences out of the twenty-two, representing 27.27% of the all clones. In other cases, by studying the seasonal and spatial trends of fungal populations, Merryweather and Fitter (1998) reported the dominance of *Scutellospora dipurpurescens* in the roots of *Hyacinthoides non-scripta* in autumn and winter while *Acaulospora* and *Glomus* were dominant in spring. Dominance of *S. dipurpurescens* will be explained by its positive correlation with shoot P content and carbohydrates availability for the fungus. This important contribution to the absorption of P in Gigasporaceae at the expense of the other groups of fungi was noted by Maherali and Klironomos (2007). This is due to their ability to produce root outside a large amount of hyphae able to explore a large volume of soil (Hart and Reader, 2002; Maherali and Klironomos, 2007).

Also, this strong presence of the genus *Gigaspora* in the roots of cowpea could be explained on one hand by its strong presence in the soil and on the other hand by the composition of root exudates. Indeed, Redmond et al. (1986) and D'Arcy-Lameta (1989) demonstrated that flavonoids are produced exclusively by leguminous plants.

These compounds have often been associated with the attraction of *Gigaspora margarita* and *Glomus* spp. spore germ tubes by plant roots (Tsai and Phillips, 1991; Bécard et al., 1992). This can promote root colonization by these AMF, promoting spore germination.

As demonstrated by Lee et al. (2008), the results have also shown that the primer AML2 was not specific to *Glomeromycota*. Based on the fact, coupled with NS31, it has amplified DNA of other organisms and DNA of plant, as attested by the blast result (18 clones in soil from Ngothie site and 30 in soil from Diokoul site were identified as *Mucuna pruriens* (tribe of Phaseoleae). Therefore, there is a very high probability of amplifying non-specific DNA products during PCR, especially with environmental material from field soils (Clapp et al., 2002). However, other primers such as AML1 (Helgason et al., 1998) were not exclusive of *Glomeromycota*, amplifying other taxa like parasitic fungi (Redecker, 2000; Lee et al., 2003).

Our results on the mycorhization rate, spore density and Shannon diversity indices might be explained by soil properties from the two sites. It is long recognized that very high or very low phosphorus levels may reduce mycorrhization and/or sporulation. The lower rate of mycorrhization and the low sporulation in soil from Diokoul site could be due to low soil P level in comparison with Ngothie soil. These results are in accordance with those of Pierart (2012) and Lagrange (2009) which showed that the very low P level inhibits the mycorrhization.

Apart from soil phosphorus, the difference noted in the diversity of AMF at the two study sites might be due to the surrounding flora. This flora is composed of an herbaceous stratum and shrub stratum. The shrub stratum is mainly composed of *Faidherbia albida* whose density is greater on the site of Ngothie (in Dek soil) than in that of Diokoul (in Dior soil). These acacia trees are usually associated with a great diversity of arbuscular mycorrhizal fungi (Belay et al., 2013) that might allow active AMF dispersal due to the growth speed of the root system (Diop et al., 1994).

Also, a slight modification of species composition can be noted with the presence of *Andropogon gayanus* only on the site of Ngothie. This difference in floristic composition might influence the diversity of arbuscular mycorrizal fungi (Diallo et al., 1998).

Conclusions

The results of this study indicate that cowpea plant is associated with a range of AMF belonging to eight genera and four families. Also, the results showed that a species of *Gigaspora* close to uncultured *Gigaspora* EF447242 was largely dominant in roots of cowpea (27% of clones in Ngothie site and 50% in Diokoul site). However, the family of Glomeraceae was the most represented and the most diverse with 53.3% of AMF species. The results suggested that ecological site conditions may influence AMF diversity and colonization rate *in planta*.

Conflict of interests

The authors did not declare any conflict of interest.

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Natural circulation of human enterovirus in Maputo city, Mozambique

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The human enteroviruses (HEV) are responsible for a large diversity of infections affecting humans. Most infections are asymptomatic, but these viruses can cause a wide spectrum of diseases, including severe cases involving the central nervous system. The aim of this study was to isolate and identify human enteroviruses in natural circulation in children less than 15 years of age admitted at the Mavalane General Hospital in Maputo City, Mozambique. In this study, 178 stool samples were processed, obtained during November 2011 to February 2012. Samples were inoculated onto cell culture and the isolates were identified as enterovirus by conventional RT-PCR in the 5' non-coding region followed by partial VP1 sequence. Twenty-six (26) out of the 45 cell-culture positive samples were constituted by *Enterovirus* (14.6% of the total 178 samples). EV-29 was the serotype most prevalent. The results show the importance of maintaining the cell line Hep2C in the diagnosis and *Enterovirus* circulating in the Maputo city, Mozambique.

Key words: Enterovirus, Stool, Cell culture, cell line Hep2C, Hospital Geral de Mavalane.

INTRODUCTION

The human enteroviruses (HEV) belong to the genus *Enterovirus* and *Picornaviridae* family. These agents infect millions of people worldwide each year, resulting in a wide variety of clinical conditions ranging from unapparent infection, undifferentiated fevers, common cold to serious diseases such as aseptic meningitis, hand-foot-mouth disease, acute hemorrhagic conjunctivitis, myocarditis, encephalitis and paralytic poliomyelitis (Pallansch and Roos, 2001). Children are more susceptible to infection and transmission occurs either by the fecal-oral or respiratory tract. The virus can be excreted in the feces for several weeks (Pallansch and Roos, 2001).

There are more than 80 species of HEV divided into four group, designated HEV- A to D based on the genetic characteristics of the VP1 capsid gene (Oberste et al., 2006; Knowles et al., 2011). In general, the 5' and 3' noncoding regions (NCRs) are highly conserved, and the most variable region of the genome lies within the genes encoding the capsid proteins exposed on the virus surface, VP1, VP2, VP3, and VP4 (Hogle et al., 1985; Racaniello, 1988; Oxman, 1999).

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Molecular epidemiology of enteroviruses (EVs) is poorly known in Southern Africa, particularly in Mozambique. Therefore, the aim was to isolate and identify human enteroviruses from stool samples of children less than 15 years of age receiving medical attention in Mavalane General Hospital in Maputo city, Mozambique and to understand the natural circulation of HEV.

MATERIALS AND METHODS

Ethics statement

National Bioethics Committee on Health of Mozambique approved the study with the reference: 280/CNBS/11. All study participants were under 15 years old and parents or guardians authorized written consent for their participation by signing an informed consent form where each party stayed with a copy.

Patients and specimens

A cross-sectional study involves enrolling a total of 178 stool specimens from children under 15 years old. Clinical specimens were collected from pediatric patients admitted in general hospital in Maputo City, Mozambique in the period from November 2011 to February 2012, with at least three or more of these signs and symptoms: fever, vomiting/nausea, difficulty breathing, cough, diarrhea, headaches and joint pain. Patient information's were recorded on a standard questionnaire including demographic and clinical details. Specimens were transported in dry ice to the Enterovirus Laboratory, Oswaldo Cruz Institute/Fundação Oswaldo Cruz/Rio de Janeiro, Brazil.

Virus isolation

Fecal suspensions were treated with chloroform as previously described (WHO, 2004). Each specimen extract (0.2 ml) was inoculated in duplicate tubes of the three cell culture lineages, RD, L20B and Hep2C, as recommended (WHO, 2004). The tubes were incubated at 37°C and examined daily for evidence of cytophatic effect (CPE) and these were accompanied by negative control. Positive cultures were harvested and kept frozen (-20°C) until further analysis.

Molecular characterization and Enterovirus genotyping

Isolates showing characteristic enterovirus CPE were subject to *in vitro* amplification by RT-PCR using enterovirus primers as previously described (Dos Santos et al., 2012). The Pan-EV pair of primers (PanEVF and PanEVR) used in one-step conventional RT-PCR, are broader reactive for the human enterovirus genus targeting conserved nucleotide sequences at the 5'-NCR of the EV genomes. Briefly 1.0 µl of sample was added to 3.2 µl reaction mixture A (containing 2.5 µl of 10× PCR buffer and 0.7 µl of reverse primer at 50 pmol/µl). The reaction mixture was first incubated at 95°C for 5 min and then chilled in ice for 5 min. The reaction mixture B containing 0.5 µl of each dNTP´s 10 mM, 0.15 µl of RNASin at 5 U/µl, 0.7 µl of foward primer, 0.2 µl of transcriptase reverse at 20 U/µl, 0.2 µl of Taq DNA polymerase at 5 U/µl, 19.05 µl of water were added to mixture A. The tubes were placed in thermo cycler (Veriti TM 96-well Thermal Cycler) and cycled as follows: 42°C for 30 min, 30 cycles at 95°C for 45 s, 55°C for 45 s and 72°C for 45 s, and these were accompanied by negative and positive control of the reaction, respectively.

All positive samples in Pan-EV were subjected to Pan-PV to test for the presence of polioviruses, as previously described (WHO, 2004). All isolates was identify by partial VP1 sequence (Oberste et al., 1999; Oberste et al., 2003). After edition, sequences were compared with those located at GenBank using the Blast Program 2.2.27 for confirmation of the viral identity and species determination (Altschul et al. 1990).

A phylogenetic three was constructed based on MEGA 4.0.2 program (Kumar et al., 2001) using the Neighbor-Joining reconstruction method (Saitou and Nei, 1987). The genetic distances were estimated to using Kimura-Two parameter model (Kimura, 1980). The robustness of the branches in the phylogenetic tree was statistically evaluated by 1,000 bootstrap replicates (Felsenstein, 1985).

All samples negative by RT-PCR PAN EV were subjected to PCR to search Adenovirus members because in cell line Hep2C showed a highly suggestive adenovirus cytopathic effect for virus isolation in cell culture. The Adenoviruses were screened through a PCR reaction using specific primers previous described (Xu et al., 2000).

RESULTS

A total of 45 (25.3%) samples had viral isolation in RD, L20B or Hep2C cell lines although it was not homogenous. The EV was identified in 14.6% of the samples (26/178). Molecular typing based on partial VP1 sequence showed a wide diversity of human enterovirus with 16 types detected (Table 1). The HEV-B was the most prevalent. Echovirus 29 (E29) serotype was presented in 5/26 (19.2%) of the EV samples, followed by EC99 in 3/26 (11.5%). The male and female ratio for enterovirus positivity was 1.8:1. Among the 26 participants who had enterovirus positive samples, 18 were from the age group of 0-24 months. The reported signs and symptoms (fever and vomiting/nausea, coughing) of participants show that the clinical manifestations were similar and occurred irrespective of the enterovirus group, HEV-A, B or C, detected (data not shown).

Two samples (32 and 163) were identified Poliovirus sabin like. The complete sequence of the gene encoding the protein VP1 of the two samples identified as Poliovirus Sabin like was performed to detect mutations that may be associated with reversion to virulence of the vaccine strains poliovirus. The sample 32 was identified as Poliovirus Sabin like 2 while sample 163 Poliovirus Sabin like 3 with 99.7% identity with the vaccine strain. Both samples were collected from children at one month of life, the first of which had record of having received polio vaccine and the second had no history of having received the vaccine.

The enteroviruses were distributed along all months of sample collection as follows: November (n=9, 34.6%); December (n=5, 19.2%); January (n=4, 15.4%); February (n=8, 30.8%) and E-29 was the only virus detected in all months (data not shown).

Adenovirus PCR reaction and run electrophoretic run gave a fragment of 134 pb in 20 of the 23 samples. The samples positive for adenovirus was 11.2% (20/178)

Table 1. Enterovirus serotypes identified, and socio-demographic data of children younger than 15 years old.

Species and type		Cell line			Sex	Type of feed	Reason for hospitalization
		RD	L20B	Hep2C			
HEV-A	3						
Coxsackievirus A5	2	CPE	NCPE	CPE	M	3	Malnutrition
Coxsackievirus A10	1	CPE	NCPE	NCPE	M	2	GEA
HEV-B	14						
Coxsackievirus B4	1	CPE	NCPE	CPE	M	3	Malaria
Coxsackievirus B6	1	CPE	NCPE	CPE	F	3	Malaria
Echovirus 7	1	CPE	NCPE	CPE	M	2	Malnutrition
Echovirus 11	2	CPE	NCPE	CPE, NCPE	M, F	3	Malnutrition, BPN
Echovirus 13	2	CPE	NCPE	NCPE	M	2, 3	Malnutrition, BPN and GEA
Echovirus 21	1	CPE	NCPE	CPE	M	3	Malaria
Echovirus 25	1	CPE	NCPE	CPE	F	3	Malaria
Echovirus 29	5	CPE	NCPE	NCPE	M, F	3	Malnutrition, Malaria
HEV-C	9						
Coxsackievirus A13	3	NCPE	NCPE	CPE	M, F	2, 3	Malaria, BPN
Coxsackievirus A20	1	NCPE	NCPE	CPE	M	2	BPN
Enterovirus C99	3	NCPE	NCPE	CPE	M	3	BPN
Poliovirus Sabin like 2	1	CPE	NCPE	NCPE	M	1	BPN
Poliovirus Sabin like 3	1	CPE	CPE	CPE	M		

F = Female; M = Male; Type of feeding: 1 = Only breast milk; 2 = Mixed (breast milk and other food; 3 = Diversified (not breast milk); Cell lines: RD= Human Rhabdomyosarcoma; L20B= Transgenic mouse cell lines (L20B and Lα) expressing the human poliovirus surface receptor; Hep2C= Human larynx epidermoid carcinoma; CEP = cytophatic effect; NCEP= non cytophatic effect; BPN = Bronchopneumonia; AGE = Acute gastroenteritis,; HEV-A = Human Enterovirus Specie A; HEV-B= Human Enterovirus Specie B; HEV-C = Human Enterovirus Specie C; PV* = *Poliovirus vaccine strain Sabin like.*

(data not shown) and only two samples (75 and 128) showed co-infection between enterovirus and adenovirus and both the species HEV-B.

DISCUSSION

This is the first study that reports the natural circulation of *Enterovirus* in Mozambique. The isolation in cell culture shows a low permissive cell line RD for members of the species EV-C and confirms the interest of maintaining the cell line Hep2C in the diagnosis for monitoring the circulation of *Enterovirus* as evidenced by Bessaud and colleagues (2012).

The results of the molecular identification demonstrate the existence of serotypes; the species HEV-B (54%), and within this specie, E29 was most identified. In fact, the HEV-B was the most identified enterovirus in previous studies conducted in Taiwan and Central African Republic (Lo et al., 2010; Bessaud et al., 2012).

Overall, the frequency of human enteroviruses was 14.6%, and this data is also consistent with previous report, conducted in Brazil, Australia, Korea and Egypt that reported 15-17.6% (Santos et al., 2002; Kelly et al., 2006; Roh et al., 2009; Afifi et al., 2009). Nevertheless, some studies have reported prevalence above 20% in England and Pakistan (Iturriza-Gomara et al., 2006; Saeed et al., 2007).

A higher frequency of enterovirus isolation in male children was observed although this finding was consistent with the larger number of male participants (1.8:1). Enterovirus infection has been reported more frequently in males compared to females (Moore and Morens, 1984; Gondo et al., 1995; Dechkum et al., 1998).

The phylogenetic analysis of the two samples Poliovirus Sabin like had 99.7% nucleotide identity with strain Poliovirus Sabin 2 and 3 isolated by Rezapkin and colleagues (2002). However, for the sample 32, although the participant had received dose of trivalent oral polio vaccine, this fact can be explained by more efficient replication of live attenuated poliovirus type 2 in the gastrointestinal tract, among serotypes that comprise the vaccine (Modlin, 1995).

In sample 163, according to the information collected during the survey, the participant did not receive the vaccine poliomyelitis. This event is predictable in countries or areas where the trivalent oral polio vaccine is used, which is one of the advantages of oral vaccination by enabling the expansion of vaccine virus in other subjects and the environment, conferring protection by

inducing production of antibodies not only serum but also an intestinal resistance and speed with which people vaccinated develop a long immunity (Melnick, 1996; da Silva et al., 2005).

However these findings reinforce the necessity of epidemiological surveillance of poliovirus in Mozambique. In 2011 there was two cases of virus circulation derived polio vaccine (cVDPV) serotype 1 in the region center of the country although the present study was conducted in the southern region of Mozambique (Conceição et al., 2011; MISAU, 2012).

The overall period of study and sample collection was short (November, 2011 to February, 2012), which did not allow a seasonal analysis, which would be important in future studies.

Most (70%) of the enterovirus cases were detected in children up to 24 months of age. In fact, these age groups (0-24 months/old) are subject to different susceptibility, disease severity and prognosis of infections and the peak of enterovirus infections occur in the first two years of age (Pallansch and Roos, 2001).

In summary, this finding shows the importance of maintaining the cell line Hep2C in the diagnosis and existence of serotypes of human enteroviruses in natural circulation in Maputo city.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

The effect of nano-TiO_2 and plant extracts on microbial strains isolated from Theban ancient Egyptian royal tomb painting

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Mural paintings in ancient Egyptian tombs in West Thebes have been suffering from several deterioration factors and symptoms such as variations of temperatures and relative humidity, salts efflorescence and crypto-florescence, crackling and bio-deterioration effects, which assimilate in insects, algae, actinomycetes, etc. Other causing factors are bacteria and fungi, which accelerate mechanical weathering, chemical changes and aesthetic deterioration, like the penetration of mycelium below plaster layers, decomposition, disintegration, alterations and discoloration. These micro-organisms can excrete organic and inorganic acids, alkaline compounds, chelating, enzymes substances and pigments. Three fungi strains (*Fusarium oxysporum, Rhizopus stolonifer* and *Aspergillus flavus*) and two bacteria strains (*Staphylococcus warnei* and *Micrococcus luteus*) were isolated from the royal Theban tombs paintings (west Thebes, Luxor, Egypt). This work aimed to access the presence of microorganisms and their effect on mural paintings deterioration; it also studies their treatment methods, such as nanoparticles (TiO_2 NPs) and *Sesbania sesban* and *Ricinus communis* plant extract (PE). The applied doses of NPs and PE did not cause any observable alterations or color changes to pigments and binding media (arabic gum) used in the paintings. TiO_2 NPs 160 ppm and 100 mg of plant extracts were the efficient concentration level in eliminating microbial growth. The causes of the different efficacy of the treatments are observed, as well as the potential risks of recolonization by viable cells left behind after treatment.

Key words: Bio-deterioration, Plant extracts, Wall painting, West Thebes tombs-Nanoparticles.

INTRODUCTION

Bio-deterioration can be defined as “any undesirable change in a material brought about by the vital activities of organisms” (Allsopp, 2011). Bacteria, actinomycetes and fungi are constantly causing problems in the conservation of mural paintings tombs because of their biodeteriorative potential. The Thebes town is one of the largest and famous archaeological sites in the world. It lies about 900 km South of Cairo on the banks of the

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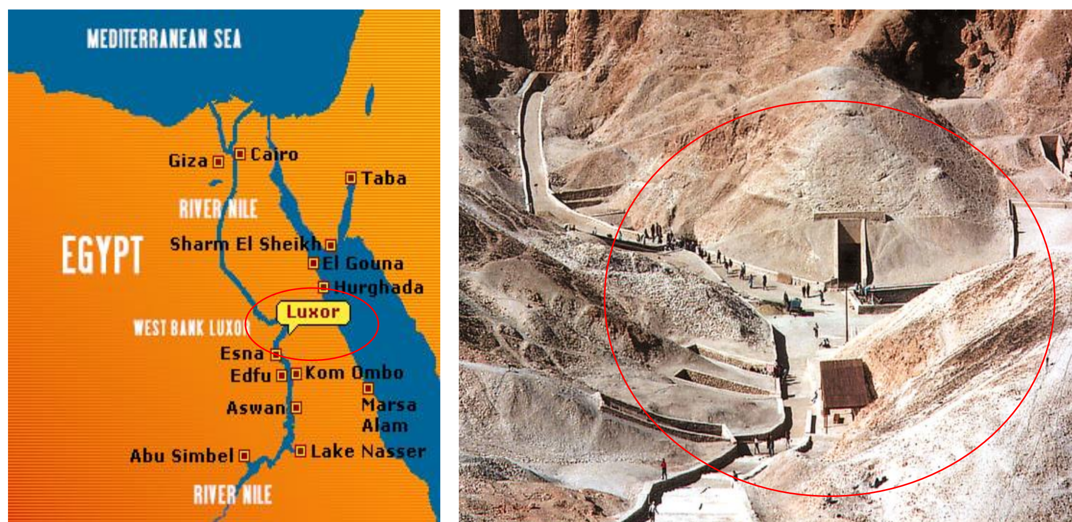


Figure 1. Plan of Luxor and west bank Luxor.

River Nile. The tradition of wall painting is very old, dating back to prehistoric times (Garg et al., 2010). Royal tombs in Thebes (west Bank, Modern Luxor) are as considered one of the most important archaeological features in Egypt and the world over. That is, where this archaeological site is the most important period of Egyptian history, tombs were constructed for the Pharaohs and powerful nobles of the New Kingdom (the Eighteenth to the Twentieth Dynasties of Ancient Egypt). Wall painting generally classified as tempera or fresco depending on the technique of execution, possesses a layered structure consisting of support, ground and paint layer (Garg et al., 2010). Wall painting supports the growth of microorganism commonly involved in bio-deterioration, contributing to the destruction of paint; their deterioration constitutes a loss affecting a significant part of the world's cultural heritage (Pepe et al., 2010). The growth of fungi on wall paintings manifested most commonly by discoloration or deterioration of the surfaces and microbial popullation causes discoloration of pigments, physical decay when fungal hyphae grow either on or below the surfaces, cause chemical decay of paintings through their metabolites and enzyme production like gluconic, citric, oxalic, malic etc (Pepe et al., 2010; Hideo, 2000; Dornieden et al., 2000; Garg et al., 2010). Biodegradation and bio-deterioration is a serious risk to cultural heritage, which needs the application of effective and fast methods in order to identify the microorganisms involved in this process and to assess their biodegradation and bio-deterioration ability (Pangallo et al., 2009). Their biological attack occurs at favourable temperature and relative humidity conditions for the development of microorganisms and spores present on the substratum. Each colonizer agent has different ways to compromise the structure in function of the substrates (Nugari et al., 1993; Borrego et al., 2010). Nanoparticles have antimicrobial effect such as silver nano particles, copper nanoparticles, zinc oxide nanoparticles and titanium oxide nano particles etc (Stoimenov, 2002; Ip et al., 2006; Ren et al., 2009; Zhang et al., 2007). In this study we used two methods to inhibit the isolated microbial from the surface of wall painting: the first involves using titanium dioxide nanoparticles with a different level of concentration and the second method entails using two tradition plant extracts- Sesbania (*Sesbania sesban*) and Ricinus (*Ricinus communis*).

MATERIALS AND METHODS

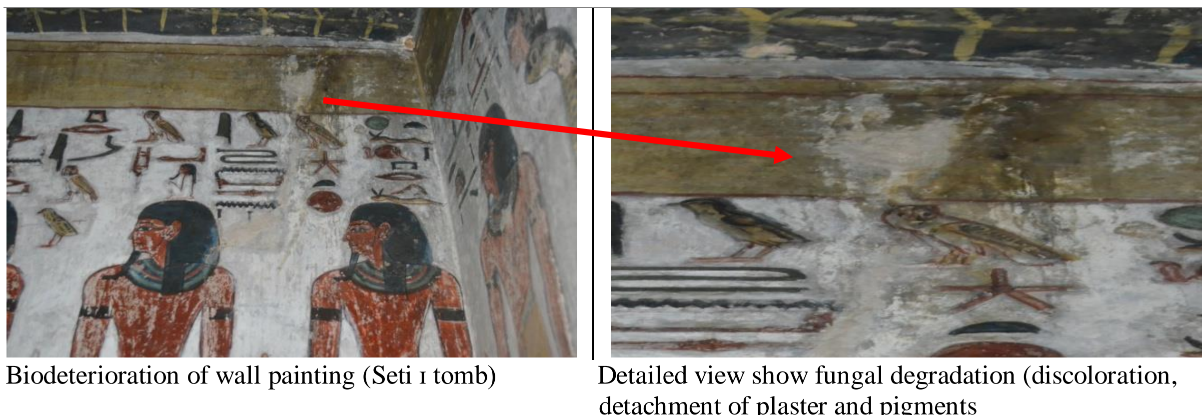
Samples and site description

Samples for the study were taken from different parts of the royal tombs such as Tausert and Setnkht, Seti ɪ., Ramsis v., Ramsis vi. (Figure 1) according to scientific and not destructive methods. The biological samples were taken by sterile cotton swap and incubated for 7 days; the isolated fungi were identified according to morphological and spore structures (Barnett et al., 1972; Sun et al., 1978).

Three isolates of fungi and two of bacteria were collected from different tombs; 900 km Southeast of Cairo, during March 2014 by using the sterile cotton swap method. Samples were taken from paintings of yellow, red, black, blue color and stone surfaces in investigated tombs (Figure 2). They were inoculated on nutrient agar and potato dextrose agar, and incubated for a period of 5 days at 30°C.

Preparation of plant extracts

The fresh plant leaves were brought to the laboratory and washed under running tap water; they were air dried and then homogenized to fine powder and stored in airtight bottles. The dried powder of *S. sesban* and *R. communis* was successively mixed with methanol for



Biodeterioration of wall painting (Seti ɪ tomb)

Detailed view show fungal degradation (discoloration, detachment of plaster and pigments

Figure 2. Biodeterioration of wall tomb painting.

Table 1. Microbial inhibition zone (mm) of plant extracts concentration (mg/ml).

Test organism	Sesbania extract concentration (mg/ml)				Ricinus extracts concentration (mg/ml)			
	20 mg	40 mg	80 mg	100 mg	20 mg	40 mg	80 mg	100 mg
F. oxysporum	9	12	13	13	8	10	13	13
R. stolonifer	10	10	12	13	7	10	11	12
A. flavus	8	9	11	12	9	12	12	13
S. warnei	8	9	12	14	8	12	12	14
M. luteus	10	11	12	15	10	11	12	13

72 h. The extracts were dried under reduced pressure using rotator evaporator to get the crude. A dark green semi-solid mass was obtained. It was stored below 4ºC until further used. The alcohol extract was prepared by adding 10 g of crushed leaves of sesbania or ricinus in 50 ml of methanol separately. It was allowed to stand for 24 h after which it was filtered using a Whatman No. 1 filter paper. The filtrate was directly used as crude extract with 100% concentration. Further dilutions were made by adding appropriate amount of methanol.

Microbial culture and media

The microbial cultures were prepared in microbiology agriculture lab and used in the present study. The bacteria rejuvenated in nutrient broth at 37°C for 18 h and then stocked at 4°C in nutrient agar (Khante et al., 2008). Subcultures were prepared from the stock for bioassay. The inoculum size of the bacterial culture was standardized according to the National committee for Clinical Laboratory Standards guideline. The bacterial culture was inoculated into sterile nutrient broth and incubated at 37°C. The fungi medium is Potato Dextrose Agar PDA (200 g potato infusion, 20 g agar + 20 g Dextrose+1 L distilled water) and the bacterial medium is nutrient agar NA (0.5% peptone, 0.3% beef extract/yeast extract, 1.5% agar, 0.5% NaCl, 1 L distilled water).

Preparation of disc for antimicrobial activities

The sterile blotting paper disc (5 mm) was soaked in the diluted extract in different concentrations (20, 40, 80 and 100%). The prepared disc was dried in controlled temperature to remove excess of solvent. The modified paper disc diffusion (Delignette-Muller and Flandrois, 1994) was employed to determine the antimicrobial activity of aqueous extract of the herbal preparations. Inoculum was spread over the agar plate using a sterile cotton swab in order to obtain uniform microbial growth. Then the prepared antimicrobial discs were kept over the lawn and pressed slightly along with control. Streptomycin 10 µg/disc was used as positive control. The plates were incubated for 48 h at 37°C. The antimicrobial activity was evaluated and diameter of inhibition zones was measured. Experiment was carried out in triplicate and the averages diameter of zone of inhibition was recorded. The antibacterial activity was classified as highly active (>10 mm), mildly active (7-10 mm) and slightly active (6-7 mm); and less than 6 mm was taken as inactive (Chandra et al., 2011).

RESULTS AND DISCUSSION

Effect of plant extracts on microbial strains

The antimicrobial activity of methanol extracts of *S. sesban* stem portion was studied against *Staphylococcus warnei* and *Micrococcus luteus* by zone of inhibition method. The dried leaves of *S. grandiflora* are used in some countries as tea which is considered to have antibiotic properties (Gupta et al., 2008). The zone of Inhibition study results are depicted in Table 1. The result indicates that after 48 h methanol extracts (100 mg/ml) showed zone of inhibition of 15 mm against *M. luteus,* and 14 mm against *S. warnei*. The fungal group zone of

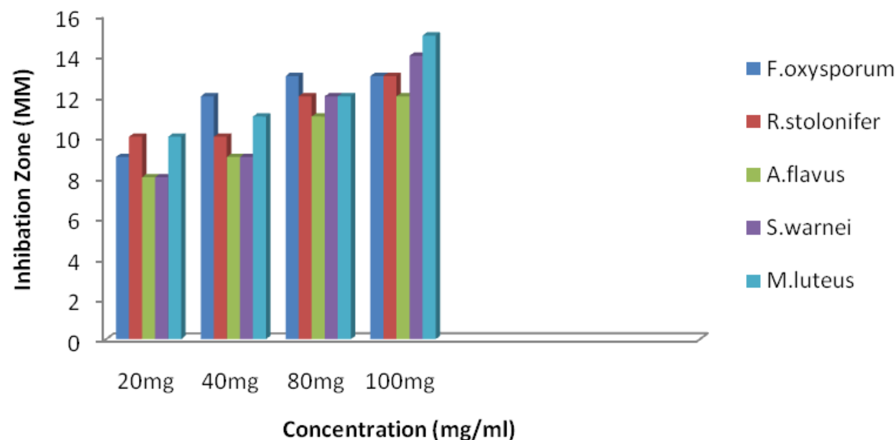


Figure 3. Effect of different sesbania concentration on microbial inhibition zone.

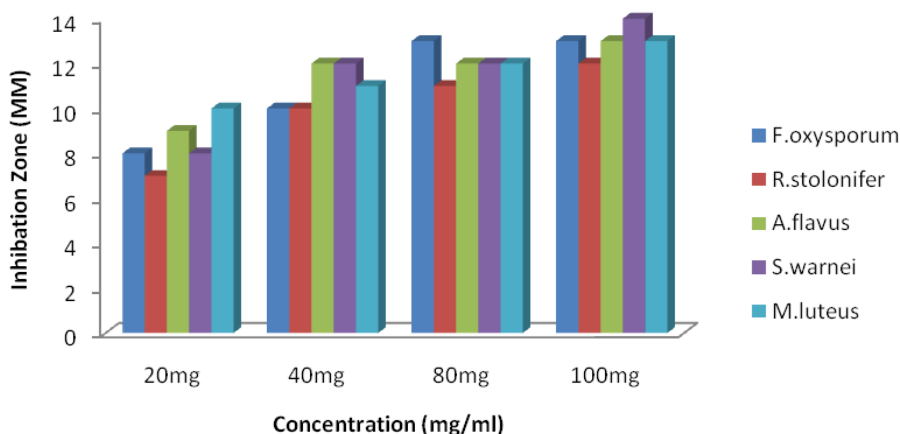


Figure 4. Effect of different Ricinus concentration on microbial inhibition zone.

inhibition (100 mg/ml), after 72 h, was 12.3 mm against *Fusarium oxysporum;* 12.1 mm against *Rhizopus stolonifer* and 12.6 mm against *Aspergillus flavus.* The graphical representation of zone of inhibition vs different concentration of plant extracts after different time intervals (48 h for bacteria and 72 h for fungi) are shown in Figures 3 and 4 respectively.

According to Sandeep et al. (2014), cloroform and methanol extracts of stem of *S. sesban* (25 mg/ml) equally inhibit *P. aeruginosa* after 48 h. Both chloroform and methanol extracts at a concentration of 100 mg/ml were more effective against *B. subtilis* than other two bacterial strains after 48 h. No antibacterial activity was observed for pet ether ethanol and water extracts in a range of 25 to 100 mg/ml. The methanol leaf extract of *R. communis* exhibited maximum antimicrobial activity, and water extract showed minimum activity against four bacteria (*S. aureus*, *B. subtilis*, *P. aeruginosa* and *K. pneumonia)* and two fungal strains (*A. fumigatus* and *A. flavus).* These results are in agreement with the previous work which showed that in plants most of the compounds having antimicrobial potential are soluble in methanol (Chandrasekaran et al., 2004), and low activity of water extract is also reported by Ashafa et al. (2008).

R. communis showed good activity against *M. luteus, S. warnei, F. oxysporum, R. stolonifer* and *A. flavus*. The antimicrobial assay revealed that the methanol extracts of leaves of *R. communis* possess good zone of inhibition, whereas alcohol extract has antimicrobial activity only in higher concentration (Table 1). Significant susceptibility was recorded by most of the organisms tested with methanol extract of leaves of *R. communis*, which showed a comparatively reduced susceptibility pattern (Figure 5).

The susceptibility pattern exhibited by the tested organisms to these extracts could be exploited for probably medicinal purposes in chemotherapy among humans. With the current spread of antibiotic resistance almost at geometric scale (Olayinka et al., 2004), proper attention should be given to such plants to reap the

Figure 5. Inhibition zone of antibacterial activity of TiO_2 nanoparticles and plant extracts at different.

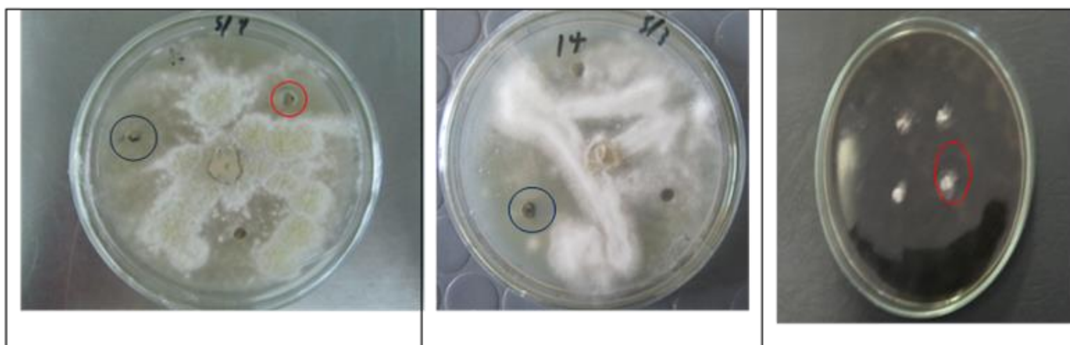


Figure 6. Inhibition zone of antifungal activity of TiO_2 nanoparticles and plant extracts at different concentrations.

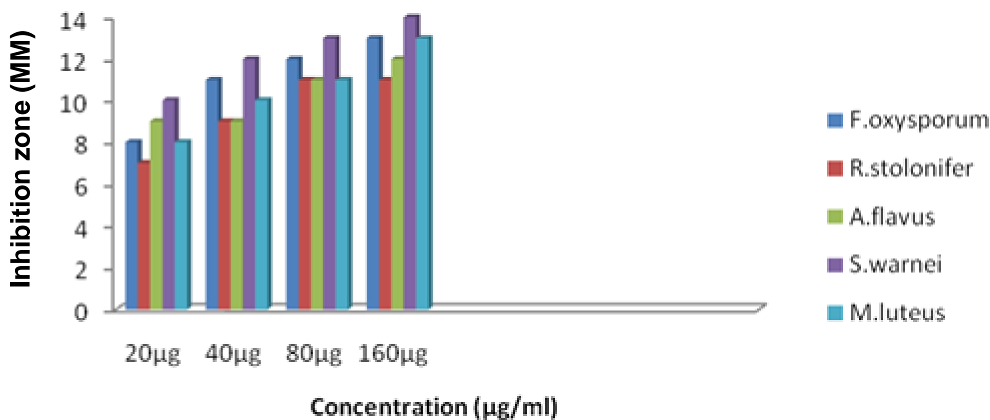


Figure 7. Effect of different nano TiO_2 concentration on microbial inhibition zone.

potential antimicrobial benefits inherent in them. However, actual antimicrobial ingredients need to be extracted and identified; also its tolerable levels in the human body as well as any toxic effects on human and animal tissues need to be investigated accordingly.

Effect of Nano-TiO_2 on microbial growth

The effect of chemical synthesized TiO_2 nanoparticles on the growth of microbial strains is shown in Figures 6 and 7. The antimicrobial activity is probably derived, through

Table 2. Microbial inhibition zone (mm) of Nano TiO_2 concentration (µg/ml).

Test organisms	20 µg	40 µg	80 µg	160 µg
F. oxysporum	8	11	12	13
R. stolonifer	7	9	11	11
A. flavus	9	9	11	12
S. warnei	8	12	13	14
M. luteus	8	10	11	13

the electrostatic attraction between negatively charged cell membrane of microorganism and positively charged nanoparticles (Hamouda et al., 2001; Dibrov et al., 2002; Dragieva et al., 1999), interaction of metal ions including titanium with microbes (Amro et al., 2000) and orientation of TiO_2 (Wang et al., 2007).

Chemical TiO_2 treatments had significant inhibitory effect on the growth of microbes during 24 and 72 h of incubation. The growth of the medium was investigated as the number of microbes after contact with nano-particles. The growth inhibition of the microbe by both treatments recorded as a function of time suggested significant differences in antimicrobial activity of the nanoparticles (Table 2).

Chemically synthesized TiO_2 NPs has a stronger inhibitory effect on microorganisms. TiO_2 NPs at a concentration 20, 40, 80 and 160 inhibited growth of bacterial and fungal strains, whereas the effect was much less at lower concentration (Kon and Rai, 2013). The efficacy of antimicrobial in terms of zones of inhibition (mm) was measured against five different fungal and bacterial strains (Table 2). The decreasing order of the average antimicrobial activity of NPs against fungal group was observed to be *F. oxysorum* > *A.flavus* > *R. stolonifer*. The average antimicrobial activity of the various NPs concentrations against bacterial group and fungal group inhibition zone ranged from 8–14 and 8-13 mm, respectively. Increasing concentration of TiO_2 nanoparticle decreases the growth of microbes, and the concentration at which growth stopped altogether was high with TiO_2 chemically synthesized nanoparticle. According to Adams et al. (2006), nanoparticles inhibited growth of gram-positive by 90% but gram-negative was much more resistant.

The antimicrobial ability of nano-TiO_2 might be referred to their small size which is 250 times smaller than a bacterium. This makes them easier to adhere with the cell wall of the microorganisms causing its destruction and death of the cell. Also, metal nano-particles are harmful to bacteria and fungi (Chwalibog et al., 2010). Nano-TiO_2 stimulates biofilm production and aggregate within this bio-film. They bind closely to the surface of microorganisms causing visible damage to the cells, and demonstrating good self-assembling ability. Nano-TiO_2 possesses well-developed surface chemistry, chemical stability which makes them easier to interact with the microorganisms (Nirmala and Pandian, 2007). The particles interact with the building elements of the outer membrane and might cause structural changes, degra-dation and finally cell death. The effectiveness of TiO_2 can be explained on the basis of the oxygen species released on the surface of TiO_2, which cause fatal damage to microorganisms (Sunada et al., 1998). The generation of highly reactive species such as OH^-, H_2O_2 andO_{2-} is explained as follows. Since TiO_2 with defects can be activated by both UV and visible light, electron-hole pairs (e^-h^+) can be created. The holes split H_2O molecules (from the suspension of TiO_2) into $OH^-$and H^+. Dissolved oxygen molecules are transformed to superoxide radical anions ($\bullet O^{-2}$), which in turn react with H+ to generate ($HO_2\bullet$) radicals, which upon subsequent collision with electrons produce hydrogen peroxide anions (HO^{2-}). They then react with hydrogen ions to produce molecules of H_2O_2. The generated H_2O_2 can penetrate the cell membrane and kill the bacteria (Fang et al., 2006). Since the hydroxyl radicals and superoxide are negatively charged particles, they cannot penetrate into the cell membrane and must remain in direct contact with the outer surface of the bacteria cell of the bacteria; however, H_2O_2 can penetrate into the cell (Blake et al., 1999). The mechanisms of TiO_2 nanoparticles depend on the bactericidal effect which is a result of mechanical interactions on cell walls and/or membranes of microorganisms in the presence of photocatalysis.

Conclusion

In this work we have reported the evidence of toxic effects of Titanium dioxide nanoparticles (TiO_2 NPs) and plant extracts on different pollution microorganisms. The antimicrobial activity of ethanol extracts was evaluated by disc diffusion plate method. All the extracts were evaluated in concentration range from 20 to 100 mg/ml. Wall painting in the royal tombs (Tausert - Setnkht and Seti I) suffers from microbial enzymes degradation and needs urgent treatment. The lab test was performed by using various concentrations of TiO_2 NPs; 160 ppm was the efficient concentration level in eliminating microbial growth. TiO_2 NPs and plant extracts did not cause any observable alterations or color changes to pigments and binding medium (Arabic gum) used in wall paintings. The order of bacterial strains inhibition of methanol extract after 48 h is as follows: *S.warnei* >*M. luteus*> *F.oxysporium*> *A. flavus* > *R. stolonifer*. So, it is concluded that Sesbania and Ricinus extracts possess good antibacterial activity. The antibacterial activity may be due to the presence of flavonoids and phenolic compounds.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

The cultivation of microalgae *Cyanobium* sp. and *Chlorella* sp. in different culture media and stirring setting

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Microalgae can serve as a source of biocompound with high nutritional and commercial potential, such as fatty acids, vitamins and pigments. The microalgae composition is influenced by the physical and chemical conditions of the culture medium and environment in which they are grown, and the composition can be manipulated to produce specific biomolecules. The lack of stirring or the employment of stirring inefficient in the microalgae cultivation, limits the development of the culture because may have areas of stagnant culture, in which the cells do not receive light, which affects photosynthesis and cellular growth. The biomass concentration too is directly influenced by concentration of each nutrient culture medium. Hence, the need to add macronutrients and micronutrients in the ideal concentration and correct form as regards mass concentration and absorption availability. The objective of this study was to assess the growth of the microalgae *Cyanobium* sp. and *Chlorella* sp. in different culture media and stirring settings. For this, the cultures were carried out in Erlenmeyer flasks and Raceway type bioreactors, respectively, for 10 days at 30°C, with illumination of 41.6 µmol m^{-2} $s^{-1,}$ and with a 12 h light/dark photoperiod. The maximum biomass concentrations (0.56 and 0.66 g L^{-1}), specific growth rate (0.30 and 0.17 d^{-1}) and productivities (0.12 and 0.09 g L^{-1} d^{-1}), were observed in *Cyanobium* and *Chlorella*, respectively, when they were grown in BG11 medium with 0.40 g L^{-1} $NaHCO_3$. Regarding the stirring, the best responses were obtained when the assays were carried out with stirring by two submersible pumps, with maximum biomass concentrations of 1.21 g L^{-1} for the *Cyanobium* sp. and 0.93 g L^{-1} for the *Chlorella* sp.

Key words: BG11 medium, H/2 medium, ASM medium, Raceway, submersible pump.

INTRODUCTION

Microalgae have been studied in biotechnology research due to their nutritional, economic and ecological importance. These microorganisms can use solar energy efficiently to transform wastewater, CO_2 and additional nutrients into a biomass (Batista et al., 2013). The biomass produced can be exploited as a source of biocompounds with high nutritional and commercial value, some of which are used in the formulation of foods

and feeds and some of which can be used to help minimize problems such as human malnutrition (Toledo-Cervantes et al., 2013; Ho et al., 2011).

Microalgae cultivation is strongly influenced by the setting of stirring and components of the culture medium, since both factors can maintain the maximum absorption of nutrients for the microorganism. According to Borowitzka (1999), microalgae require stirred systems to allow high rates of growth because a good stirring increases the absorption of nutrients and allows better oxygen removal from the medium. In addition, a good stirring promotes amount of light essential, for in the absence of any other limiting factor, the cell concentration increases with light intensity until reaching maximum biomass concentration that is denominated "saturation level" (Bezerra et al., 2012).

The bioreactor employed for microalgae cultivation has direct influence on the kinetic results of this culture. Raceway ponds are the most widely used and economical one for the large scale cultivation. One of the major advantages of open ponds is that they are easier to construct and operate than most closed systems. One disadvantage is the biomass productivity limited due to poor utilization of light and possibility of contamination as compared to the closed bioreactor (photobioreactor) (Vasumathi et al., 2012). The depth of the bioreactor must take into account the need to provide light to the cells and the need to maintain a liquid column suitable for mixing and to avoid large ionic exchange due to evaporation (Borowitzka, 1999). In bioreactors without stirring, the light limits the development of the culture. These bioreactors may have areas of stagnant culture, in which the cells do not receive light, which affects photosynthesis and cellular growth (Pulz, 2001).

The culture medium has a direct influence on cellular growth, as well as on the biomass composition of microalgae (Richmond, 2004). In this context, microalgae, like other microorganisms, require sources of carbon, nitrogen, phosphorus and other micronutrients (Kumar et al., 2010), and the nutrients that make up the medium must be dissolved (available) for biochemical use by the microalgae. This way, the stirring of the bioreactor and the cultivation medium are two important factors to be studied.

The objective of this study was to assess the growth of the microalgae *Cyanobium* sp. and *Chlorella* sp. with different culture media and stirring settings.

MATERIALS AND METHODS

Selection of nutritional conditions

The studied microalgae were *Chlorella* sp. (Henrard et al., 2014) and *Cyanobium* sp. (Henrard et al., 2011). The culture media used to cultivate the *Cyanobium* microalgae were ASM, BG11, BG11+$NaHCO_3$, H/2 and H/2+$NaHCO_3$, while BG11, BG11+$NaHCO_3$, H/2, H/2+$NaHCO_3$, MBM, MBM+$NaHCO_3$, MC and MC+$NaHCO_3$ were used for the *Chlorella* sp.

The ASM medium contained (g L^{-1}): $NaNO_3$ (0.17), $MgSO_4$ (0.049), $MgCl_2$ (0.041), $CaCl_2$ (0.029), K_2HPO_4 (0.0174), Na_2HPO_4 (0.0356); H_3BO_3 (0.00248), $MnCl_2$ (0.00139), $FeCl_3$ (0.00108), $ZnCl_2$ (0.00034), $CoCl_2$ (0.00002), $CuSO_4$ (0.00186), and Na_2EDTA (0.00744) (Gorham et al., 1964). The BG11 medium contained (g L^{-1}): $NaNO_3$ (1.50), $K_2HPO_4 \cdot 3H_2O$ (0.04), $MgSO_4 \cdot 7H_2O$ (0.075), $CaCl \cdot 2H_2O$ (0.036), $C_6H_{11}FeNO_7$ (0.006), EDTA disodium magnesium (0.001), Na_2CO_3 (0.02), $C_6H_8O_7$ (0.006), H_3BO_3 (0.00286); $MnCl_2 \cdot 4H_2O$ (0.00181) $ZnSO_4 \cdot 7H_2O$ (0.000222), $Na_2MoO_4 \cdot 2H_2O$ (0.00039), $CuSO_4.5H_2O$ (0.000079), $Co(NO_3)_2 \cdot 6H_2O$ (0.0000494) (Rippka et al., 1979). The BG11 medium was modified by adding 0.40 g L^{-1} $NaHCO_3$.

The H/2 medium contained (g L^{-1}): $NaNO_3$ (0.075), $NaH_2PO_4 \cdot H_2O$ (0.005), $FeCl_3 \cdot 6H_2O$ (0.00315), $Na_2EDTA \cdot 2H_2O$ (0.00436), $CuSO_4 \cdot 5H_2O$ (0.0098), $Na_2MoO_4 \cdot 2H_2O$ (0.0063), $ZnSO_4 \cdot 7H_2O$ (0.022), $CoCl_2 \cdot 6H_2O$ (0.010), $MnCl_2 \cdot 4H_2O$ (0.18), Vitamin B_{12} (0.001), Biotin (0.001), Thiamine (0.20) (Guillard, 1975; Guillard and Ryther, 1962). The H/2 medium was modified by adding 0.40 g L^{-1} $NaHCO_3$. The MBM medium contained (g L^{-1}): KNO_3 (0.25); $CaCl_2$ (0.01); $MgSO_4 \cdot 7H_2O$ (0.075); K_2HPO_4 (0.075); KH_2PO_4 (0.175); NaCl (0.025); $FeSO_4 \cdot 7H_2O$ (0.02), H_3BO_3 (0.00286), $MnCl_2 \cdot 4H_2O$ (0.00181), $ZnSO_4 \cdot 7H_2O$ (0.000222), $CuSO_4 \cdot 5H_2O$ (0.00079) and $NaMoO_4$ (0.000015) (Watanabe, 1960). The MBM medium was modified by adding 0.40 g L^{-1} $NaHCO_3$. The MC medium contained (g L^{-1}): KNO_3 (1.25); $MgSO_4 \cdot 7H_2O$ (1.25); KH_2PO_4 (1.25); $FeSO_4 \cdot 7H_2O$ (0.02), H_3BO_3 (0.00286), $MnCl_2 \cdot 4H_2O$ (0.00181), $ZnSO_4 \cdot 7H_2O$ (0.000222), $CuSO_4 \cdot 5H_2O$ (0.000079) and $NaMoO_4$ (0.000015) (Watanabe, 1960). The MC medium was modified by adding 0.40 g L^{-1} $NaHCO_3$.

The culturing was carried out in duplicate, under controlled conditions in a controlled temperature chamber at 30°C, and the illumination of 41.6 µmol m^{-2} s^{-1} was provided by 40 W daylight-type fluorescent lamps with a 12 h light/dark photoperiod (Reichert et al., 2006) for 10 days. The microalgae were grown in closed Erlenmeyer flask photobioreactors (0.50 L) with a working volume of 0.40 L and continuous stirring by injection of compressed air. The initial concentrations of the cultures of *Cyanobium* sp. and *Chlorella* sp. were 0.10 and 0.20 g L^{-1}, respectively. After selecting the culture medium with the best results, the different stirring settings were studied.

Study of the different stirring settings

To evaluate the different methods of stirring, *Cyanobium* sp. and *Chlorella* sp. were grown in open Raceway type bioreactors (0.70 m long, 0.20 m wide and 0.075 m deep) (Radmann et al., 2007) with 5 L of working volume (Henrard et al., 2014).

The stirring was continuous using eight different configurations: rotating blades at 15 rpm (Figure 1A); rotating blades and air injection through 2 porous curtains (900 mm each), in a spiral, attached to the bottom of the bioreactor (Figure 1B); rotating blades and air injection through 3 elongated porous stones (150 mm each) (Figure 1C); rotating blades and air injection through 6 cylindrical porous stones (30 mm each) (Figure 1D); 2 submersible pumps with a flow of 60 L h^{-1} each (Figure 1E); 1 submersible pump with a flow of 60 L h^{-1} (Figure 1F); 2 porous curtains (600 mm each), attached to the bioreactors with air injection (Figure 1G); 6

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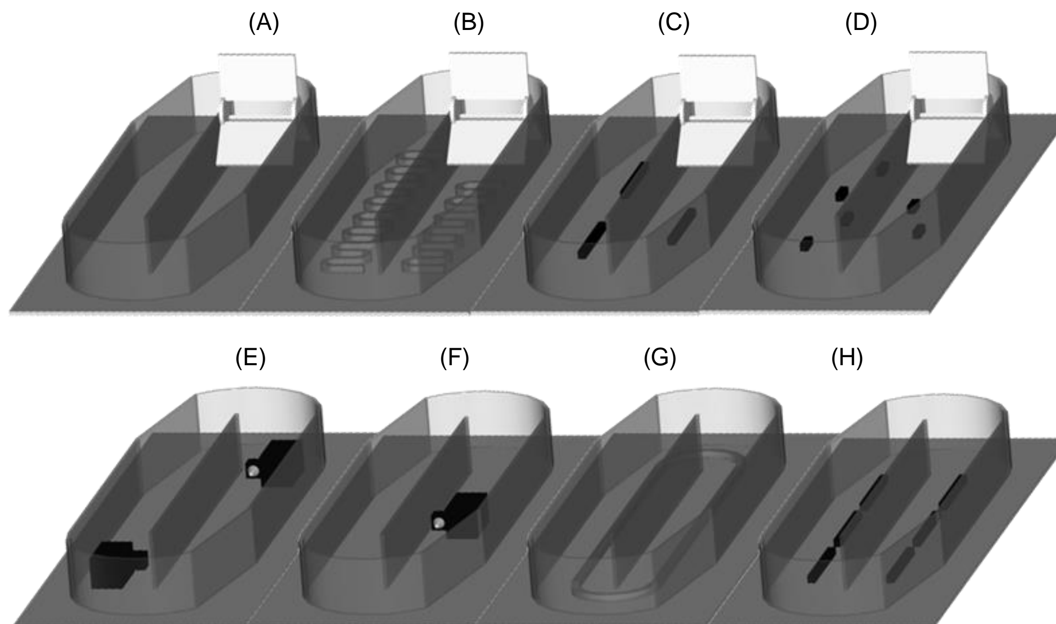

Figure 1. Schematic drawing of each Raceway type bioreactor, with its stirring settings: (A) rotating blades at 15 rpm; (B) rotating blades and porous curtains (900 mm each), in a spiral; (C) rotating blades and 3 elongated porous stones (150 mm each); (D) rotating blades and 6 cylindrical stones (30 mm each); (E) 2 submersible pumps with a flow of 60 L h^{-1} each; 1 submersible pump with a flow of 60 L h^{-1}; (G) 2 porous curtains (600 mm each) and (H) 6 elongated porous stones (150 mm each).

elongated porous stones (150 mm each) with air injection (Figure 1H). Figure 1 presents the bioreactors of the cultures with their respective stirrings.

The culturing was carried out in duplicate, under controlled conditions in a temperature-controlled chamber at 30°C, under illumination of 41.6 µmol m^{-2} s^{-1} provided by 40 W daylight-type fluorescent lamps with a 12 h light/dark photoperiod (Reichert et al., 2006) for 10 days. The initial concentrations of the *Cyanobium* sp. and *Chlorella* sp. cultures were 0.35 and 0.20 g L^{-1}, respectively. The medium culture selected for this stage was the BG11 with addition of 0.4 g L^{-1} of $NaHCO_3$.

Analytical measurements

The samples were collected every 24 h to measure the biomass concentration, calculated using the optical density at 670 nm in a spectrophotometer (FEMTO 700 Plus, São Paulo – SP - Brazil) with a calibration curve based on the association between the optical density and the dry weight of biomass for each strain (Costa et al., 2002). The pH was also monitored every 24 h using a digital pH meter (Quimis Q400H, Diadema - SP - Brazil).

Studied responses

The daily values of biomass concentration were used to calculate the maximum specific growth rates, maximum biomass concentration and maximum biomass productivity. The maximum specific growth rate (μ_{max}, d^{-1}) was calculated by exponential regression of the logarithmic phase of the cellular growth curve.

The maximum biomass concentration (X_{max}, g L^{-1}) is the maximum value of cellular concentration obtained. The biomass productivity (P, g L^{-1} d^{-1}) was obtained according to the equation $P = (X_1 – X_0) (t – t_0)^{-1}$, in which X_1 is the biomass concentration at time t (d) and X_0 (g L^{-1}) is the biomass concentration at time t_0 (d). P_{max} is the maximum value of biomass productivity found during the cultivation.

Statistical analysis

The results obtained from the experimental data for the microalgae were compared using analysis of variance (ANOVA), followed by Tukey's test, with a confidence interval of 95%.

RESULTS AND DISCUSSION

Selection of nutritional conditions

The choice of culture medium is essential for high biomass production through microalgae cultivation. Culture media can be defined based on previous studies with strains of known microalgae. However, if the nutritional requirements of a strain are not known, a trial and error approach is necessary, using different culture media that may promote the growth of a wide range of microalgae (Mutanda et al., 2011; Scott et al., 2010).

The highest biomass concentrations for *Cyanobium* sp. and *Chlorella* sp. (0.56 and 0.66 g L^{-1}, respectively) were obtained in the BG11 culture medium with the addition of 0.40 g L^{-1} of sodium bicarbonate ($p \leq 0.05$). In all of the assays, the adaptation phase (lag) was missing from the growth profile observed in the microalgae (Figures 2 and

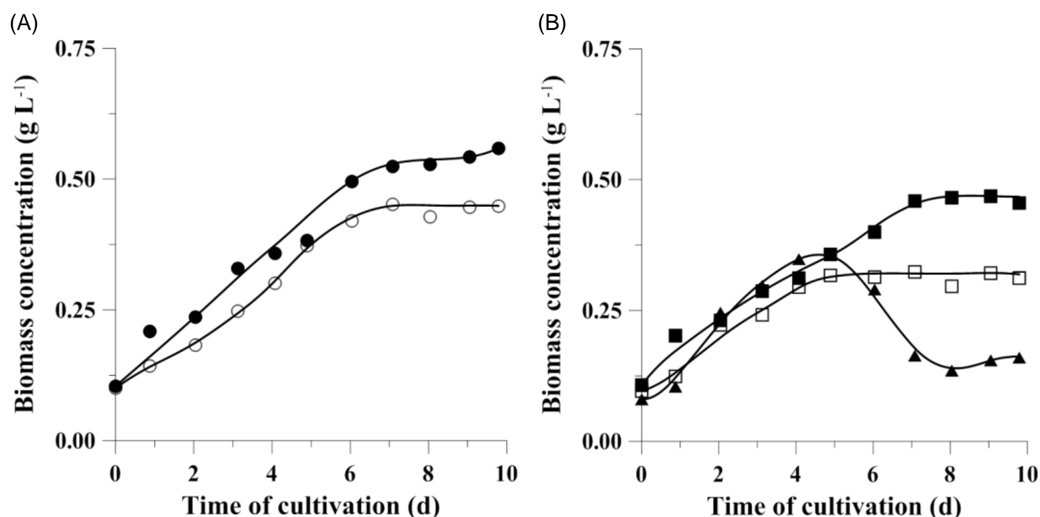

Figure 2. Growth profiles of the assays carried out with different media for *Cyanobium* sp.: (A) BG11 (○), BG11 + 0.40 g L^{-1} $NaHCO_3$ (●); (B) ASM (▲), H/2 (□) e H/2 + 0.40 g L^{-1} $NaHCO_3$ (■).

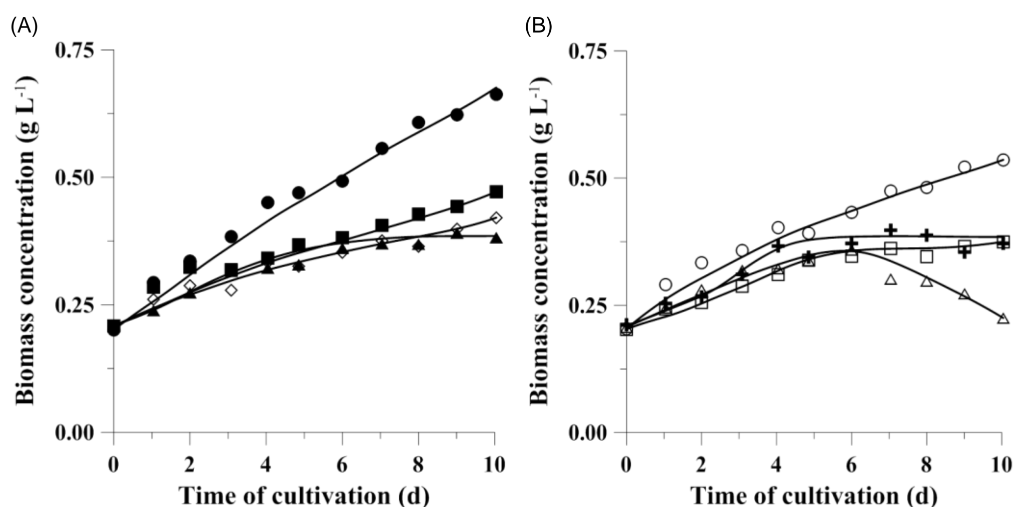

Figure 3. Growth profiles for the assays carried out with different culture media for *Chlorella* sp.: (A) BG11 + 0.40 g L^{-1} $NaHCO_3$ (●), H/2 + 0.40 g L^{-1} $NaHCO_3$ (▲), MBM + 0.40 g L^{-1} $NaHCO_3$ (■) and MC + 0.40 g L^{-1} $NaHCO_3$ (◊); (B) BG11 (○), H/2 (Δ), MBM (□) and MC (+).

3), despite the fact that the microalgae were not adapted before the assays.

The assays were 10 days long and the *Cyanobium* sp. showed cellular growth until the 7th day of cultivation with standard BG11, BG11 + $NaHCO_3$ (Figure 2a) and H/2 + $NaHCO_3$ (Figure 2b). When grown in ASM medium, the growth phase continued until the 5th day, reaching a maximum biomass concentration of 0.35 g L^{-1}, followed by cellular death (Figure 2B). For these two nutritional conditions (ASM medium and H/2 medium without $NaHCO_3$), the biomass concentrations of *Cyanobium* sp. were statistically equal ($p = 0.18$) (Table 1).

Chlorella sp. presented cellular growth during the entire 10 days of culture when maintained in BG11 medium, with and without bicarbonate, and in MBM with sodium bicarbonate (Figure 3A and B). Of these nutritional conditions, the biomass concentration was superior ($p < 0.05$) in the cultures grown in standard BG11 medium (Table 1).

The standard H/2 medium led to a cell death phase in *Chlorella* sp. after six days of culture (Figure 3B), leading to a lower maximum biomass concentration ($p < 0.05$) than in *Cyanobium* grown in H/2 medium with sodium bicarbonate. This attenuated growth may have occurred because of the limitation or depletion of nutrients essential for cellular development (such as carbon) because

Table 1. Maximum biomass productivity (P_{max}), maximum biomass concentration (X_{max}) and maximum specific growth rate (μ_{max}) for *Cyanobium* sp. and *Chlorella* sp., when grown in different culture media (ASM, BG11, H/2, MBM and MC).

Culture medium	P_{max} (g L^{-1} d^{-1})	X_{max} (g L^{-1})	μ_{max} (d^{-1})
***Cyanobium* sp.**			
ASM	0.08^{a}	0.35^{a}	0.22^{a}
BG11	0.05^{b}	0.45^{b}	0.25^{b}
BG11 + $NaHCO_3$	0.12^{c}	0.56^{c}	0.30^{c}
H/2	0.06^{d}	0.32^{a}	0.28^{d}
H/2 + $NaHCO_3$	0.11^{e}	0.46^{b}	0.18^{e}
***Chlorella* sp.**			
BG11	0.08^{a}	0.53^{a}	0.09^{a}
BG11 + $NaHCO_3$	0.09^{b}	0.66^{b}	0.17^{b}
H/2	0.04^{cd}	0.36^{c}	0.09^{a}
H/2 + $NaHCO_3$	0.03^{c}	0.39^{c}	0.09^{a}
MBM	0.04^{ce}	0.37^{c}	0.10^{c}
MBM + $NaHCO_3$	0.07^{f}	0.47^{d}	0.07^{d}
MC	0.04^{de}	0.40^{c}	0.12^{e}
MC + $NaHCO_3$	0.05^{g}	0.42^{cd}	0.08^{f}

Superscript letters equal within each column refer to statistically equal means ($p > 0.05$).

the composition of this culture medium has a low nutrient concentration.

The nutrients that are present in the culture medium must be provided in sufficient quantities so that the algal growth rate is maintained at peak levels (Vasumathi et al., 2012). Carbon is the nutrient required at high concentrations for microalgae. This demand is high because the carbon forms carbon skeletons for the subsequent synthesis (Grobbelaar, 2004) of proteins, lipids and carbohydrates within cells. The origin of the total inorganic carbon dissolved in microalgal cultures is HCO_3^-, the predominant species. The concentration of HCO_3^- in the medium determines the rate of formation of CO_2. If there is a low concentration of HCO_3^- in the medium, the growth of microalgae may be limited by the lack of this nutrient (Richmond, 2004)

The results of this study are consistent regarding the addition of sodium bicarbonate because the cell concentration of the cultures with both species of microalgae was increased by adding this inorganic salt in the medium. According to Hughes and Benemann (1997), one alternative to minimize production costs, is to substitute the carbon source in the growth medium with carbon dioxide that originates from the burning of fossil fuels, for the photosynthetic and autotrophic growth of microalgae.

According to Huang et al. (2009), in addition to nutrients such as nitrogen, phosphorus, potassium and carbon, trace elements are also important for the growth of autotrophic and heterotrophic microorganisms. The results obtained in this study demonstrate the influence of the composition of the culture medium that was used, in line with Huang et al. (2009).

When the microalgae *Cyanobium* and *Chlorella* were grown with ASM and H/2 media, respectively, both of them presented cellular growth until the 5th day of cultivation; however, at the 6th day, they began to enter a cellular decline phase because these culture media are usually used for maintenance of the inoculum. The nutrients made up small amounts of the total volume ($mL_{nutrient}$ L_{medium}^{-1}), and their composition did not include a carbon source, which is a key nutrient for the cellular growth of microalgae.

The better maximum specific growth rates (0.30 and 0.17 d^{-1}) and maximum biomass productivity (0.12 and 0.09 g L^{-1} d^{-1}) for *Cyanobium* sp. and *Chlorella* sp., respectively, were obtained when the microalgae were grown in BG11 medium with sodium bicarbonate (Table 1). Comparing the initial concentrations of the experiments for *Cyanobium* (0.10 g L^{-1}) and for *Chlorella* (0.20 g L^{-1}) shows that the biomass productivity and specific growth rates were higher ($p \leq 0.05$) when the initial biomass concentration of the culture was 0.10 g L^{-1}. Maintaining high productivity with increasing biomass concentration is important from an economic standpoint and is one of the challenges of growing microalgae. Increased cell density adversely affected the incidence of light and, in discontinuous cultivation; this implies a drop in the osmotic pressure of the medium, due to the consumption of nutrients. Both factors have a potential effect on the productivity and growth rate (Vonshak et al., 1982).

Nitrogen is the second most important nutrient and varies from 1 to 10% in the composition of microalgae (Grobbelaar, 2004). A low concentration of nitrogen in the culture medium encourages the accumulation of lipids and polysaccharides in the cells. With an abundant supply of nitrogen, the cultures tend to produce increasing concentrations of proteins and chlorophyll in the cells. When there is a low concentration of nitrogen available for the microalgae, the rate of cellular growth is reduced (Lourenço et al., 2004).

The ASM, BG11, H/2, MBM and MC media contain 0.17 g L^{-1} $NaNO_3$; 1.50 g L^{-1} KNO_3; 0.075 g L^{-1} $NaNO_3$; 0.25 g L^{-1} KNO_3 and 1.25 g L^{-1} KNO_3, respectively. The largest biomass concentrations were obtained in the BG11 and MC media, which contained maximum amounts of nitrate. These values are in agreement with Grobbelaar (2007), who reported that one of the most important factors to achieve high biomass productivity and concentration is the nutritional content of the medium. The study of the influence of nutritional parameters for the growth of microalgae is of great importance because the use of certain nutrients in the culture medium can increase production costs and

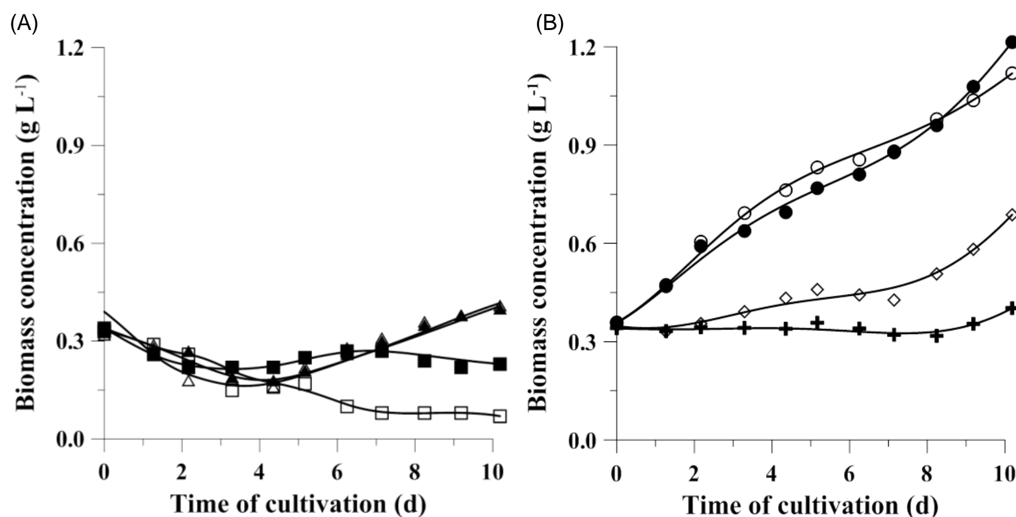

Figure 4. Growth profiles for *Cyanobium sp.* cultivated with the following stirring settings (A): rotating blades (■), rotating blades and porous curtain (□), rotating blades and elongated stones (Δ), rotating blades and cylindrical stones (▲); (B): 2 submersible pumps (●), 1 submersible pump (○), porous curtain (◊) and elongated stones (+).

Table 2. Maximum biomass productivity (P_{max}), maximum biomass concentration (X_{max}) and maximum specific growth rate (μ_{max}) for *Cyanobium* sp. and *Chlorella* sp., when grown in different stirring settings.

Stirring settings	*Cyanobium* sp.			*Chlorella* sp.		
	X_{max} (g L^{-1})	P_{max} (g L^{-1} d^{-1})	μ_{max} (d^{-1})	X_{max} (g L^{-1})	P_{max} (g L^{-1} d^{-1})	μ_{max} (d^{-1})
Rotating blades	-	-	-	0.48^{a}	0.064^{a}	0.071^{a}
Rotating blades and porous curtain	-	-	-	$0.45^{a,b}$	0.048^{b}	0.086^{b}
Rotating blades and elongated stones	0.41^{a}	0.008^{a}	0.150^{a}	$0.44^{a,b}$	0.082^{c}	0.068^{a}
Rotating blades and cylindrical stones	0.40^{a}	0.007^{a}	0.140^{a}	0.43^{b}	0.029^{d}	$0.092^{b,c}$
2 submersible pumps	1.21^{b}	0.108^{b}	0.183^{b}	0.93^{c}	0.111^{e}	0.263^{d}
1 submersible pump	1.12^{c}	0.113^{c}	0.176^{b}	0.71^{d}	0.067^{f}	0.133^{e}
Porous curtains	0.68^{d}	0.034^{d}	*N.E.P	0.29^{e}	0.016^{g}	0.041^{f}
Elongated stones	0.41^{a}	0.008^{a}	*N.E.P	0.24^{f}	0.014^{h}	0.022^{g}

Superscript letters equal within each column refer to statistically equal means ($p > 0.05$); *N.E.F: No exponential phase of growth.

influence the growth and/or composition of the biomass (Sassano et al., 2007).

Study of the different stirring settings

In the cultures with *Cyanobium* sp. (Figure 4A), only the assays with submerged pumps had no adaptation phase (lag), and presented cellular growth during the 10 days of cultivation. In the assay carried out with stirring only by rotating blades, the cultivation had remained constant with no cellular growth or decline. In the experiment with rotating blades and porous curtains, a cellular decline was observed. Insufficient stirring may influence the development of cells, leading them to a regimen of low light incidence, which impairs their growth (Hosaka et al., 1995). The retention of biomass in the porous curtain probably hinders the aeration and homogenization of the culture, and prevents cells from receiving enough light for photosynthesis and growth.

In the assays with *Cyanobium* sp. (Figure 4B), which was carried out with aeration and elongated porous stones, no decline of biomass concentration was observed. However, biomass precipitation occurred in the bioreactor, which slowed growth, and this differed from the experiments that were stirred with 1 or 2 submersible pumps and porous curtains. The cultures that used a porous curtain presented a biomass growth of 0.68 g L^{-1} (Table 2), but precipitation of the biomass was also detected, indicating that the stirring was insufficient to

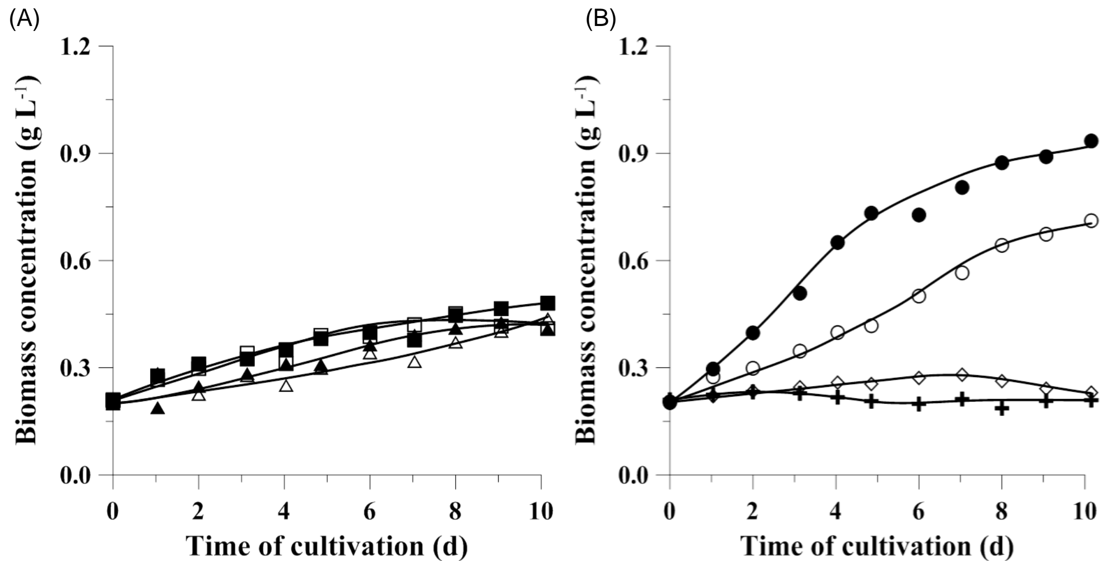

Figure 5. Growth profiles for *Chlorella sp.* cultivated with the following stirring settings: (A): rotating blades (■), rotating blades and porous curtain (□), rotating blades and elongated stones (Δ), rotating blades and cylindrical stones (▲); (B): 2 submersible pumps (●), 1 submersible pump (○), porous curtain (◊) and elongated stones (+).

maintain homogenization of the cultures. These data are consistent with those of Vonshak (1997), who stated that when high luminosity is present, photoinhibition may occur, which is the loss of photosynthetic capacity due to damages caused by light intensities above the level required for photosynthesis. On the other hand, in bioreactors with insufficient stirring, the development of the culture is limited by a lack of luminosity (Borowitzka, 1999).

The maximum biomass concentrations of the *Cyanobium* cultures with 1 or 2 submersible pumps were significantly different ($p \leq 0.05$): 1.12 and 1.21 g L^{-1} (Table 2), respectively. The maximum specific growth rates (0.17 and 0.18 d^{-1}) (Table 2) for *Cyanobium* sp. were obtained with 1 and 2 submersible pumps, respectively, with no significant difference ($p > 0.05$). The maximum biomass productivity (0.11 g L^{-1} d^{-1}) was observed when the microalgae was grown with just 1 submersible pump, which was statistically different ($p \leq 0.05$) from the other stirring settings (Table 2). The stirring of the microalgae cultures is related to specific factors that influence cellular growth. The stirring prevents the formation of cellular clusters and ensures enough light incidence for the cells (Grima et al., 1996), enabling the uptake of atmospheric CO_2 and liberation of O_2 from the interior of the liquid medium. Furthermore, stirring homogenizes the levels of nutrients and gases in the medium (Jiménez et al., 2003).

In cultures carried out with stirring by air injection through elongated porous stones and porous curtain (Figure 5B), the microalgae *Chlorella* sp. presented no cellular growth during the 10 days of cultivation. When *Chlorella* sp. was stirred by rotating blades, the biomass concentration has achieved 0.48 g L^{-1}. The microalga grew throughout the entire cultivation but did not differ from the cultures with stirring using rotating blades with porous curtains and rotating blades with elongated stones (Figure 5A).

The greater the intensity of light to which cells are exposed during the day, the greater the rate of respiration in the dark period, and this respiration can consume up to 35% of the biomass produced in the light period (Torzillo et al., 1991). Therefore, the nocturnal loss of biomass in non-dense cultures that receive high light intensities (as occurs at the beginning of the cultures), contributes to keeping the cell density at a low level, and growth is slow during this period. Likely, when the *Chlorella* was stirred by just rotating blades or compressed air through elongated stones and porous curtains, it did not present formation of biomass during the entire culture due to a lack of stirring, which led to low productivities and possible consumption of part of the biomass produced during the light phase.

The higher responses for *Chlorella* sp. were obtained from stirring with 1 and 2 submersible pumps (Figure 5B), which generated significantly different ($p \leq 0.05$) maximum biomass concentrations of 0.71 and 0.93 g L^{-1}, maximum specific growth rates of 0.13 and 0.26 d^{-1} and maximum biomass productivities of 0.07 and 0.11 g L^{-1} d^{-1}, respectively. Several factors influence the productivity, including the inefficient conversion of light into biomass, accumulation of oxygen in the culture, biomass consumption during respiration, the presence of nutrients in the culture medium and photoinhibition (Stewart and

Hessami, 2005). The maximum biomass concentrations are important for obtaining cell densities that are high enough to make it economically viable to produce microalgae cultures with high operating costs. This way, the results of this study demonstrate that the highest biomass concentrations for *Cyanobium* and *Chlorella* were obtained when they were cultivated with submersible pumps: an increase of 95% in biomass productivity was obtained in these cultures as compared to those stirred with porous curtains and rotating blades with elongated stones.

Conclusions

Cyanobium sp. and *Chlorella* sp. has achieved maximum biomass concentrations (0.56 and 0.66 g L^{-1}, respectively), maximum biomass productivity (0.12 and 0.09 g L^{-1} d^{-1}) and the maximum specific growth rates (0.30 and 0.17 d^{-1}) in BG11 medium supplemented with 0.40 g L^{-1} of sodium bicarbonate. Thus, the medium selected for the studied with different stirring settings was the BG11 with addition of $NaHCO_3$. In this study, *Cyanobium* sp. and *Chlorella* sp. had achieved maximum biomass concentrations (1.21 and 0.93 g L^{-1}, respectively) and maximum specific growth rates (0.18 and 0.27 d^{-1}, respectively) when stirred with two submersible pumps. The maximum biomass productivity of 0.11 g L^{-1} d^{-1} was achieved for *Cyanobium* sp. (with one submersible pump) and *Chlorella* sp. (with two submersible pumps). These results show that the proper selection of nutrients for microalgae and the continuous stirring of the culture improve the efficiency of biomass production, providing light energy and nutritional conditions for cellular multiplication during the entire cultivation period.

Conflict of interests

The authors did not declare any conflict of interest.

ACKNOWLEDGEMENTS

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Full Length Research Paper

Presence of pathogenic *E. coli* in ready-to-be-eaten salad food from vendors in the Kumasi Metropolis, Ghana

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This study was carried out to assess the distribution of *E. coli* O157:H7 in salad foods from restaurants and street food vendors within the Kumasi Metropolis from January to April, 2013. A detailed and well-structured questionnaire was first administered to 500 vendors with emphasis on knowledge of personal hygiene and salad food preparation. A total of 270 salad foods were aseptically sampled from vendors and transported on ice to the laboratory to determine the presence of total coliforms and *E. coli* (*E. coli* O157:H7) using standard microbiological methods. Out of the total samples analysed, all the samples were found to contain some counts of total coliforms and *E. coli*. Mean logcfu/g of total coliforms and *E. coli* were found to be 6.35 ± 0.09 and 5.1 ± 0.1, respectively. Three (3) samples showed positive to *E. coli* O157:H7 giving a prevalence of 1.1%. The low prevalence still suggests that *E. coli* O157:H7 is still a public health concern especially ready to be eaten salad foods since a relatively low infectious dose could be fatal. Although, street food vending has a positive impact on food supply and livelihoods of the ordinary people by providing cheap and affordable means of green-leafy or vegetable food in the Kumasi Metropolis, it poses a health risk which can result in serious health implications for consumers.

Key words: *E. coli* O157:H7, hygiene, restaurant, vendors, salad food.

INTRODUCTION

Public announcements and awareness of the intake of vegetables have increased massively (Chadrasekhar et al., 2003). While there are many reasons to include these nutritious foods into our daily diets, their accessibility and portability cannot be underplayed. Eating a diet rich in vegetables and fruits as part of an overall healthy diet may reduce risk for heart disease (He et al., 2007), including heart attack and stroke (He et al., 2006), as well as reduce certain types of cancers (World Cancer Research Fund, 2007). Outbreaks of human infections associated with the consumption of fresh or minimally processed fruits and vegetables have increased in recent

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years (Mensah et al., 2002). Food-borne diseases are increasing and becoming wide spread, therefore, food safety has been considered as an important global health issue (Bernstein et al., 2007). One of the vehicles of food-borne diseases is through the consumption of raw vegetables such as salad foods (Mensah et al., 2002). Research has shown that vegetables are increasingly eaten as salad foods by the urban population in Ghana and estimated that, each day; about 800,000 people in the cities of the country consume wastewater irrigated salad foods (Seidu and Drechsel, 2010). The street food industry plays a very important role in meeting food requirements of commuters and urban dwellers in many cities and towns of developing countries, as it feeds thousands of people daily with a large range of food that are relatively cheap and easily accessible (Tambekar et al., 2008). Studies conducted in Accra revealed that salad foods had a high level of microbial contamination (Mensah et al., 2002) which suggests a high risk of microbial infection. Many studies have been carried out on other microbial pathogens but little is known about the occurrence of Entero-haemorragic *Ecsherichia coli*, especially in salad food especially in Ghana. In Ghana, diarrhoea has been recognized as one of the major causes of hospital attendance and 16% of deaths in African children younger than five years are directly attributable to diarrhoeal diseases (Bruce et al., 2005). Despite the commitment and dedication of various agencies to improved food safety systems, it has not been widely implemented which raises much concerns about the probable role street and other vending food play in food poisoning (Kosek et al., 2003; Soyiri et al, 2008)

The aim of this study was thus to assess the level of contamination of ready to-be-eaten salad foods by Total coliforms, the normal *E. coli* strain and entericpathogenic *E. coli* 0157:H7, from various food vendors and the relationship between salad consumption and the prevalence of microbial contamination by *E. coli* and total coliform within the Kumasi Metropolis.

MATERIALS AND METHODS

Kumasi was selected as the study area, which is located in the forest zone and is about 270 km north of the national capital, Accra. Its location is between latitude 6.35° to 6.40° and longitude 1.30° to 1.35°, at elevation which ranges between 250-300 m above sea level with an area of about 254 km^2 (KMA Development Plan, 2006-2009). Kumasi is divided into ten (10) sub-metropolitan areas namely Bantama, Subin, Manhyia, Oforikrom, Tafo, Nhyiaeso, Kwadaso, Suame, Asokwa and Asawase.

The study area is unique and centrally placed which makes it possible for people from all parts of the country to converge for trading and engage in other equally important activities. The metropolis falls within the wet sub-equatorial type with an average minimum temperature of about 21.5°C and a maximum average temperature of 30.7°C. The average humidity is about 84% at 0900 GMT and 60% at 1500 GMT (KMA Development Plan, 2006).

Urban agriculture is the main activity practiced by farmers in the metropolis with about 90% of farmers using wastewater for irrigating their crops throughout the year due to the lack of potable water for irrigation purposes (Ghana Statistical Service, 2010).

The farmers cultivate vegetable crops such as lettuce, cabbage, spring onions, green pepper, carrots among others.

Study population

About 500 salad food vendors were included in the study from all the 10 sub-metros within the metropolis and 270 salad food samples were collected. The study was carried out from January to April, 2013; where salad food vendors were interviewed using a detailed questionnaire where information such as the educational level, training on salad food preparation, knowledge of personal hygiene among others were captured while some observations were made. The vendors included restaurants/cafeteria managers and street food sellers.

Sampling and analysis

The inclusion criteria were food vendors who serve salad in addition to the food and are located within the Kumasi metropolis. In most cases, salad consisted of slices of lettuce, onion- spring onion or round bulb-like onion, cabbage, cucumber and carrot. After a month of questionnaire administration, triplicate samples of salad foods were collected in sterile plastic bags kept on ice and transported to the laboratory for analysis.

Triplicates samples of salad food ready for consumption were thoroughly mixed together to form a composite and then 1 g of the composite sample was weighed and added to 9 ml of saline. The content was then pulsified for sixty seconds (60 s) to wash away the surface contents of the samples and thus form a stock solution or diluent. Diluent was used to inoculate Eosine Methylene Blue Agar (EMBA) plates and incubated at 44°C for 24 h. Plates showing growth of *E. coli* colonies (blue black with metallic sheen appearance) were sub cultured onto Sorbitol MacConkey agar (SMA) plates and incubated at 44°C for 24 h. Colonies that showed colourless appearance were purified (Baron et al., 1994). Pure colonies were then serologically confirmed with *E. coli* prolex™ latex agglutination test kit from Oxoid, England. One drop of *E. coli O157:H7* antisera were dispensed onto a reaction card and a loop full of the pure colony of the test organism was then added. This was then carefully emulsified into a smooth suspension. The card was carefully rocked in a circular motion. If agglutination occurred within 1 min then the test organisms were considered positive for *E. coli O157:H7*. A similar reaction was carried out as a control using normal saline instead of the *E. coli O157:H7* antisera. Quantifications were based on colony forming units (CFU) (Oxoid Limited, 2012).

The specificity and sensitivity of this protocol was 100 and 99%, respectively.

Sample of questionnaire

Food vendors' questionnaire on salad preparation and handling within the Kumasi Metropolis is shown below:

Date of interview:

Interview start:
End:

Name of interviewer Tel.
No.

Suburb:

Type of eating place: A. Hotel [ ] B. Restaurant [ ] C.Cafeteria [ ] D. Street food [ ]

Do they serve salad? A. Yes [ ] B.No [ ]

(Inclusion criteria is facilities where salad is served)

Name of respondent
Tel. No.

1. Age of respondent:

2. Gender: A. Male [ ] B. Female [ ]

3. Marital status: 1. Single [ ] 2. Married [ ] 3. Divorced [ ] 4. Widowed [ ]

4. Educational level: Can you read and write? Yes ( ) No ( )

A. Primary [ ] B. Secondary [ ] C. Tertiary [ ] D. No answer [ ]

5. How long have you been working as a food vendor? A. <1 yr ( ) B. 1-2 year [ ] C. 3-5 years [ ] D. 6-10 years [ ] E. More than10 years

6. Are vegetables good for our health? 1. Yes [ ] 2. No [ ]

If yes, why? ....

If No, why?...

7. What type(s) of vegetable(s) do you use to prepare your salad?
A. Cabbage [ ] B. lettuce [ ] C. green pepper [ ] D. Carrots [ ] E. Other [ ] F. cabbage and lettuce G. cabbage, lettuce and carrots [ ]

If other, Specify…………………

8. Where do you buy raw vegetables? A. Farm [ ] B.Wholesale [ ] C. Retail Market [ ] D. Other source

If other source specify…………………………

If farm, where is it located? ……………………………

If a wholesale market, where is it located?.................................

If a retail market, where is it located?...

Do you always obtain your vegetables from the same place?

A. Yes [ ] B. No [ ]

9. Where do you store the raw vegetables before salad preparation?

A. On the floor [ ] B. Refrigerator [ ] C. On table [ ] D. Sack [ ]

10. How do you wash your raw vegetables?

A. Water only [ ] B. Water and salt only [ ] C. Water and vinegar only [ ] D. Water with Salt and vinegar solution [ ] E. Chlorine tablets [ ] F. Potassium permanganate [ ] G. Do not wash [ ] H. Other

If other specify………………………………………………

11. How long do you soak the vegetables? A. Less than 1 minute [ ] B. 1–2 minutes [ ] C. 3–4 minute [ ] D. 5–10 minutes [ ] More than 10 minutes [ ] E. Do not know [ ]

12. Have you had any training on salad preparation? A. Yes [ ] B. No [ ]

If yes where? A. School [ ] B. Friends [ ] C. Relatives [ ] D. Other [ ]

If other, specify………………………………………..

13. Do you use running tap water or a bowl of water? A. Running [ ] B. Bowl [ ]

14. Where do you cut your vegetables? A. Chopping board [ ] B. Ordinary table [ ] C. any plane surface [ ] D. Other [ ]

If other, specify…………………………………………………

15. How often do you clean your Cutting surface? 1. Everyday [ ] 2. Every Two days [ ] E. Once a week [ ] F. Other [ ]

If other, specify…………………………………………………

16. How often do you change you cutting surface? A. 1-3 months [ ] B. 4- 6months [ ] C. 7-12months [ ] D. More than 12months

17. Do you wash your hands before food preparation? A. [ ] Yes [ ] B. No

If yes, how? A. With water only [ ] B. With soap and water [ ]

19. Do you wash your hands after visiting the toilet? A. Yes [ ] B. No [ ]

If yes, how? A. With water only [ ] B. With soap and water [ ]

21. How do you preserve left over vegetable salad?
A. Refrigerator [ ] B. Leave it uncovered [ ] C. Kitchen cupboard [ ] [ ]D. other

If other, Specify…………………………………..

Observational checklist

Observational checklist used is shown in Table 1.

RESULTS AND DISCUSSION

Most outbreaks of food borne diseases have been implicated with consumption of unrestricted wastewater irrigated vegetables (Gould et al., 2013). Two hundred and seventy (270) salad food samples prepared from wastewater irrigated vegetables were analyzed for *E. coli* O157:H7, total coliforms and faecal indicator *E. coli* from both restaurants/cafeteria and street food vendors in Kumasi. The overall prevalence of *E. coli* O157:H7 was found to be 1.1% with mean log cfu/g of 1.1 ± 0.3 and within the sub-metro where *E. coli* O157:H7 occurred, the prevalence was 13.0% which confirms the study reporting that wastewater irrigated lettuce is contributing to the

Table 1. Observational checklist.

Observation	Yes (1)	No (2)	Remarks
Near a toilet facility			
Near a refuse dump			
Near a storm water drain/gutter			
Water used for cleaning utensils looks dirty			
Flies can be seen around facility			
Cutting board for vegetables is dirty			
Unprocessed vegetables are lying on the floor			

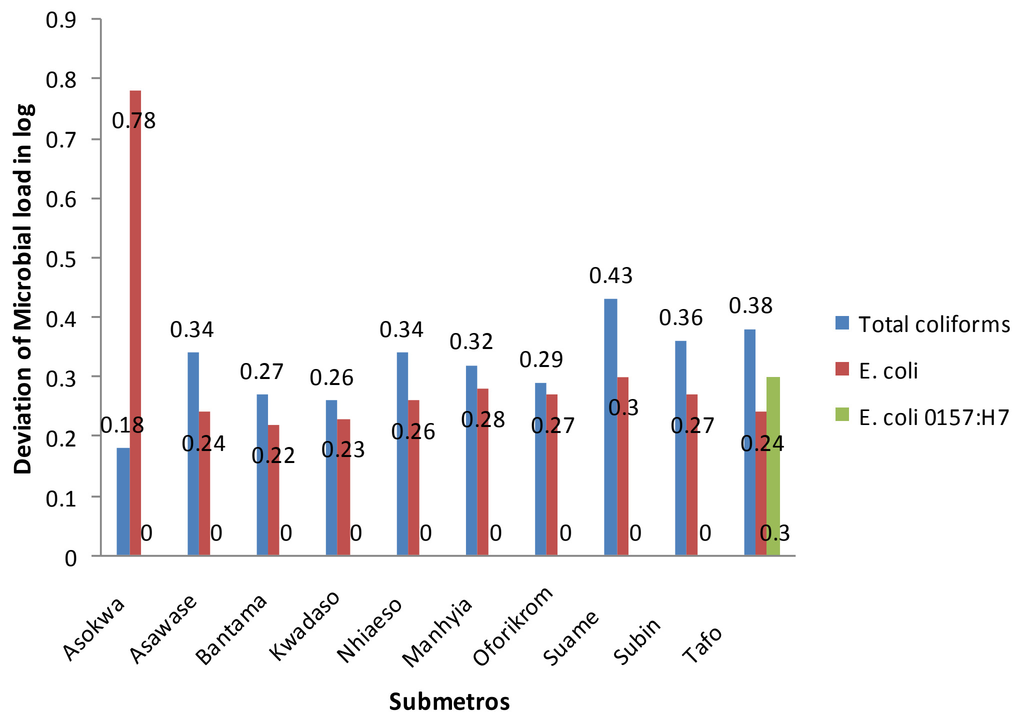

Figure 1. Mean $\log_{10}$ counts of total coliforms and *E. coli* and *E. coli 0157:H7* in ready-to-be-eaten salad food from vendors in the Kumasi Metropolis.

transmission of *E. coli* O157:H7 in Kumasi, Ghana (Seidu et al., 2014). Among the 270 food samples analyzed, 42 were sampled from restaurants and cafeterias which were all negative for *E. coli* O157:H7 which thus imply a null prevalence rate. This study therefore noted that hygiene practices among restaurants and cafeteria food vendors' rates higher compared with street food vendors. However, all the samples had some levels of total coliforms and faecal indicator *E. coli* which also indicates that salad foods sold in Kumasi contains microbial pathogens (Seidu et al., 2014). The mean log cfu/g of total coliforms and faecal indicator *E. coli* from both restaurants/cafeteria and street foods were found to be 2.9±0.34, 2.4±0.30 and 2.8 ± 0.25, respectively. The sub metro that recorded the highest total coliform result was Oforikrom (Figure 1) with mean log cfu/g of 6.8± 0.9 which happens to be one of the sub metros with quiet a number of farmers growing vegetables like lettuce, cabbage, green pepper, carrot and spring onion among others with the main source of water for irrigation been untreated wastewater from drains which have been noted by other studies as possible source of *E. coli* O157:H7 (Tanaro et al., 2010). From Table 2, Total coliform and E. coli have significant values as 0.999 and 0.991 respectively which are all greater than the alpha value (0.05) we conclude that the test is not significant. This implies that the mean log/cfu of Total coliform and E. coli present in salad foods is the same throughout the ten sub-metros.

On the other hand, since the significant value (0.011) of

Table 2. Chi-Square Test of significance of microorganisms between sub-metros.

Microorganism	Degrees of freedom	Chi-Square Value	Significant value	Alpha value
Total Coliform	8	0.800	0.999	0.05
E. coli	8	1.200	0.991	0.05
E. coli 0157:H7	8	6.400	0.011	.005

Table 3. ANOVA table of significant difference between means.

Medium of microbes	Sum of squares	Degrees of freedom	Mean square	P-Value	Alpha value
Within means	52.104	3	17.368	0.093	0.05
Between means	30.396	6	5.066		
Total	82.500	9			

E. coli O157:H7 is less than alpha value (0.05) we conclude that the test is significant. This implies that the mean log/cfu of *E. coli* O157:H7 is not the same throughout the ten sub-metros.

From Table 3, since the p-value (0.093) is greater than the alpha value (0.05) we have enough evidence to reject the null hypothesis Ho:u1=u2=u3 hence we conclude that the test is not significant. This implies that the mean log/cfu of the three different microorganisms in the salad foods is the same.

Farmers from this study mostly use manure from livestock which serves as a reservoir of pathogen and therefore stands as a route for pathogen transmission (Oliveira et al., 2012). During the study faeces of livestock grazing around irrigation fields were also common to sight, which get washed into these irrigation water bodies when there is a fall increasing the pathogen contamination to a lager extent (Karesh et al., 2012). It was also noted during interactions with the food vendors that, most of the vegetables cultivated by these farmers are the main source of vegetables salad food vendors close-by and around the vicinity use for salad food preparations. About 90.4% of the vendors buy their raw vegetables from the retail market within the metropolis and 6.4% obtain theirs directly from the farms which they claim is the cheapest means of acquiring their raw materials for salad food preparation. From this study, it was observed that majority of the salad food vendors (SFV) fall within the ages 20-30 recording a percentage of 32.6 as against 26-45 years recorded by Monney et al. (2014) and the smallest group is within the age range 51-55 giving a percentage of 1.0 and about 70.4% of the SFV are female which is a little lower than 95% reported by Monney et al., (2014) and this is attributed to the fact that in the Ghanaian culture, food preparation is considered as the female's job. It was again noted that about 48.4% of the SFV have had secondary education which puts them in a better position to be taken through training of salad food preparation and made it less difficult to deal with in explaining the bases of the study to them. During the study it was also recorded that about 61.4% of the respondents have had training for the job they were engaged, from vocational training schools and friends who are into the salad food vending business. 63.1% of the vendors stated that during their training they were taught to use water and salt to wash their vegetable before preparing, which has been the routine practice; 18.7% use vinegar and water only, 0.2% use potassium permanganate and 9.85% blended water, salt and vinegar for washing. It has been noted from this study that not only disinfection can reduce pathogen load from fresh vegetables but rather pathogen load reduction involves several other factors (Bermudez- Aguirre and Barbosa-Canovas, 2013). Microbial contamination of vegetables can occur through various factors which include soil, irrigation water, various farming practices such as manure application (Amoah et al., 2005), poor sanitary conditions on farm, poor post-harvest handling of vegetables by market women and unhygienic salad food preparation (Amoah et al., 2007). Poor conditions of vending sites could also serve as major vehicles of microbial contaminations because it was observed from this study that, with the restaurant operators who observed a bit of standard level of hygienic conditions, there were lower level of microbial loads recorded compared to the street food vendors.

The study realized from the pattern of response that, 8.2% SFV use ordinary water for washing the vegetables with the idea of removing some levels of microbial load and these are people without any form of training in the job they are handling as well as other contributing factors like weak and inconsistent local bye-laws and logistical constraints (Monney et al., 2014).

Conclusion

Regardless public education and other training programmes organised occasionally by the Food and Drugs Authority and concerns by municipal authorities

such the Kumasi Metropolitan Assembly, salad contamination by food-borne pathogens remains a major public health issue. Almost all the vegetables used for salad food preparations within the city of Kumasi are obtained for wastewater irrigated farms either directly from the farmers or the retail centers. As such, these salad foods contain high levels of microbial pathogens which were isolated in this study. Effort should therefore be made to improving our irrigation systems and adopt new technologies practiced elsewhere since most of the vegetables produced in Kumasi are irrigated with wastewater from the drains and streams. As the structured questionnaire of the study showed, an important element will be to raise public health awareness on options to reduce health risks through proper and effective vegetable washing at the various restaurants or cafeteria as well as street food vendors, which is necessary in and as part of the efforts of addressing salad contamination by food-borne pathogens in the Kumasi Metropolis. Eventually, regular laboratory examinations of the microbial quality of salad foods prepared and sold in restaurants and streets has to remain the target, notwithstanding public awareness on the need to insist on salad foods of good quality as well as improving agricultural practices such as wastewater irrigation by treating these them well before irrigation.

Conflict of interests

The authors did not declare any conflict of interest.

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