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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.

Pelczar JR, Harley JP, Klein DA (1993). Microbiology: Concepts and Applications. McGraw-Hill Inc., New York, pp. 591-603.

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Full Length Research Paper

Growth measurement of *Escherichia coli* by differential scanning calorimetry

Esmeralda Rangel-Vargas[1], Carlos A. Gómez-Aldapa[1], José R. Villagómez-Ibarra[1], Eva M. Santos-López[1], Jesus Rodriguez-Miranda[2] and Javier Castro-Rosas[1]*

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Using differential scanning calorimetry (DSC) and plate count (PC), growth of *Escherichia coli* was measured in four initial *E. coli* cell concentrations cultured in tryptic soy broth (TSB) at 35°C. The calorimeter measured the energy released and/or absorbed by *E. coli* cells during growth and expressed it as thermograms. The resulting thermograms accurately reflected cell growth and activity, and exhibited a close correlation with growth values recorded by PC. Heat release decreased notably in the final stage of the logarithmic growth phase, and no signal was generated once the stationary phase had begun. DSC accurately quantified growth in *E. coli* at different initial cell concentrations grown in TSB at 35°C. Depending on initial *E. coli* concentration, DSC detected growth starting after just a few minutes and up to five hours. The detection limit of calorimeter was 4.3 ± 0.4 $\log_{10}$ CFU. DSC is an effective alternative method for measuring bacterial growth in TSB in real time. This is the first report of DSC use for measuring bacterial growth.

Key words: Differential scanning calorimetry (DSC), *Escherichia coli,* thermograms.

INTRODUCTION

Plate count and turbidimetric (e.g. Bioscreen C) are the most commonly used techniques to monitor microbial growth (Huchet et al., 1995). Plate count is widely used to estimate viable cell numbers in culture medium (Madrid and Felice, 2005), but has the disadvantage that it requires 24 to 48 h.

Techniques based on turbidity measurement are easy and fast, use small volumes and can be monitored in real time. However, they are useless for measuring microbial growth in opaque samples (McClure et al., 1993; McKellar and Knight, 2000). In addition, with turbidimetric is required a large numbers of cells (10^6-10^7 cells) for detect differences in optical density (Biesta-Peters et al., 2010).

Calorimetry involves use of a thermal sensor to directly measure the heat emitted or absorbed by a material.

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Microcalorimetry and differential scanning calorimetry (DSC) are based on material thermal response and have been used to study some aspects of microorganisms. They are generally distinguished by measurement cell size and calorimeter specifications (von Stockar and Marison, 1989). Microcalorimetry has been used to measure bacterial metabolism (Braissant et al., 2010a; 2010b) whereas DSC has been used to measure microbial thermal death (Lee and KaletunÇ, 2002; Honglin et al., 1993). Microcalorimetry can measure changes in heat released and/or absorbed during bacterial growth (Boe and Lovrien, 1990; Braissant et al., 2010a; 2010b; Von et al., 2009), and has been used to monitor growth of *E. coli* in broth under different culture conditions. Sugar degradation during *E. coli* growth generates heat that can be measured by a microca-lorimeter sensor, producing thermograms with exother-mal peaks (Belaich and Belaich, 1976a; 1976b; Dermoun and Belaich, 1979; 1980; Braissant et al., 2010a; 2010b). A distinct disadvantage with microca-lorimetry is the need for large sample volumes. Microcalorimetry for studying cellular metabolism has been limited to the use of batch and flow microca-lorimeter and has required large numbers of cells (10^5 - 10^6 cells) for reproducible measurements (Braissant et al., 2010a). In addition, most cases, microcalorimetry requires an initial equilibration time of 1 h, during which data cannot be collected (Braissant et al., 2010a). DSC offers a more sensitive method for studying cellular metabolism in living cell. In a Differential scanning calorimeter a sample (in crucible) and a suitable reference material (in crucible) are heated in separate chambers at a constant rate. A feedback system maintains an essentially zero temperature difference between the chambers and provide an output that measures the excess power requirement of one chamber relative to the other one. The differential scanning calorimeter requires only 1 minute for tempe-rature equilibration of crucibles. A limitation of DCS is the low throughput: just one sample crucible per DSC per day.

Differential scanning calorimeters function within a wide temperature range (-45 to 700°C), have a <0.04 µW signal resolution, require small sample volumes and can analyze liquid, solid, turbid and/or opaque samples. Bacterial growth has not been measured previously using DSC, although this technique is a promising alternative for real-time monitoring of bacterial growth in opaque, turbid and/or solid samples that cannot be monitored with turbidimetric methods. In food microbiology, DSC is potentially promising since it can directly measure microbial growth in food, in real time and continuously. It also makes it possible to use bacterial growth heat generation data in predictive equations with applications in predictive microbiology or in food biotechnology for monitoring microbial fermentations.

The present study objective was to measure *E. coli* growth in tryptic soy broth at 35°C by both differential scanning calorimetry (DSC) and plate count (PC), and compare the resulting data.

MATERIALS AND METHODS

Bacterial strain

The strain used was *E. coli* ATCC (American Type Culture Collection) 25922 preserved in inclined blood agar base under refrigeration. Before use, the strain was activated three times in trypticase soy broth (TSB) and incubated for 18 h at 37°C.

Inoculum preparation

E. coli cultured for 18 h at 37°C in TSB was washed twice with isotonic saline solution by centrifuging at 1507 *g* for 20 min each time and then resuspended in TSB at pH 7. Decimal dilutions were then done in TSB at pH 7 to produce flasks containing 100 ml TSB with final bacterial concentrations of 4 x 10^7, 4000, 40 or 4 colony-forming units (CFU)/ml.

Calorimetry and bacterial count

An 822e/400 (Mettler-Toledo) calorimeter was used, and calibrated with indium. Samples (40 µl) were taken of each bacterial suspension and placed in previously sterilized 40 µl aluminum crucibles for thermal analysis (ME-00026763, Mettler-Toledo). These were hermetically sealed with a press and placed in the calorimeter. A sample of sterile, uninoculated TSB was used to verify device baseline. Temperature was maintained at 35°C for 14 h. The resulting thermograms were analyzed to record initial signal (heat) generation (that is, onset), maximum signal generation (peak) and signal termination (final), as well as total heat generated based on the area below the curve (Toro-Vazquez et al., 2003). The onset, peak, and final temperature for the different transitions were determined using the first derivative of the heat capacity of the sample calculated with the DSC software library. Thus, onset and final temperature were established as the temperatures where the first derivative of the heat capacity of the sample initially departed from or returned to the baseline, respectively. In contrast, peak was established as the temperature where the first derivative of the heat capacity of the sample crossed the baseline (i.e. the inflexion point of the transition curve). The enthalpy (total heat generated) was calculated by integration of the corresponding exothermal peak. Integration was done between the respective onset and final temperature with the DSC software (Toro-Vazquez et al., 2003). Three replicates were done per experiment.

In a parallel procedure, different sealed crucibles (one for each time-point) containing inoculated TSB with the different initial inoculums level were incubated for 14 h at 35°C and the standard plate count technique applied (FDA, 2010), briefly, in the sampling time, a crucible was taken and punched. The culture was taken from crucible with a micropipette and serial dilutions (1:10, 1:100, 1:1000, etc) were made with peptone diluent. Dilutions were plated in trypticasein soy agar (TSA) to quantify *E. coli* growth. The CFU data were $\log_{10}$-transformed and bacterial growth results from the DSC and plate count techniques were compared. Three replicates were done per experiment.

Statistical analysis

A completely random design was used and significant differences ($p<0.05$) calculated with an analysis of variance (ANOVA) and a

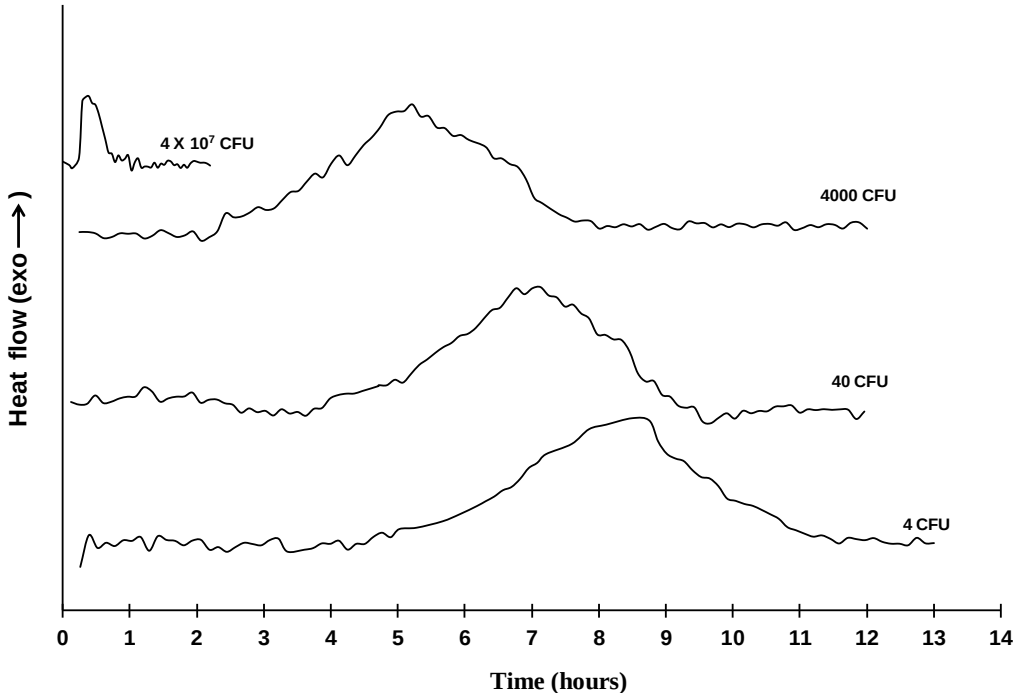

Figure 1. DSC thermograms of *E. coli* growth for four different initial inoculum concentrations in TSB at 35°C and pH 7.

Table 1. Time in minutes at signal detection onset, peak heat generation and final signal detection, and total heat generated during growth of four *E. coli* initial inoculum concentrations.

Initial concentration (CFU)	Onset	Peak	Final	Heat released (Integral [mJ])
4	*290 ± 20[a1]	522 ± 28[a]	693 ± 31[a]	163 ± 13[a]
40	233 ± 72[b]	437 ± 26[b]	581 ± 11[b]	150 ± 71[a]
4000	136 ± 24[c]	297 ± 14[c]	450 ± 12[c]	113 ± 29[a]
4×10^7	14 ± 0.3[d]	28 ± 6[d]	43 ± 1[d]	8 ± 2[b]

*Mean ± SD of three replicates, [1] Different letters in the same column indicate statistically significant difference ($p < 0.05$).

Duncan test (Statistica 8, StatSoft, Inc., 2007). Simple correlation coefficient (r) between initial inoculum concentration and total heat output for *E. coli* growth in TSB were determined with a statistical analysis system (Statistica 8, StatSoft, Inc., 2007).

RESULTS AND DISCUSSION

Thermograms illustrate microorganism growth by generating a curve with three main points: onset, peak and final signal detection (Figure 1). In thermograms, signal onset is related to heat generation, which can be associated with microbial activity. Samples with different initial cell concentrations produced different responses, with higher concentrations producing more rapid heat generation (Figure 1). *E. coli* grew well in TSB at low oxygen levels and under these conditions the thermograms showed it to exhibit a hyperbolic growth pattern (Figure 1). This coincides with a previous report of hyperbolic growth in *E. coli* when metabolizing glucose and with use of a calorimetric pump (Boe and Lovrien, 1990). This can also be observed in the onset, peak and final time results (Table 1). At an initial concentration of 4 CFU, onset was at 4.83 ± 0.33 h, while at 40 CFU it was 3.88 ± 1.19 h, at 4000 CFU it was 2.27 ± 0.4 h and at 4 $x10^7$ CFU it was 0.23 ± 0.005 h. As mentioned above, a higher initial concentration produced a more rapid thermal response, probably due to greater metabolic activity (Table 1). In addition, final time occurred more

rapidly (0.71 ± 0.02 h) at the highest initial concentration ($4x10^7$) than at the lowest (4 CFU; 11.55 ± 0.51 h). There are not previous reports about DSC used for growth measurement of bacteria to compare our data. However, there are data obtained with microcalorimetry. Although a direct comparison between DCS and microcalorimetry is not possible, it is useful to have results from the previous study with microcalorimetry as a reference.

Our results agrees with microcalorimetry results in which both onset and final signals occurred more rapidly at higher initial concentrations of *Lactococcus lactis* (Kobanova et al., 2012). In addition, our results are in agreement with the studies made with microcalorimetry and reported by Maskow et al. (2012), who observed that initial rapid heat generation depends of the initial inoculum concentration.

In the other hand, in a microcalorimeter, the heat released by the microbial cultures during growth has been found to correlate quantitatively with biomass generation, uptake of substrates or oxygen (Birou et al., 1987). Metabolic events, such as shifts from one substrate to another, change of limitations, inhibitions and overflow metabolism would cause characteristic changes in the heat evolution curves (Braissant et al., 2010b; von Stockar and van der Wieler, 1997; Yi et al., 2000). It is possible that during the growth of *E. coli* in crucibles of DCS occur to changes in oxygen concentration and affect the behavior of *E. coli* and biomass generation and the heat released. However, in the present study we did not evaluate the effect of changes in oxygen concentration in *E. coli* behavior.

Initial *E. coli* concentration significantly ($p < 0.05$) affected detection of exothermal signal onset, peak and final (Figure 1) generated by *E. coli* metabolic activity. However, except for the $4x10^7$ CFU initial concentrations, no difference ($p > 0.05$) was detected in total heat detected for the 4, 40 and 4000 CFU initial concentrations (Table 1). This coincides with a report of lack of difference ($p > 0.05$) in total heat detected by microcalorimetry between initial *L. lactis* concentrations ranging from 10^2 - 10^4 CFU (Kobanova et al., 2012). However, total heat values were significantly lower ($p < 0.05$) at very low (10^0-10^1) and very high (10^5-10^6) concentrations compared to those of the 10^2 - 10^4 CFU concentrations (Kobanova et al., 2012).

The low total detected heat value in the present study at the $4x10^7$ CFU concentrations versus the other initial concentrations suggests that cellular multiplication was minimum in the TSB contained in the crucibles during incubation at 35°C. In addition, the rapid heat release observed in this broth (0.23 h) is probably related to high enzymatic activity (e.g. sugar degradation) rather than cellular multiplication. Indeed, a series of biochemical reactions known to occur before the lag phase can lead to heat generation (Zhang et al., 1993). In the present study, it is therefore probable that the very high initial *E. coli* concentration ($4x10^7$) allowed heat generated by lag phase reactions to be detected by the calorimeter. This hypothesis would agree with the rapid heat generation over a short time reported at a high initial cell concentration for *E. coli* grown in culture broth at 25°C and monitored by calorimetric pump (Boe and Lovrien, 1990). These authors state that caloric yield is a measure of metabolic capacity which in turn is dependent on and proportional to cell number and mass.

To estimate cell concentration during thermal monitoring in the calorimeter, *E. coli* growth in TSB inoculated with the same initial cell concentrations was measured simultaneously by the plate count (PC) technique. Final cell concentrations measured in the PC trial did not differ, independent of initial inoculum concentration (Figure 2). At higher initial cell concentrations, maximum cell concentration from microbial growth was reached more rapidly (Figure 2). This indicates that growth rate was lower at higher initial concentration. The 4×10^7 CFU initial inoculum concentration exhibited the lowest multiplication rate, supporting the hypothesis proposed above to explain this inoculum's thermal behavior. Indeed, heat production in bacterial cultures is directly linked to microorganism multiplication (Marison and Stockar, 1985).

To estimate cell concentrations at onset, peak and final signal detection, as well as total heat generated, plate count values were related to the thermograms based on initial inoculum concentration (Table 2). For the 4, 40 and 4000 CFU initial concentrations, onset was detected when the *E. coli* had begun to grow and had reached a concentration of 4 $\log_{10}$ to 4.5 $\log_{10}$ CFU, but this occurs in different times. In other words, the calorimeter detection limit is 4.3 ± 0.4 $\log_{10}$ CFU for *E. coli* at 4 to 4000 CFU initial concentrations (Table 2).

Microcalorimeters have a higher detection limit. For example, in a study of *L. lactis* growth in culture broth the microcalo-rimeter detection limit was 10^5 cells (Kobanova et al., 2012). In the present study, by contrast, the calorimeter detected a heat signal within the first few minutes for the 4×10^7 initial concentration, which was associated with enzymatic activity rather than cell multiplication.

Cell concentrations at the peak signal detection had reached an advanced logarithmic phase: 7.2 $\log_{10}$ CFU for the 4 CFU initial concentrations, 6.82 $\log_{10}$ CFU for the 40 CFU, and 6.92 $\log_{10}$ CFU for the 4000 CFU (Table 2). Heat release was no longer detected when growth had concluded the logarithmic phase, at 8.5 to 8.8 $\log_{10}$ CFU. The drastic drop in heat release observed as the *E. coli* entered the final stage of the logarithmic phase can be attributed to a decrease in growth rate as the cultures neared stationary phase (Ramírez et al., 2005), and a consequent reduction in the amount of heat generated. In bacteria, a lack of nutrients, among other reasons, causes cellular multiplication rates to decrease notably and almost stop as they enter the stationary phase (Ramirez et al., 2005). In addition, the amount of heat

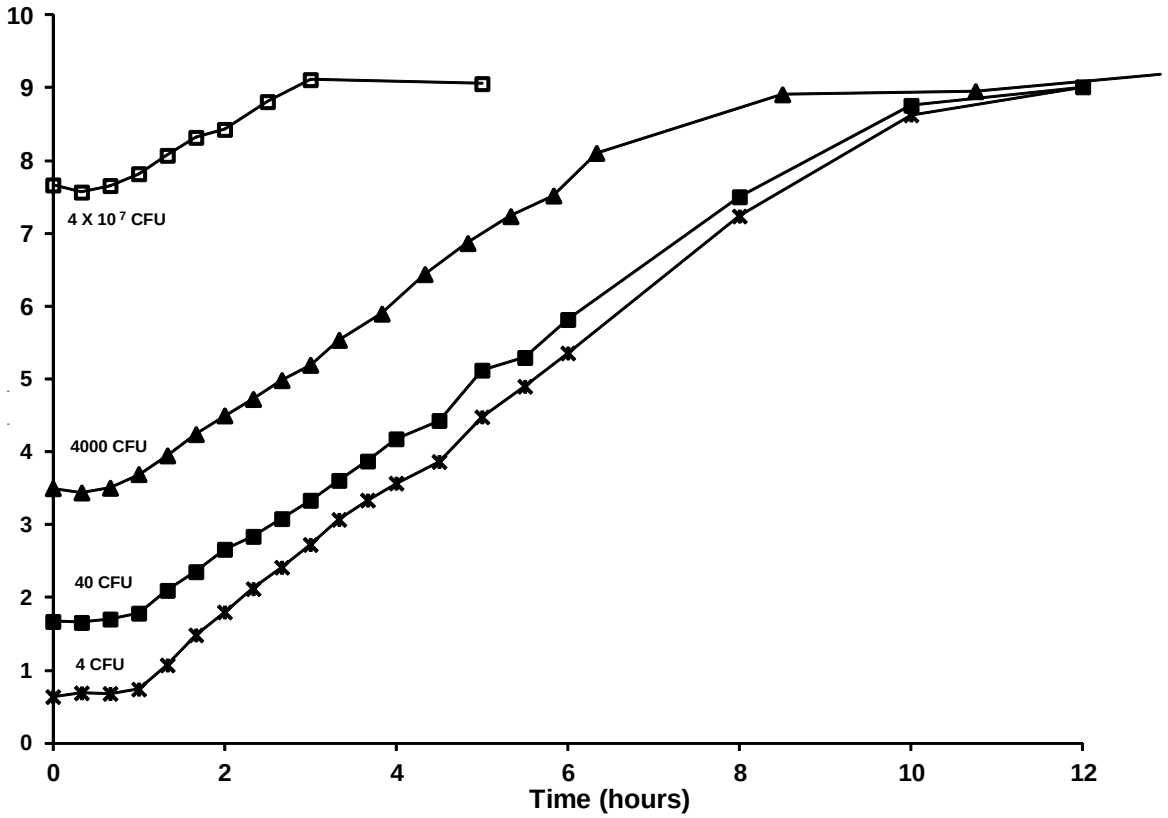

Figure 2. *E. coli* growth curves from plate counts for four different initial inoculum concentrations in TSB at 35°C and pH 7.

Table 2. Bacterial concentration (log_{10} CFU) at signal detection onset, peak heat release and final signal detection during growth of four initial inoculum concentrations of *E. coli*.

Initial inoculum concentration (CFU)	Cell concentration (CFU)		
	Onset	Peak	Final
4	4.3 ± 0.3^{a1}	7.2 ± 0.4^{a}	8.8 ± 0.2^{a}
40	3.9 ± 0.5^{a}	6.9 ± 0.2^{ab}	8.5 ± 0.5^{a}
4000	4.7 ± 0.2^{ab}	6.9 ± 0.6^{ab}	8.6 ± 0.3^{a}
4×10^{7}	7.0 ± 0.07^{c}	7.6 ± 0.1^{ac}	7.5 ± 0.4^{b}

*Mean ± SD of three replicates, [1]Different letters in the same column indicate statistically significant difference ($p < 0.05$).

released by a bacterial cell depends on its cellular activity, the types of energy and carbon sources it has access to (especially their degree of reduction), the biomass production coefficient, physical conditions such as temperature and pH, microorganism respiration and culture medium chemical composition (Chang-Li et al., 1988). The reduced growth rate of *E. coli* as it entered the stationary phase in the present study manifested as a progressive decrease in heat generated until the calorimeter ceased to detect a change in heat flow. This is more visible when the thermograms for a low initial concentration (4 CFU) are compared point-by-point with the corresponding PC values (Figure 3). In the PC, growth was detected at 4.83 h and 4.3 log_{10} CFU. In the DSC, the highest heat flow was detected during the logarithmic growth phase, the peak occurred at the end of this phase and the stationary phase is clearly visible as the point at which heat flow is stable. Finally, although no difference ($p>0.05$) was detected in total heat output during the growth of *E. coli* for 4, 40 and 4000 CFU of initial inoculum concentrations (Table 1), a negative correlation ($r=0.845$) between initial inoculum (including

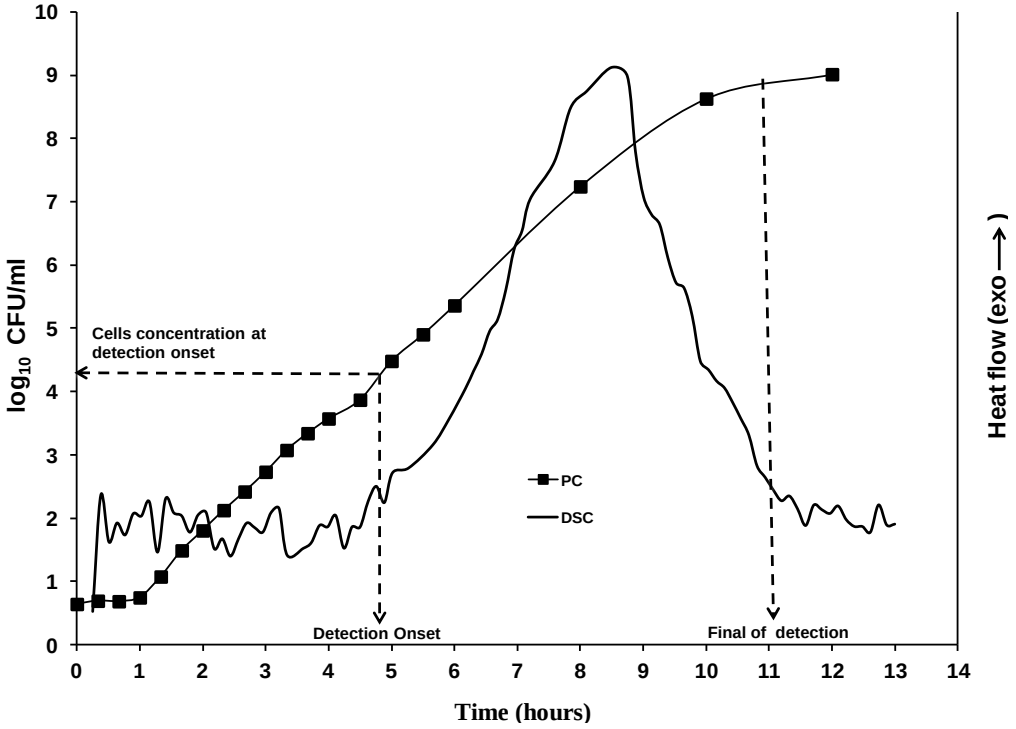

Figure 3. Comparison of DSC thermogram and plate count (PC) growth curve for *E.coli* growth at a 4 CFU initial concentration in TSB, at 35 °C and pH 7.

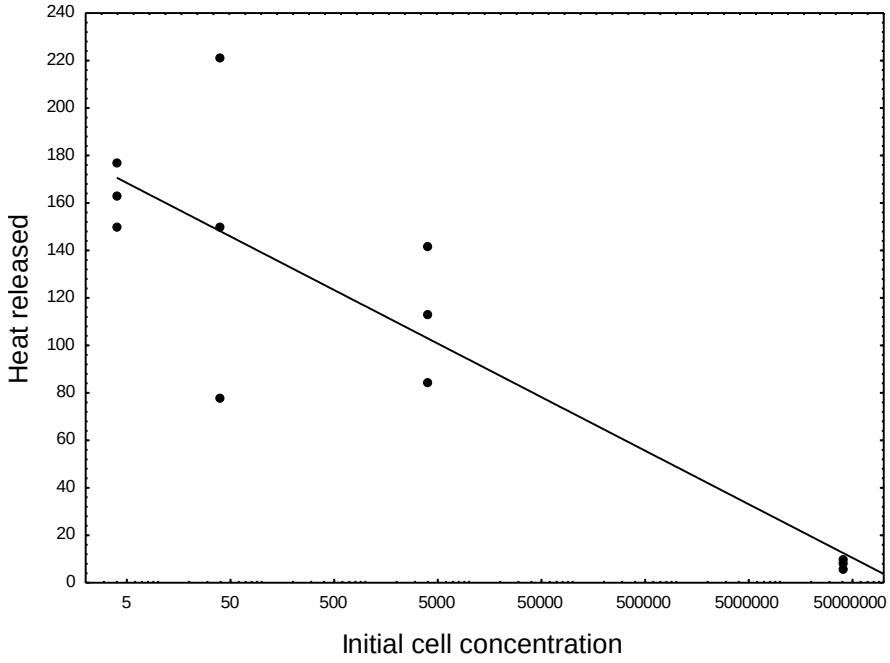

Figure 4. Correlation between initial inoculum concentration and total heat output for *E.coli* growth in TSB at 35°C and pH 7 (r=0.845).

4 x10^7 CFU) and total heat output was observed (Figure 4).

DSC effectively monitored growth in *E. coli* at different initial inoculum concentrations. It is a promising alterna-

tive technique for real-time monitoring of bacterial growth and has the potential to record different growth-related parameters. The calorimeter is a powerful tool for studying metabolic processes in living bacteria because it provides data on metabolism. Thermograms readily lend themselves to monitoring general microbial activity, and observing and pinpointing metabolic events such as product formation with subsequent oxidation, medium limitations and inhibition. The fast response of the differential scanning calorimeter and its direct correlation to plate count results suggests its use for generating convenient and rapid quantification of microbial growth kinetics. This is the first report of DSC use to measure real-time bacterial growth. It effectively measured growth of *E. coli*. Measurement of cellular growth by DSC can help in modeling cell behavior, and may contribute to creating mathematical models that could predict the behavior of microorganisms on various substrates and under different growth conditions.

Conflict of Interests

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

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Status of root-knot nematode (*Meloidogyne* species) disease in vegetable crops of some districts of central plain region of Chhattisgarh State, India

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The root-knot nematodes (*Meloidogyne* species) are economically important pathogens, especially infesting vegetable crops. In the tropics and subtropics, *Meloidogyne incognita* causes an estimated yield loss of 5 to 43% in vegetable crops. In the present study, root-knot nematode disease was surveyed in various vegetable crops in the central plain region of Chhattisgarh State. Roots and rhizosphere soil samples were collected from 44 farmlands representing five districts of Chhattisgarh during mid cropping season. Nematodes were extracted by Cobb's sieving and decanting method, followed by modified Baermann's funnel technique. Out of 44 samples, 24 showed the root-knot nematodes infection with an overall incidence of 54.54%. The percent incidences of disease ranged between 30 and 80% in five districts. Out of 13 species of vegetable crops surveyed, nine were observed to be infected. The most frequently occurring species was *Meloidogyne incognita* (63.33%), followed by *Meloidogyne arenaria* (20%) and *Meloidogyne javanica* (16.67%). Maximum frequency was observed in *Lageneria siceraria* (100%), and least was *Musa paradisiaca, Daucus carota* and *Amaranthus tristis* (50%). The mean population density of 2nd stage nematodes ranged between 766 and 9076 nematodes/200 cc soil and 10 g roots in samples.

Key words: Root-knot nematode, survey, *Meloidogyne* spp., incidence, frequency, Chhattisgarh.

INTRODUCTION

The state of Chhattisgarh is situated between 17-23.7°N latitude and 80.40-83.38°E longitude in the central eastern part of India with a population of more than 20 million and total geographical area of 136.03 thousand sq. km. It is divided into three agro-ecological regions on the basis of topography; they are the Northern Hills, the Central Plains and the Bastar Plateau. The central plain also known as Chhattisgarh plain includes the districts of Raipur (capital), Mahasamund, Dhamtari, Durg, Rajnandgaon, Kabirdham, Bilaspur, Korba, Janjgir and a part of Kanker (Narharpur and Kanker block) along with a part of Raigarh. Its richness in biotic and abiotic diversities as well as socio-economic conditions, favor the cultivation of various horticultural crops like vegetables, temperate, tropical and high elevation fruits. In spite of being known as the rice bowl of the country its

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different soil types support vegetable cultivation. Chhattisgarh stands in 15th position in vegetable cultivation for the year 2008-2009 in India (Health Vision Research, 2009-10).

The root-knot nematodes (*Meloidogyne* spp.) are one of the most economically important plant pathogens especially in vegetable crops (Kalaiarasan, 2009), causing an estimated yield loss which varies from 5 to 43% (Sasser, 1979). The genus *Meloidogyne* attacks nearly every crop and has been reported to cause an annual loss of Rs. 547.5 million in Cucurbits (Jain et al., 2007; Chandra et al., 2010). There are three common species: *Meloidogyne incognita*, *Meloidogyne javanica* and *Meloidogyne arenaria,* among which *M. incognita* is widespread in the tropics and subtropics (Sasser, 1979).

Some preliminary works have been documented on the prevalence and community analysis of root-knot nematode infections in vegetable crops in some districts of the state of Chhattisgarh (Sao et al., 2008; Sahu et al., 2011). Occurrences of some plant parasitic nematodes, such as, *Aphelenchoides besseyii*, *Rotylenchulus reniformis*, *Radopholus similis*, *M. incognita* and *Rotylenchulus reniformis* are also reported (Khan et al., 2010). In this area, farmers bear a loss of productivity in vegetable crops due to lack of knowledge concerning these pests. However, no work has been documented on the incidence of the major *Meloidogyne* spp. in the districts of the central plains. The present study comprises of a survey of root-knot nematode diseases prevalent in various vegetable crops of the central plain region of Chhattisgarh state.

MATERIALS AND METHODS

A field survey of various villages belonging to the five districts of central plains of Chhattisgarh was conducted during September-February, 2011-2012 and 2012-2013 to determine the root-knot nematodes (*Meloidogyne* spp.) associated with various vegetable crops. Diseased fields were selected on the basis of above ground symptoms of the crops such as, wilting, slow growth, stunting and yellowing of leaves. Soil and root samples were collected by digging from a depth of 10-15 cm at the rate of one unit sample per acre crop area. Each unit sample was a composite of 20 cores obtained from four corners and centre of the field. Root/soil sub samples (prepared from the unit samples) were stored in polythene bags and kept at 4°C in a refrigerator for not more than seven days. In all, 44 soils and root samples were collected during mid cropping season.

Extraction of the nematodes was done by Cobb's sieving and decanting method followed by modified Baermann's funnel technique (Goodey, 1957; Southey, 1985) and the nematode suspensions collected. Infected roots were stained in Lacto phenol acid fuchsin (Himedia, India) and observed for the presence of nematodes. Species identification (Eisenback et al., 1981) was done by preparation of perineal sections of the females after dissecting out from the galled roots observed under Leica compound microscope with camera attachment. The estimation of nematode population per 10 g root sample and 200 cc soil sample was done in a multi-chambered counting dish under a stereoscopic binocular microscope (ZEISS, STEMI 2000C) and the incidence, occurrence and frequency was calculated using the formulae (Norton, 1978):

$$\text{Disease incidence} = \frac{\text{Number of sample with root-knot nematodes infection}}{\text{Total number of sample surveyed}} \times 100$$

$$\%\ \text{Occurrence of species} = \frac{\text{Number of sample with a species}}{\text{Total no. of samples observed}} \times 100$$

$$\text{Absolute frequency} = \frac{\text{No. of sample containing a species}}{\text{No. of sample collected}} \times 100$$

$$\text{Relative frequency} = \frac{\text{Frequency of occurrence of species}}{\text{Sum of frequency of all samples}} \times 100$$

RESULTS

Incidence and occurrence of root-knot nematode disease (district wise)

In all, 44 samples belonging to 19 farmlands and 13 different vegetable crop species were collected during survey. Out of these, only nine crop species exhibited the presence of the root-knot nematodes. District wise variations in the incidence of the root-knot disease were observed to exist with the highest percent incidence in Raipur and Bemetara (80), followed by Dhamtari (57.14), Rajnandgaon (33.33), while the lowest incidence was observed in district Durg (30) (Figure 1).

The percentage incidence in this investigation was based on the direct examination of root-knots of vegetable crops. A more detailed root-knot nematodes disease survey in five districts of Chhattisgarh central plain region is expected to reveal many more crops to be susceptible.

The populations tentatively identified included *M. incognita, M. javanica* and *M. arenaria* (Figures 2 and 3) from the infected roots which occurred both singly or concurrently. *M. incognita* happened to be the most abundant species in the sampled area, single infections of which ranged from 10 to 40% in all surveyed districts. *M. javanica* alone (4.76 to 10%) and *M. incognita* + *M. arenaria* (9.52 to 10%) concurrently were found in Dhamtari and Durg, *M. arenaria* (8.33 to 20%) alone in Dhamtari and Raipur, *M. incognita* + *M. javanica* (40%) in Bemetara, and *M. javanica* + *M. arenaria* (25%) were found in Raipur (Figure 4).

Incidence of root-knot nematode disease (crop wise)

Crop wise variations in the incidences of root-knot nematode infections were observed with an overall incidence of 54.54% (Figure 5). A hundred percent incidence was observed in *Lageneria siceraria* (Table 1), followed by *Momordica charantia*, *Cucumis sativus* and *Capsicum annum* (67%), *Lycopersicon esculentum* (64%), and *Solanum melongena* (60%), while least incidence was observed in *Musa paradisiaca, Daucus*

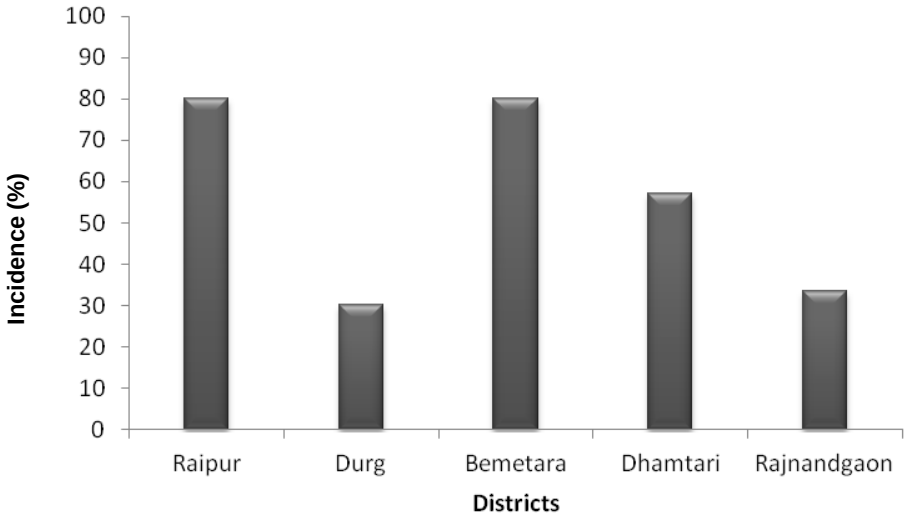

Figure 1. Percent incidence of root-knot nematode infection in some five districts of central plain region of Chhattisgarh, India.

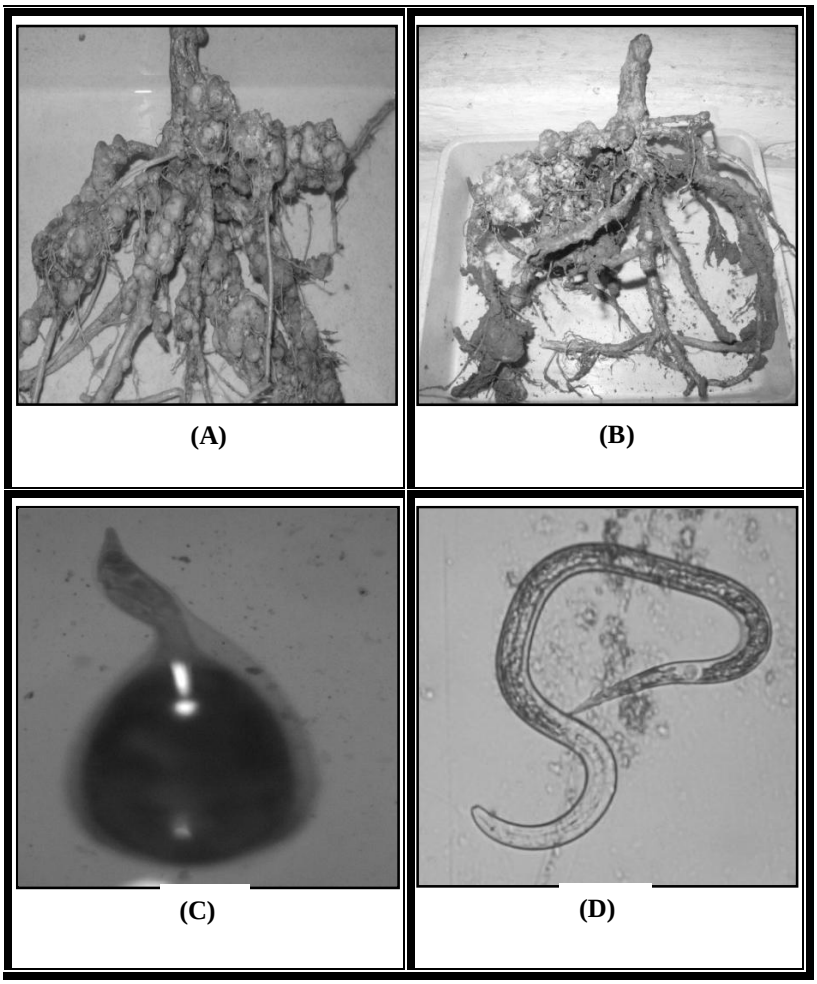

Figure 2. Infected root samples and root-knot nematodes collected from vegetable crop fields; (A) heavily infected bitter gourd roots, (B) heavily infected bottle gourd roots, (C) *Meloidogyne* adult female nematode (Magnification; 200X) and (D) *Meloidogyne* 2nd stage juvenile (100X).

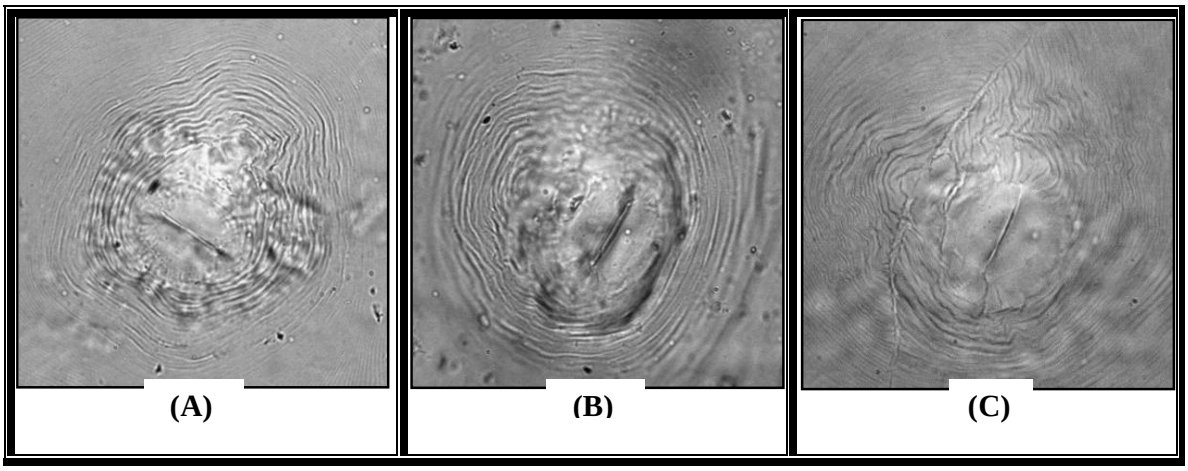

Figure 3. Perineal patterns (400X) of three species of the genus *Meloidogyne* collected from root samples of vegetable crops: (A) *Meloidogyne incognita*, (B) *Meloidogyne javanica* and (C) *Meloidogyne areneria*.

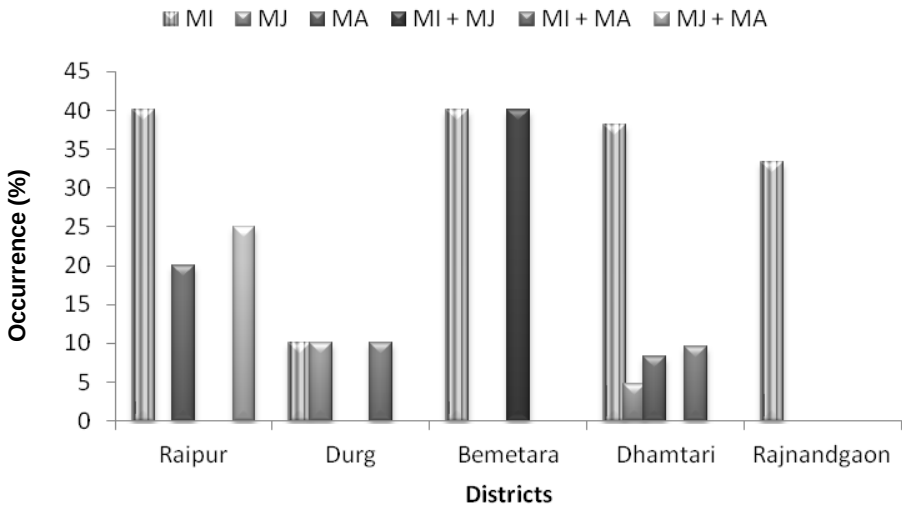

Figure 4. Percent occurrence of *Meloidogyne* species (district wise) in surveyed diseased vegetable crops of central plains region of Chhattisgarh. MI, *Meloidogyne incognita*; MJ, *Meloidogyne javanica*; MA, *Meloidogyne arenaria*.

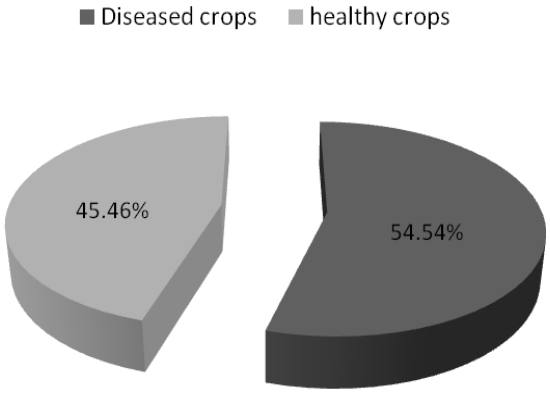

Figure 5. Overall crop wise incidence of root-knot nematode disease in central plains regions of Chhattisgarh.

carota and *Amaranthus tristis* (50%). The other crops such as *Vigna unguiculata*, *Phaseolus vulgaris, Glycine max* and *Zinziber officinale* were non-infected (Table 1).

Occurrence and frequency of root-knot nematode species

Occurrence and frequencies of root-knot nematodes (*Meloidogyne* spp.) associated with vegetable crops in the five districts of central plain of Chhattisgarh are presented on Tables 2 and 3. The most frequently occurring root-knot nematode in crop-roots of this area was *M. incognita* (63.33%), followed by *M. arenaria* (20%) and the least frequent was *M. javanica* (16.66%). Table 3 also showed the distribution and overall percent

Table 1. Cropwise percent incidence of root-knot nematode infection in vegetable crops of central plain region of Chhattisgarh (RKN- root-knot nematode, NI- non-infected).

Family	Crop	Common name	RKN incidence (%)
Cucurbitaceae	*Lageneria siceraria*	Bottle gourd	100
Cucurbitaceae	*Momordica charantia*	Bitter gourd	67
Cucurbitaceae	*Cucumis sativus*	Cucumber	67
Solanaceae	*Capsicum annuum*	Chili	67
Solanaceae	*Lycopersicon esculentum*	Tomato	64
Solanaceae	*Solanum melongena*	Brinjal	60
Apiaceae	*Daucus carota*	Carrot	50
Musaceae	*Musa paradisiaca*	Banana	50
Amaranthaceae	*Amaranthus tristis*	*Amaranthus*	50
Fabaceae	*Vigna unguiculata*	Cow Pea	NI
Fabaceae	*Phaseolus vulgaris*	Bean	NI
Fabaceae	Glycine max	Soybean	NI
Zingiberaceae	*Zingiber officinale*	Ginger	NI

Table 2. Absolute and relative frequency of *Meloidogyne* species associated with vegetable crops of central plain region of Chhattisgarh.

Root-knot nematodes (*Meloidogyne* spp.)	Absolute frequency	Relative frequency
Meloidogyne incognita	46.34	63.33
Meloidogyne javanica	12.19	16.66
Meloidogyne arenaria	14.63	20
Total	73.17	100

Table 3. Occurrence and distribution of *Meloidogyne* species in roots of vegetable crops belonging to five districts of the central plain region of Chhattisgarh (+ present, - absent, RKN- root-knot nematode).

District	Village	Crop	RKN disease	*M. incognita*	*M. javanica*	*M. arenaria*
1. Raipur	Gatapar	*L. esculentum*	+	+	-	-
	Mohandi	*S. melongena*	+	-	-	+
		D. carota	+	+	-	-
	Kopedih	*S. melongena*	-	-	-	-
		C. annum	+	-	+	+
2. Dhamtari	Khursenga	*L. esculentum*	+	+	-	-
		M. charantia	-	-	-	-
		C. sativus	-	-	-	-
		P. vulgaris	-	-	-	-
	Birejhar	*L. esculentum*	+	+	-	-
		S. melongena	+	+	-	+
		S. melongena	+	+	-	-
		C. sativus	+	+	-	-
		M. charantia	+	+	-	-
	Mura	*L. esculentum*	-	-	-	-
		S. melongena	+	+	-	-
		A. tristis	+	-	+	-
		P. vulgaris	-	-	-	-
	Chatoud	*L. esculentum*	+	+	-	-

Table 3. Contd.

		C. sativus	+	-	-	+
		S. melongena	-	-	-	-
	Ganeshpur	*S. melongena*	+	+	-	-
		L. esculentum	-	-	-	-
		C. annum	-	-	-	-
	Kotgaon	*S. melongena*	+	+	-	+
		P. vulgaris	-	-	-	-
3. Durg	Kotni	*M. charantia*	+	+	-	-
		L. siceraria	+	+	-	+
		S. melongena	-	-	-	-
	Kanharpuri	*L. esculentum*	-	-	-	-
	Patharia	*M. paradisiaca*	+	-	+	-
	Gomchi	*V. unguiculata*	-	-	-	-
		Z. officinale	-	-	-	-
	Kumhari	*M. paradisiaca*	-	-	-	-
		A. tristis	-	-	-	-
		D. carota	-	-	-	-
4. Rajnandgaon	Bijanapur	G. max	-	-	-	-
	Dhara	*L. esculentum*	+	+	-	-
		L. esculentum	-	-	-	-
5. Bemetara	Semaria	*L. esculentum*	+	+	-	-
		L. esculentum	+	+	+	-
	Pendritarai	*C. annum*	+	+	-	-
	Berla	*L. siceraria*	+	+	+	-
		S. melongeno	-	-	-	-
Overall percent occurrence			54.54%	43.18%	11.36%	13.63%

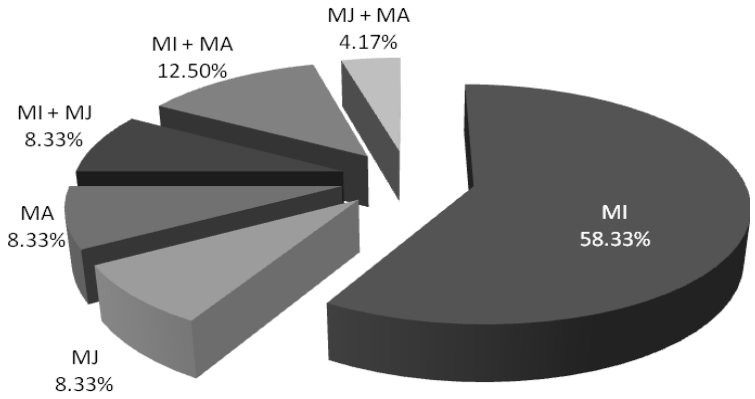

Figure 6. Percent occurrence of *Meloidogyne* species (single and concurrent) in vegetable crops of central plains region of Chhattisgarh. (MI- *Meloidogyne incognita*, MJ- *Meloidogyne javanica*, MA- *Meloidogyne arenaria*).

occurrence of *M. incognita* (43.18%), followed by *M. arenaria* (13.63%) and *M. javanica* (11.36%) in surveyed villages of study areas.

Of all associated *Meloidogyne* species, *M. incognita* constituted 58.33%, *M. javanica* 8.33% and *M. arenaria* 8.33% singly, while, concurrent infections were observed in some crops, such as; *M. incognita* + *M. javanica* (8.33%), *M. incognita* + *M. arenaria* (12.5%) and *M. javanica* + *M. arenaria* (4.17%) (Figure 6).

Out of 44 samples, 24 samples collected were diseased with the mean nematode population density (NPD) per 200 cc of soil and 10 g of roots was observed

Table 4. Nematode population density per 200 cc soil+10 g roots of diseased vegetable crops belonging to five districts of the central plain region of Chhattisgarh (±SE- standard error).

District	Village	Crop	Nematodes/200cc soil and 10 g roots ± SE
1. Raipur	Gatapar	*Lycopersicon esculentum*	1439±64.14
	Mohandi	*Solanum melongena*	4327±312.25
	Kopedih	*Daucus carota*	5715±541.12
		Capsicum annum	5536±464.91
2. Dhamtari	Khursenga	*Lycopersicon esculentum*	1859±164.20
	Birejhar	*Lycopersicon esculentum*	1447±192.42
		Solanum melongena	766±118.32
	Mura	*Solanum melongena*	2257±317.59
		Cucumis sativus	1857±201.21
		Momordica charantia	870±102.36
		Solanum melongena	1160±412.97
		Amaranthus tristis	1446±124.29
	Chatoud	*Lycopersicon esculentum*	1758±86.76
		Cucumis sativus	1669±97.34
	Ganeshpur	*Solanum melongena*	1462±264.75
	Kotgaon	*Solanum melongena*	5093±101.53
3. Durg	Kotni	*Momordica charantia*	9076±281.38
		Lageneria siceraria	7983±244.39
	Patharia	*Musa paradisiaca*	5306±203.75
4. Rajnandgaon	Dhara	*Lycopersicon esculentum*	2786±153.66
5. Bemetara	Semaria	*Lycopersicon esculentum*	5528±464.58
		Lycopersicon esculentum	6298±291.17
	Pendritarai	*Capsicum annum*	6428±377.68
	Berla	*Lageneria siceraria*	6582±296.78

to vary among the sampling sites (Table 4) ranging from 766 to 9076 which is significantly analyzed (ANOVA F<5 and P<0.001). In *M. charantia* it was highest between 870 and 9076 with average 4973. *L. esculentum* showed 1439 to 6298 NPD with average 3016. NPD of 766 to 5093 with average estimated 2511 was observed in *S. melongena*, 6582 to 7983 with average 7282 in *L. sicereria*, 6428 to 5536 with average 5982 in *C. annum*, 1669 to 1857 with average 1763 in *C. sativus*, 1446 in *Amaranthus*, 5306 in *M. paradisiaca* and 5715 in *D. carota*.

DISCUSSION

The results of the present study show the current status of the incidence and occurrence of root-knot nematodes (*Meloidogyne* spp.) in some districts of central plain regions of Chhattisgarh. During this survey, it was observed that the growths of vegetables plants were highly variable with presence of root galling and arrested root systems. This study provides the quantification of occurrence and density of root-knot nematodes associated with the vegetable crops of this area.

M. incognita, *M. javanica* and *M. areneria* are a closely related group causing widespread damage in developing countries (Sasser, 1979). Due to their wide host ranges and concurrent occurrences of virulent races and species, it is difficult to control these pests by management practices like rotation and resistant cultivars (Jepson, 1987; Fargette and Braaksma, 1990; Roberts, 1992). Ehwaeti et al. (1998) reported the multiplication rates root-knot nematode to be rapid on good hosts further increasing the difficulty of preventing crop damage during growing season. Among the *Meloidogyne* spp., *M. incognita* is the most common in vegetable crops world-wide where they parasitize root tissues and induce root galls causing severe damage (Barker and Olthof, 1976; Sasser, 1979; Abawi and Widmer, 2000; Davis et al., 2003; Anwar and Mckenry, 2010; Anwar et al., 2013).

The prevalence of root knot nematode infection in some districts of Chhattisgarh has been earlier reported by Sao et al. 2008, showing high average population density in tomato and bean. However, highest average population has been observed by us in bottle gourd followed by bitter gourd and cucumber. The remarkable occurrence of this nematode in bottle gourd, bitter gourd, cucumber, tomato, brinjal, carrot, amaranthus, chili and banana grown in central region clearly depicts severe damage to these crops by these pests. The reports by

Sahu et al. (2011) on the highest absolute and relative density of *M. incognita* and high prominence value of *M. javanica* among all plant nematodes of Durg district of Chhattisgarh corroborate our present work. Judging the incidence/distribution, mean population and level of damage assessed, the *Meloidogyne* spp. probably appears to be the most important species associated with damage to vegetable production in the state and may be considered to be the most important parasite of the crops under local conditions. In the present study, three species of *Meloidogyne* were recorded including- *M. incognita, M. javanica* and *M. arenaria,* all occurring both singly and concurrently in this area. Similar observations were reported by El-Sherbiny (2011) and Ibrahim and Al-yahya (2002) where *M. incognita* and *M. areneria* showed high percentage of concurrent occurrence. *M. incognita* is recorded as the most frequent and important species of root-knot nematode on locality basis and constitutes a large portion of root-knot population in central plains of Chhattisgarh. *M. areneria* ranks second in terms of frequency and is relatively less common in the region. Thus, *M. incognita* and *M. areneria* are also quite frequent and potentially damaging species of the area. Moreover, *M. javanica* is also present in the area with low frequency. *M. incognita* and *M. areneria* are the common species of this area and therefore need to be controlled efficiently. Severe infection in cucurbits and occurrence of concurrent species in some districts in our data is supported by Kayani et al. (2013) who reported the concurrent occurrence of *Meloidogyne* spp. in Pothowar region of Pakistan and indicated that cucumber was severely infested by root-knot nematodes.

Chhattisgarh is predominantly an agricultural state and central plains are one of its most fertile land areas. The species content of the region may possess an agricultural relationship history. The incidence of *Meloidogyne* spp. on vegetable crops has increased significantly as the acreage allotted to these crops has grown. Our results are in accordance with observations made by Sasser (1989) who conducted a survey of important genera of plant parasitic nematodes including *Meloidogyne* and observed its remarkable representation in most regions of the developed and developing countries all over the world. The findings of this study also clearly demonstrate that *M. incognita* is widely distributed across central plain region of the Chhattisgarh state and involves a wide host range.

Conflict of Interests

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

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Full Length Research Paper

Bioprocessing of sugarcane factory waste to production of Itaconic acid

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This paper aimed at advances in industrial biotechnology over potential opportunities for economic utilization of agro-industrial residues sugarcane bagasse. Sugarcane bagasse, which is a complex material, is the major by-product of the sugarcane industry. The present work has been taken up with a view of exploring the possibilities of using agriculture by products as a source for the production of itaconic acid from various fungi. *Aspergillus oryzae, Aspergillus niger, Aspergillus flavus and Penicillium* sp., were selected and optimized for itaconic acid production in solid state fermentation using cheaper raw material sugarcane baggasse powder. *A. niger* produced the highest itaconic acid level (8.241 ±1.5 mg/kg) in solid state fermentation at 35°C. Optimum fermentation medium and pH for itaconic acid production were found to be 3.5. This article shows the recent developments on processes and products developed for the value addition of sugarcane bagasse through the biotechnological means.

Key words: Sugarcane bagasse, itaconic acid, fermentation medium.

INTRODUCTION

In the developing and developed countries, year after year there has been an increasing use of organic acids particularly itaconic, gluconic, lactic, fumaric and kojic acids. Itaconic acid ($C_5H_6O_4$) is an unsaturated dicarboxylic acid, crystalline, relatively non-toxic with a melting point of 167 to 168 ºC and density of 1.632 (Milsom and Meers, 1985). The property that makes itaconic acid a uniquely valuable compound is the conjunction of the two carboxyl groups and the methylene group. The methylene group is able to take part in adding polymerization giving rise to polymers with many free carboxyl groups that confer advantageous properties (Mattey, 1992). Itaconic acid is known to be produced on a commercial scale using only *Aspergillus terreus* and *Aspergillus itaconicus* (Milsom and Meers, 1985). In the cane sugar industry, as in many other industries, control and disposal of wastes is of major concern. There are two important reasons for increased attention to these problems: First, the greatest possible recovery, use, and reduction of wastes is necessary for most economical production in small as well as in large plants. Second, protecting the Nation's limited water resources for maximum use is essential to our health and continued economic growth. Thus, wastes which cannot be

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Table 1. Itaconic acid production by solid state fermentation.

Substrate	Itaconic acid (mg/Kg) of fermented substrate
Aspergillus niger MTCC 281	8.241 ± 1.5
Aspergillus flavus MTCC 277,	5.102 ± 2.0
Aspergillu oryzae MTCC 634	2.307 ± 2.0
Penicillium Chrysogenum MTCC 6795	5.062 ± 2.0

eliminated must be disposed of in a manner which will not impair the usefulness of stream waters for other beneficial purposes. In the present study, attempts were made to develop a potent strain for improved production of itaconic acid. Alternatively, with a view to reducing the substrate costs, cheap and abundantly available substrates such as fruit wastes, which had not been reported earlier and corn starch were used.

MATERIALS AND METHODS

Microorganism and culture conditions

Aspergillus niger MTCC 281 strain *Aspergillus flavus* MTCC 277, *Aspergillu oryzae* MTCC 634 and *Penicillium chrysogenum* MTCC 6795 was used in this study (Yahiro et al., 1995; Dwiarti et al., 2002). The strain was maintained on potato dextrose agar (PDA) slants and sub cultured. The spore suspension was prepared by adding sterile distilled water containing 0.1% Tween 80 to a fully sporulated culture. The spores were dislodged using a sterile inoculation loop.

Production medium

Fifty gram (50 g) of sugarcane bagasse powder was mixed in 100 ml of distill water (Bressler and Braun, 2000), sodium nitrate 3.0 g, dipotassium hydrogen potassium phosphate 1 g, magnesium sulfate 0.8 g, ferrous sulfate 0.01 g, potassium chloride 0.5 g. The pH of the solution was adjusted to desired value using nitric acid at room temperature. This medium was sterilized in an autoclave at 121°C at 15 psi for 20 min (Xu et al., 1989; Nubel and Rakajak, 1964). For shake flask fermentation, a different microbial culture was inoculated to the production medium in a 500 ml shaking flask and cultured on a rotary shaker. The sample was collected at an interval of 24 h (Walinky, 1984). The collected sample was used for the determination of itaconic acid and glucose consumed was estimated. The qualitative analysis of itaconic acid was measured by ultraviolet-visible (UV) spectrophotometer at 210 nm (Kautola et al., 1985; Welter, 2000).

Effect of pH

The pH of the solution was adjusted to desired value by the addition of nitric acid and sodium hydroxide to pH values 2.0, 2.5, 3.0, 3.5 and 4.0.

Effect of temperature

The production media for itaconic acid was optimized at four different temperatures The temperatures are 25, 30, 35, 40 and 45ºC. Best yield of itaconic acid was studied in these five temperatures.

High-performance liquid chromatography (HPLC) analysis

Itaconic acid was analysed using a HPLC, Shimadzu Corp., Kyoto, Japan (Md. Rafi et al., 2012) consisting of a LC-10ATVp pump, SCL 10A system controller and a variable Shimadzu SPD-10 A Vp UV VIS detector and a loop injector with a loop size of 20 µl. The peak area was calculated with a CLASSVP software. Reverse-phase chromatographic analysis was carried out in isocratic conditions using a C-18 reverse phase column (250 × 4.6 mm i.d., particle size 5 µm, Luna 5µ C-18; phenomenex, Torrance, CA, USA) at 25°C. The mobile solvent 60% Acetonitrile had a significant effect on the resolution of compounds. Detection wavelength was 280 nm. Itaconic acid was used as internal and external standards. Itaconic acid present in sample were identified by comparing chromatographic peaks with the retention time (Rt) of individual standards and further confirmed by co-injection with isolated standards. The amount of each phenolic acid is expressed as milligram per Kilogram of fresh weight unless if not confirmed.

RESULTS AND DISCUSSION

In this study sugarcane bagasse powder shows maximum yield 8.241 ±1.5 after 120 h. (Table 1.). It was concluded that the nature of the substrate, incubation time, temperature and agitation, affect the production of taconic acid on sugarcane bagasse powder. The itaconic acid fermentation process works optimally under phosphate-limited growth conditions (Nubel and Rakajak, 1964). Once the fungal biomass is established preferably after inoculation from spores, the phosphate level should be kept rather low to prevent growth. Although *A. niger* is now the mostly frequently used commercial producer of itaconic acid, other microorganisms that are not as sensitive to a particular conditions (example, substrate impurities) or which have a more favorable product composition have been found. Among the filamentous fungi, some ustilaginales species also produce itaconic acid.

Effect of pH on fermentation medium

pH is one of the most important parameters that affect the production of itaconic acid by fermentation. Fermentable medium with adjusted pH of 2.5, 3.0, 3.5, 4.0 and 4.5 were observed over a period of 15 days and maximum yield was obtained at all pH levels on Day 10 of fermentation with pH 4 yielding the highest itaconic acid concentration of 7.522±0.30 mg/kg of fermented substrate (Table 2). This is close to the findings of Rao et al. (2007) and Chandragiri and Sastry (2011) who reported the highest production of itaconic acid at pH 3.5 and 3, respectively.

Effect of Incubation temperature

Incubation temperature is one of the parameters that

Table 2. Effect of pH on fermentation medium.

pH	Itaconic acid (mg/kg) of fermented substrate
2.5	3.976 ± 0.50
3.0	6.782 ± 0.20
3.5	7.522 ± 0.30
4.0	5.023 ± 0.25
4.5	3.976 ± 0.50

Table 3. Effect of incubation temperature on fermentation medium.

Temperature (°C)	Itaconic acid (mg/kg) of fermented substrate
25	2.814 ± 0.40
30	6.291 ± 0.15
35	8.017 ± 0.50
40	5.138 ± 0.10
45	3.462 ± 0.50

Table 4. Validation data.

	SPD10Avp (280 nm)			
Name*	Retention time	Area	Height	Concentration (mg/kg)
Itaconic acid	5.000	7090	3910	1.19

affect the production of itaconic acid by solid state fermentation. Fermentable medium was incubated with 25, 30, 35, 40 and 45°C observed over a period of 15 days and maximum yield was obtained at all temperature levels on Day 10 of fermentation with temperature 35°C yielding the highest itaconic acid concentration of 8.017±0.50 mg/kg of fermented substrate (Table 3).

High-performance liquid chromatography (HPLC) determination of itaconic acid

The HPLC result showed based on the Retention time (Rt), Itaconic acid (Rt-5.000) content in sugarcane baggase fermented with *A. niger* was found to be 1.19 mg/kg given in Table 4 and Figure 1 the obtained value was compared with standard.

Conclusions

It can be concluded that bioconversion of bagasse could be economically advantageous in some cases, for example, for the production of enzymes, amino acids and drugs. Such processes require only small quantities of bagasse, which would not be difficult to obtain from the sugar factories. Sugarcane baggase powder has been shown to be suitable for the fermentative production of itaconic acid. Hence, bioprocesses which need large quantities of bagasse could eventually be considered only if surplus bagasse availability were ensured to meet such demands.

Conflict of Interests

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

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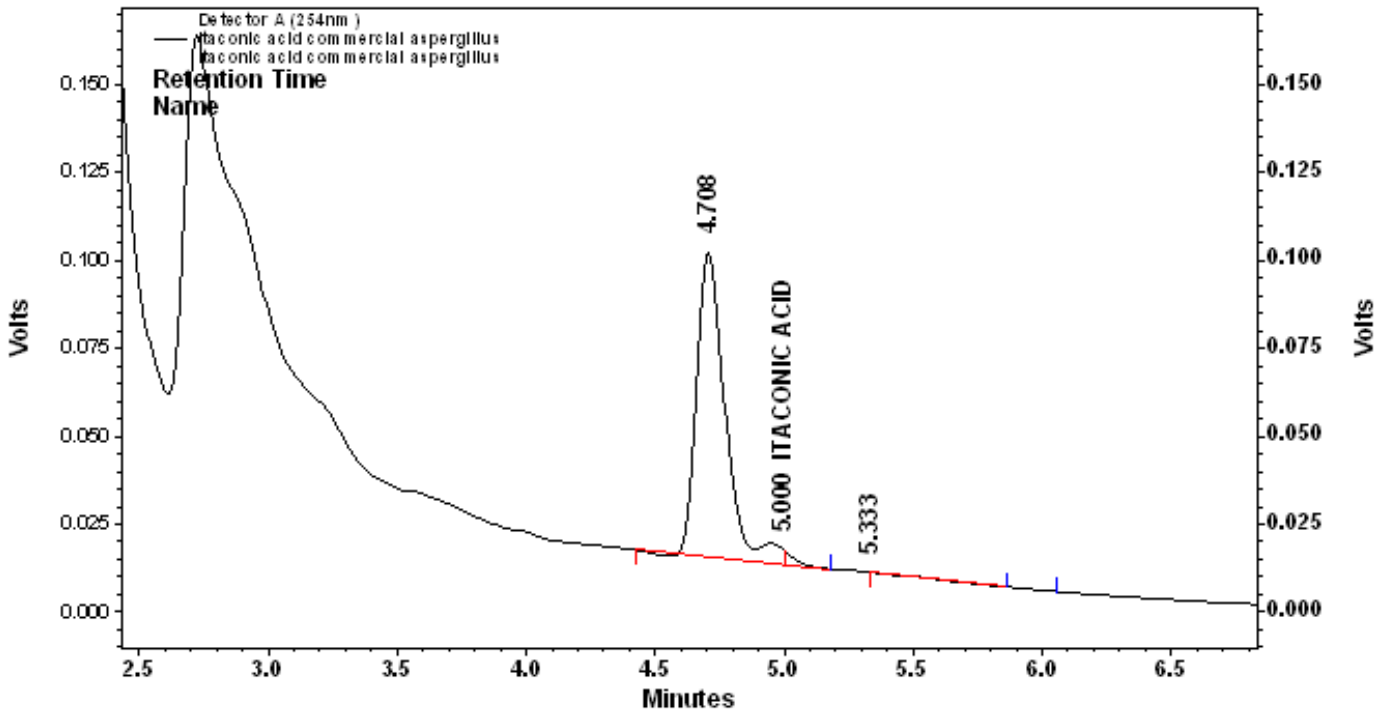

Figure 1. HPLC Chromatogram of itaconic acid in *Aspergillus niger* fermented sugarcane baggase.

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Full Length Research Paper

Antifungal resistance and herbal sensitivity of oral *Candida* isolates from HIV-infected patients in a rural community in Western Uttar Pradesh, India

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Drug-resistant *Candida* species in HIV-infected patients are the result of the selective pressure of currently used azoles. In the course of screening for active plant products against drug-resistant *Candida* species, we paid special attention on the effects of commonly available herbal plants. A total of 123 oral *Candida* isolates which were previously isolated from 172 HIV-infected patients were included in this study. *In vitro* antifungal susceptibility to fluconazole (FCZ), itraconazole (ITZ) and amphotericin B (AMB) was evaluated using Clinical and Laboratory Standard Institute (CLSI) guidelines. Antifungal potency of garlic, neem, *Aloe*, *Calendula*, *Citrus*, mint, tea and ginger extracts were tested against drug-resistant isolates. Out of 123 isolates, 26.8% were resistant to FCZ, 21.9% to ITZ and 8.1% to AMB. All drug-resistant isolates tested, were completely inhibited by garlic and neem leaves extracts with minimum fungicidal concentration (MFC) of 0.781 and 1.562 mg/ml, respectively. *Aloe* and *Calendula* extracts were also found to be effective with MFC of 3.125 mg/ml each. The observed growth inhibition zones and minimum inhibitory concentrations (MICs) showed that the isolates exhibited susceptibility. Our results provided scientific justification for the use of garlic, neem, *Aloe* and *Calendula* extracts in health products and herbal remedies against multidrug-resistant candidiasis.

Key words: Oral lesions, antifungal susceptibility, drug resistant, herbal, *Candida albicans*.

INTRODUCTION

Oral candidiasis (OC) is the most frequent human immunodeficiency virus (HIV) infection-associated oral disease and can also act as a marker for immuno-suppression (Moura et al., 2006; Chunchanur et al., 2009; Merçon et al., 2009; Moura et al., 2010; Thompson et al., 2010). Fluconazole (FCZ) is frequently used for the treatment of mucocutaneous candidiasis in patients with AIDS (Vazquez et al., 2001). In the 1990s, there was a significant increase in the prevalence of drug-resistant fungal infections due to *Candida* species in patients hospitalized for mucosal or systemic diseases. The widespread application of FCZ or related azole antifungal is postulated to promote selection of resistant subpo-pulations by shifting colonization to more naturally resistant species, such as *Candida krusei* or *Candida glabrata.* Alternatively, azole-resistant subspecies have arisen *in vivo* and *in vitro* that shows changes in the target enzyme lanosterol 14-α-demethylase, in expression

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of multidrug efflux pumps, or in both (Mathema et al., 2001). In India, despite the numerous reports of isolation of azole resistant strains of *Candida* species from patients with refractory mucosal candidiasis, only a few longitudinal prospective studies have evaluated the antifungal susceptibility of *Candida* isolates recovered from HIV-infected patients receiving long-term therapy with azole (Gautam and Garg, 2013a). Drug-resistant *C. albicans* and non-*albicans Candida* species (NACs) in immunocompromised patients are the result of the selective pressure of currently used azoles and have been well recognized as a global problem in recent years (Gautam and Garg, 2013a). As plants can produce antimicrobial compounds to protect themselves from biotic attack, the search for antimicrobial agents with different chemical structure and biological mode of action from plant sources is an alternative option in the fight against these microbes (Lai and Roy, 2004). In the course of screening for active plant products against drug-resistant *Candida* species, we paid special attention on the effects of commonly available herbal medicinal plants. Here, we reported the drug-resistant oral *Candida* isolates from HIV-infected patients in a rural community in western Uttar Pradesh, India together with antifungal activity of eight herbal plants extracts against these isolates.

MATERIALS AND METHODS

Isolates

A total of 123 *Candida* isolates, which were previously isolated and identified from HIV-infected patients (Gautam and Garg, 2013b) in the Department of Microbiology, Chaudhary Charan Singh University, Meerut, India were included in this study. Out of 123 isolates, 91 were recovered from patients with oral lesions (Group 1) and 32 were from patients without oral lesions (Group 2). Informed consent obtained from participants and procedures were performed according to institutional board of ethical committee.

Antifungal susceptibility test

The three antifungal agents used for susceptibility testing of *Candida* species isolates were fluconazole (FCZ), itraconazole (ITZ), and amphotericin B (AMB) (Sigma-Aldric, USA). Fluconazole was dissolved in sterile distilled water while ITZ and AMB were dissolved in dimethyl sulfoxide (DMSO) (Hi-Media, India) to make stock solutions. Antifungal susceptibility test using broth microdilution (BMD) method was performed as per Clinical and Laboratory Standard Institute (CLSI) (CLSI M27-A3, 2008). A completely synthetic medium, RPMI 1640 (Hi-Media, Mumbai) supplemented with glutamine but without bicarbonate, was used. Medium was supplemented to final concentration of 20 g/L (2.0%) glucose for better growth of yeast isolates. The range of concentrations tested were 0.125 to 64.0 µg/ml for FCZ and 0.0313 to 16.0 µg/ml for ITZ and AMB. The BMD test was performed using sterile, disposable multiwell microdilution plates (96 U-shaped wells) (Tarson, Mumbai). Aliquots of 100 µl of each antifungal agent at a concentration two times the targeted final concentration were dispensed in the wells of the plates. The suspension of yeasts after 48 h of incubation onto Sabouraud's dextrose agar was prepared in sterile saline, adjusted spectrophotometrically at 530 nm to match the turbidity of a 0.5 McFarland standard and was diluted in RPMI 1640 in order to obtain a final concentration of 0.5×10^3 to 2.5×10^3 CFU/ml (CLSI M27-A3, 2008). A constant volume (100 µl) of the inoculum was aseptically added to each microdilution well containing 100 µl of serial dilution of antifungal agents so as to make final desired concentration. For the members of the azole drug the MIC was defined as the lowest drug concentration that resulted in 80% growth inhibition. For AMB, this value was defined as the value in which 100% growth inhibition was observed (Brito et al., 2010). The isolates were tested in duplicate. The breakpoints used as interpretive guidelines for *in vitro* susceptibility testing of *Candida* species were based on the M 27-A3 Document of the CLSI (CLSI M27-A3, 2008). Because of the lack of consensus about the definition of MIC breakpoints for AMB, arbitrary values suggested by a previous study were used (da Matta et al., 2007). Isolates with MICs ≤ 8 µg/ml for FCZ, ≤ 0.125 µg/ml for ITZ and ≤ 1 µg/ml for AMB were considered susceptible. Isolates with MICs from 16 - 32 µg/ml for FCZ and 0.25 - 0.5 µg/ml for ITZ were considered as susceptible in a dose-dependent manner (SDD). Isolates with MICs ≥ 64 µg/ml for FCZ, ≥ 1 µg/ml for ITZ and ≥ 2 µg/ml for AMB were considered resistant. Quality control strains including *C. albicans* (ATCC 90028) and *C. parapsilosis* (ATCC 22019) were also used. These strains were included with each batch of MICs testing and results were within acceptable ranges.

Collection and processing of herbals

In vitro antifungal activities of eight herbal plants extracts were evaluated. These were garlic (*Allium sativum*), neem (*Azadirachta indica*), aloe (*Aloe vera*), *Calendula* (*Calendula arvensis*), *Citrus* (*Citrus sinensis*), mint (*Mentha spicata*), tea (*Camellia sinensis*) and ginger (*Zingiber officinale*). All were collected from crop fields and local market of Western Uttar Pradesh, India. The plant material was dried under shade and reduced to small pieces; plant material was grounded in electronic grinder to obtain fine powder. Dried herbal powder (100 g) was macerated with 1000 ml ethanol (95%) in 2000 ml Erlenmeyer flask. Flask was closed with aluminium foil and placed on orbital incubator shaker at 130 rpm for 5 days with intermittent agitation and soaking. After 5 days the mixture was filtered using Whatman's filter paper No. 1. Herbal extract was evaporated to semi solid state under vacuum at 40°C using a Buchi Rotavapour. Semi solid extract was dried in the crucible under a controlled temperature of 40°C to obtain solid extract. Prepared extract was stored in dark-coloured container at 4°C until used (Gautam and Garg, 2013a). All herbals were processed in same manner.

Antifungal activity test of herbal extracts

Agar well diffusion method

All extracts were first subjected to an antifungal susceptibility test for their growth inhibition zone diameter (in mm) following the previous agar well diffusion (AWD) method with a slight modification (Gautam and Garg, 2013a). In this method, Mueller-Hinton Agar (MHA) plates were prepared. Each plate was inoculated with test organism of 2.5×10^3 CFU/ml inoculum density using spread plate technique. Three wells at equidistance were cut aseptically with the help of sterile cork borer. Stock solution having 1250 mg/100 ml air dried powder of herbal extracts dissolved in DMSO was prepared. 100 µl of each herbal extract was dispensed into 6 mm precut wells of MHA plates. These plates were incubated at 37°C for 48 h and inhibition zone diameter was measured using Hi Antibiotic Zone Scale-C PW297 (Hi-Media, Mumbai). Data are presented in the form of inhibition zone diameter (mm) which is an average of two

independent replicates.

Minimum inhibitory concentration assay

To measure MICs of active herbal extracts, macro-broth dilution (MBD) method was used with some modifications (Gautam and Garg, 2013a). In this method, 12 sterilized glass tubes with caps were taken. Tube 1 was filled with 2 ml of double-strength culture medium (Sabouraud's Dextrose broth) containing twice the final active herbal extract concentration (6.25 mg/ml). Tubes 2 - 11 were filled with 1 ml of double strength Sabouraud's Dextrose broth. Tube 12 was considered as blank filled with distilled water. 1 ml amount was taken from tube 1 and diluted two-fold by transferring it to tube 2 with a micropipette. 1 ml sample was then removed from tube 2 and transferred to tube 3 and so on to tube 10. The last 1 ml of diluted herbal extract was then discarded. Thus, each tube 1-10 contained 1 ml of double-strength Sabouraud's Dextrose broth containing twice the final herbal extract concentrations. Each tube was inoculated with 1 ml of 5×10^3 CFU/ml yeast suspension which will give the required herbal extract concentration and inoculums density of 2.5×10^3 CFU/ml. The growth control tube (tube 11), which contained 1 ml of sterile drug-free medium, was also inoculated with 1 ml of the same inoculum suspension. Tube 12 was filled with 2 ml of sterile distilled water, from the lot used to prepare the inoculum as a sterility control for media. Final volume of each tube was 2 ml. All tubes were incubated for 24 to 48 h at 37°C after which the turbidity was observed and measured with the spectrophotometer. On the basis of optical density, the tube showing critical inhibitory points were selected and 100 µl samples from each MIC assay tube with growth inhibition was spread onto Sabouraud's Dextrose Agar (SDA) plate. Plates were then incubated at 37°C for 48 h to count number of colony forming units (CFU). CFU/ml was determined for each selected well along with control well. Data are presented in the form of MIC_{50}, MIC_{80} and minimum fungicidal concentration (MFC). The MIC_{50} was considered the lowest tested concentration with a 50% reduction in growth as compared to growth of positive control. The MIC_{80} was considered the lowest tested concentration with 80% reduction in growth as compared to growth of positive control. The highest dilution of the plant extract that retained its inhibitory effect resulting in no growth (absence of turbidity) of a microorganism is recorded as the MFC value of the extract.

Phytochemical constituents of plant extracts

Herbal extracts were subjected to qualitative chemical tests for identification of various categories of organic chemical constituents such as alkaloids, tannins, steroids, flavonoids, saponins, glycosides, terpenoids, carbohydrates, antiquinones and phenols. The phytochemical analysis was done according to standard methods (Roopashree et al., 2008; Obasi et al., 2010; Raphael, 2012). Briefly the methods are given below.

Test for alkaloids

Extracts were dissolved individually in dilute hydrochloric acid and filtered.

a) Mayer's test: Filtrates were treated with Mayer's reagent (potassium mercuric iodide). Formation of a yellow coloured precipitate indicates the presence of alkaloids.
b) Wagner's test: Filtrates were treated with Wagner's reagent (iodine in potassium iodide). Formation of brown/reddish precipitate indicates the presence of alkaloids.

Test for carbohydrates

Extracts were dissolved individually in 5 ml distilled water and filtered. The filtrates were used to test for the presence of carbohydrates.

a) Molisch's test: Filtrates were treated with 2 drops of alcoholic α-naphthol solution in a test tube. Formation of the violet ring at the junction indicates the presence of carbohydrates.
b) Benedict's test: Filtrates were treated with Benedict's reagent and heated gently. Orange red precipitate indicates the presence of reducing sugars.
c) Fehling's test: Filtrates were hydrolysed with diluted HCl, neutralized with alkali and heated with Fehling's A & B solutions. Formation of red precipitate indicates the presence of reducing sugars.

Test for glycosides

Extracts were hydrolysed with dilute HCl and then subjected to test for glycosides.

a) Modified Borntrager's test: Extracts were treated with ferric chloride solution and immersed in boiling water for about 5 min. The mixture was cooled and extracted with equal volumes of benzene. The benzene layer was separated and treated with ammonia solution. Formation of rose-pink colour in the ammonical layer indicates the presence of anthranol glycosides.
b) Legal's test: Extracts were treated with sodium nitropruside in pyridine and sodium hydroxide. Formation of pink to blood red colour indicates the presence of cardiac glycosides.

Test for saponins

a) Froth test: Extracts were diluted with distilled water to 20 ml and this was shaken in a graduated cylinder for 15 min. Formation of 1 cm layer of foam indicates the presence of saponins.
b) Foam Test: 0.5 g of extract was shaken with 2 ml of water. If foam produced persists for ten minutes it indicates the presence of saponins.

Test for phenols

Ferric chloride test: Extracts were treated with 3-4 drops of ferric chloride solution. Formation of bluish black colour indicates the presence of phenols.

Test for tannins

Gelatin test: To the extract, 1% gelatin solution containing sodium chloride was added. Formation of white precipitate indicates the presence of tannins.

Test for Steroids

2 ml of acetic anhydride was added to 0.5 g ethanolic extract of each sample with 2 ml H_2SO_4. The colour changed from violet to blue or green in some samples indicating the presence of steroids.

Test for flavonoids

a) Alkaline reagent test: Extracts were treated with few drops of sodium hydroxide solution. Formation of intense yellow colour, which

Table 1. Yeast distribution in different groups of the study population.

Species	Group 1* (n=91)	Group 2† (n=32)	Test group (n=123)
C. albicans	55 (60.4)	20 (62.5)	75 (60.9)
C. tropicalis	9 (9.9)	3 (9.4)	12 (9.8)
C. glabrata	6 (6.6)	2 (6.3)	8 (6.5)
C. parapsilosis	5 (5.5)	0	5 (4.1)
C. albicans + *C. tropicalis*	2 (2.2)	0	2 (1.6)
C. albicans +*C. krusei*	2 (2.2)	2 (6.3)	4 (3.3)
C. albicans + *C. dubliniensis*	3 (3.3)	0	3 (2.4)
C. famata	1 (1.1)	1 (3.1)	2 (1.6)
C. guilliermondii	1 (1.1)	2 (6.3)	3 (2.4)
Non *albicans Candida*	29 (31.9)	10 (31.3)	39 (31.7)

Data are shown in no. (%) of *Candida* spp. isolates; *, HIV-infected patients with oral lesions; †, HIV-infected patients without clinical signals of oral lesions.

becomes colorless on addition of dilute acid, indicates the presence of flavonoids.

b) Lead acetate test: Extracts were treated with few drops of lead acetate solution. Formation of yellow colour precipitate indicates the presence of flavonoids.

Test for terpenoids

a) Copper acetate test: Extracts were dissolved in water and treated with 3-4 drops of copper acetate solution. Formation of emerald green colour indicates the presence of diterpenes.

a) Salkowski's test: Extracts were treated with chloroform and filtered. The filtrates were treated with few drops of concentrated sulphuric acid, shaken and allowed to stand. Appearance of golden yellow colour indicates the presence of triterpenes.

Statistical analysis

All the data were analysed in the worksheet of Statistical Package for Social Science (SPSS) software (Version 16.0) and analyzed accordingly.

RESULTS

A total of 123 *Candida* isolates, which were previously isolated and identified from HIV-infected patients (Gautam and Garg, 2013b) were included in this study. Out of 123 isolates, 91 were recovered from patients with oral lesions (Group 1) and 32 were from patients without oral lesions (Group 2). *C. albicans* was the most common isolate from both groups. The frequency of isolation of *Candida* isolates in Group 1 was: *Candida albicans* 68.1%. *Candida tropicalis* 12.1%, *Candida glabrata* 6.6%, *Candida parapsilosis* 5.5%, *Candida krusei* 2.2%, *Candida dubliniensis* 3.3%, *Candida famata* 1.1% and *Candida guilliermondii* 1.1%, while in Group 2 was: *C. albicans* 68.8%, *C. tropicalis* 9.4%, *C. glabrata* 6.3%, *C. krusei* 6.3%, *C. famata* 3.1% and *C. guilliermondii* 6.3% (Table 1).

Antifungal susceptibility testing

In vitro antifungal susceptibility results of 123 isolates are summarized in Table 2. The determined MIC ranges for Group 1 isolates were 0.125 - 128, 0.031 - 4.0 and 0.031 - 2.0 µg/ml for FCZ, ITZ and AMB, respectively. The determined MIC ranges for Group 2 isolates were 0.125-64, 0.031-1.0 and 0.031-1.0 µg/ml for FCZ, ITZ and AMB, respectively. Group 1 isolates demonstrated very high FCZ MIC_{50} in which two isolates of *C. glabrata* presented values of 128 µg/ml followed by ITZ MIC_{50} in which same isolates presented values of 4 µg/ml. Out of 123 isolates, 77 (62.6%) were susceptible to FCZ, 80 (65.1%) to ITZ and 113 (91.9%) to AMB. Only 10.6 and 17.3% of all isolates were dose dependent to FCZ and ITZ,, respectively. Among all isolates, 33 (26.8%) were resistant to FCZ, 27 (21.9%) to ITZ and 10 (8.1%) to AMB. Out of 84 *C. albicans* isolates, 25.0% were resistant to FCZ, 20.2% to ITZ and 5.9% to AMB while among 39 NAC isolates, FCZ resistance accounted for 30.8%, ITZ for 25.6% and AMB for 12.8%.

Herbal activity against drug-resistant isolates

In vitro antifungal activity results of herbal plants extracts are summarized in Table 3. Using AWD method the highest growth inhibition zone diameter was recorded as 26±3.7 mm (mean) with garlic extract against all drug-resistant isolates, followed by neem extract which demonstrated 24.0±2.9 mm. *Aloe* and *Calendula* extracts demonstrated 18.2±3.5 and 17.5±3.2 mm, respectively. *Citrus* peel, Mint leaves and ginger extracts demonstrated inhibition zone diameters of 15.8±4.1, 13.3±2.9 and 13.6±1.6 mm, respectively, while tea leaves extract demonstrated only 11.1±2.2 mm.

Using MBD method, the lowest MIC_{50} value was recorded as 0.097 mg/ml with garlic and neem extracts each against all isolates tested while *Aloe* and *Calendula* extracts demonstrated MIC_{50} value of 0.390 mg/ml each. The isolates demonstrated low garlic extract, MIC_{50} in which 66.7% (22) presented values of 0.048 mg/ml. *Citrus* peel extract demonstrated MIC_{50} value of 0.390 mg/ml against all drug-resistant isolates while mint leaves and ginger extracts were comparatively less effective with MIC_{50} value of 3.125 mg/ml each. Tea leaves extract was effective only to inhibit 37.23% growth of drug-resistant isolates. Garlic and neem leaves extracts completely inhibit the growth of all drug-resistant isolates with MFC value of 0.390 and 0.781 mg/ml respectively, while 72.7%(24) isolates demonstrated low neem extract's MFC value of 0.390 mg/ml. *Aloe* leaves and *Calendula* extracts demonstrated MFC value of 1.562 mg/ml each. *Citrus* peel, mint leaves and ginger extracts were unable to inhibit complete growth of any of the drug-resistant

Table 2. Antifungal susceptibility of *Candida* spp. isolates using CLSI testing.

Test isolate	FCZ			ITZ			AMB*	
	S	SDD	R	S	SDD	R	S	R
Group 1 isolates								
C. albicans (62)	36 (58.1)	9 (14.5)	17 (27.4)	37 (59.7)	11 (17.7)	14 (22.6)	57 (91.9)	5 (8.1)
C. tropicalis (11)	7 (63.6)	1 (9.1)	3 (27.3)	8 (72.7)	1 (9.1)	2 (18.2)	11 (100)	0
C. glabrata (6)	2 (33.3)	0	4 (67.7)	2 (33.3)	0	4 (67.7)	3 (50.0)	3 (50.0)
C. parapsilosis (5)	5 (100)	0	0	5 (100)	0	0	5 (100)	0
C. krusei (2)	0	0	2 (100)	0	0	2 (100)	1 (50.0)	1(50.0)
C. dubliniensis (3)	3 (100)	0	0	3 (100)	0	0	3 (100)	0
C. famata (1)	1 (100)	0	0	1 (100)	0	0	1 (100)	0
C. guilliermondii (1)	1 (100)	0	0	1 (100)	0	0	1 (100)	0
Group 2 isolates								
C. albicans (22)	15 (68.2)	3 (13.6)	4 (18.2)	15 (68.2)	4 (18.2)	3 (13.6)	22 (90.9)	0
C. tropicalis (3)	3 (100)	0	0	3 (100)	0	0	3 (100)	0
C. glabrata (2)	1 (50.0)	0	1 (50.0)	1 (50.0)	0	1 (50.0)	2 (100)	0
C. krusei (2)	0	0	2 (100)	1(50.0)	0	1(50.0)	1(50.0)	1(50.0)
C. famata (1)	1 (100)	0	0	1 (100)	0	0	1 (100)	0
C. guilliermondii (2)	2 (100)	0	0	2 (100)	0	0	2 (100)	0
All isolates (123)	77 (62.6)	13 (10.6)	33 (26.8)	80 (65.1)	16 (17.3)	27 (21.9)	113 (91.9)	10 (8.1)

Data are shown in number (%) of *Candida* isolates; FCZ, fluconazole; ITZ, itraconazole; AMB, amphotericin B; S, susceptible; SDD, susceptible to dose dependent; R, resistant;*, no endpoint defined by CLSI.

Table 3. Growth inhibition zone diameters and MICs of the herbal extracts against 33 drug-resistant oral *Candida* isolates.

Herbal	Family	Part	GIZD* (mm)	MIC_{50} (mg/ml)	MIC_{80} (mg/ml)	MFC (mg/ml)
Garlic (*Allium sativum*)	Amaryllidaceae	Bulb	28.1 ± 3.7	0.097	0.195	0.390
Neem (*Azadirachta indica*)	Meliaceae	Leaves	24.0 ± 2.9	0.097	0.195	0.781
Aloe (*Aloe vera*)	Xanthorrhoeaceae	Leaves	18.2 ± 3.5	0.390	0.781	1.562
Calendula (*Calendula arvensis*)	Asteraceae	Petals	17.5 ± 3.2	0.390	0.781	1.562
Citrus (*Citrus sinensis*)	Rutaceae	Peel	15.8 ± 4.1	0.390	0.781	Nd
Mint (*Mentha spicata*)	Lamiaceae	Leaves	13.3 ± 2.9	3.125	Nd	Nd
Tea (*Camellia sinensis*)	Theaceae	Leaves	11.1 ± 2.2	Nd	Nd	Nd
Ginger (*Zingiber officinale*)	Zingiberaceae	Rhizome	13.6 ± 1.6	3.125	Nd	Nd

*Growth inhibition zone diameter; MIC_{50}, minimum inhibitory concentration inhibiting 50% growth; MIC_{80}, minimum inhibitory concentration inhibiting 80% growth; MFC, minimum fungicidal concentration; Nd, not detected.

isolates resulting to, no MFC value. Figure 1 summarizes the comparative antifungal activity analysis of all herbal extracts tested against 33 drug-resistant oral *Candida* isolates.

Qualitative assay of herbal extracts for phytochemical constituents

Phytochemical assay results indicated the presence of carbohydrates (reducing sugars, hexose sugars, non-reducing polysaccharides gums and mucilages), alkaloids and flavonoids as the main constituents in herbals tested (Table 4).

DISCUSSION

A variety of antifungal agents are now available for the treatment of *Candida* infections. However, worldwide reports indicated that pathogenic isolates of *C. albicans* have relatively high potentials for developing resistance

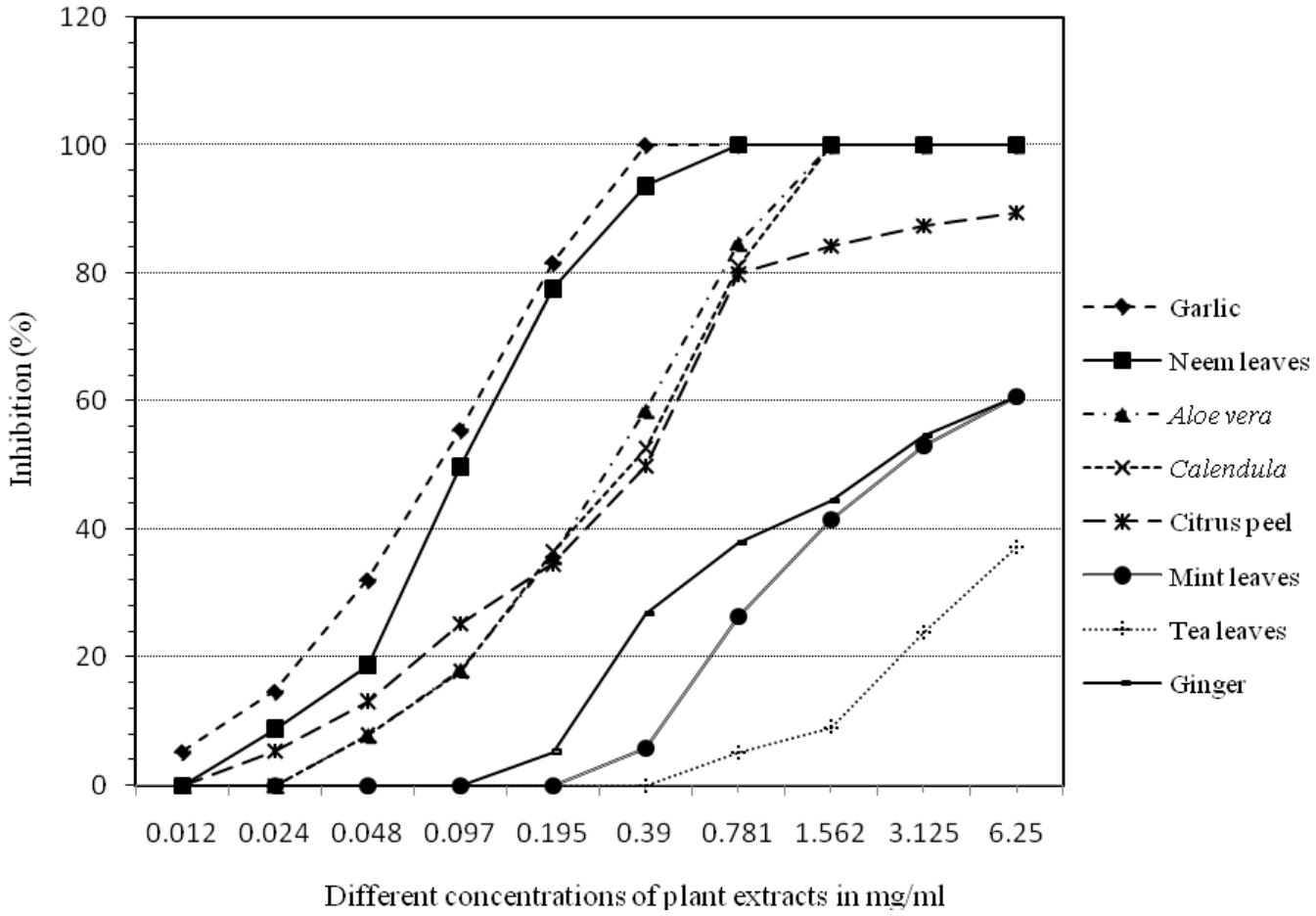

Figure 1. Comparative analysis of herbal extracts activity against drug-resistant oral *Candida* isolates.

Table 4. Phytochemical constituents of plant extracts.

Variable tested	Garlic	Neem leaves	Aloe leaves	Calendula flower	Citrus peel	Mint leaves	Tea leaves	Ginger
Alkaloids	+	+	+	+	-	+	-	-
Tannins	-	-	+	-	+	+	+	-
Steroids	+	+	-	-	+	+	-	-
Flavonoids	+	+	+	+	+	+	+	+
Saponins	-	-	-	+	+	-	-	-
Glycosides	-	+	-	-	+	-	-	-
Terpenoids	+	+	+	-		+	-	+
Carbohydrates	+	+	+	-	+	-	-	-
Phenols	-	-	-	-	+	-	-	+

(+) Indicates present; (-) indicates absent.

(Espinel-Ingroff et al., 1998). Relatively, high resistance of *C. albicans* and non-*albicans Candida* to common antifungal agents tested was observed in the present study. Although triazole agents appear to be highly effective initially, the increase of resistance to them has been reported (Ellepola and Samaranayake, 2000; Pfaller and Diekema, 2007).

Antifungal resistance

Two different populations of individuals were studied, HIV-infected patients with oral lesions (symptomatic) and without oral lesions (asymptomatic). Isolates from symptomatic patients showed higher MIC range in comparison with isolates from asymptomatic patients. All

these isolates were recovered from patients who had previously received same antifungal agent. This feature could be due to a secondary resistance produced by exposure to the drug. In past few decades, there have been numerous reports of *Candida* infections in India (Basu et al., 2003; Gugnani et al., 2003; Bharathi and Rani, 2011; Jain et al., 2011; Gautam and Garg, 2013b, c). Several studies have also reported FCZ resistance in *C. albicans* strains isolated from HIV-infected patients with oral candidiasis (Mane et al., 2010).

In the present study, 21 of 33 FCZ resistant, 17 of 27 ITZ resistant and 5 of 10 AMB resistant isolates were *C. albicans* while remainders were NACs isolates. NACs isolates showed higher resistance as compared to *C. albicans*.

Among 33 drug-resistant isolates, 80.8% (27) isolates (seventeen *C. albicans*, two *C. tropicalis,* five *C. glabrata* and three *C. krusei*) presented resistance to both azole tested. These findings suggest the possibility of a cross-resistance phenomenon between these antifungal agents. Active flux and alteration in drug target are considered to be the most important mechanisms of azole resistance.

Although the breakpoint concentrations for AMB are not clearly defined, a strain is considered to be resistant when it represents an MIC ≥ 2 µg/ml (Luque et al., 2008). According to this criterion, only 10 isolates were found resistant to AMB and this feature is difficult to analyse from a clinical point of view. These isolates were recovered from patient, who had been previously treated with FCZ. These results agree with previous reports on the existence of strains resistant to AMB which had previously been in contact with azoles. The azoles affect the cellular membrane and this could be a factor in a selection of mutants (Carrillo-Muñoz et al., 1997). Most of the resistant isolates to AMB were recovered from immunocompromised individuals and this could be related to effects on cellular membrane of yeast, in relation to the multiple treatments received for these patients (Luque et al., 2008).

Herbal sensitivity

In this study, eight herbal extracts preparations were tested against drug-resistant oral *Candida* isolates. In the present study, garlic and neem leaves extracts exhibited highest anticandidal activity against all the drug-resistant isolates tested with MFC value of 0.390 and 0.781 mg/ml, respectively. Three *C. glabrata* isolates which were resistant to all the three drugs tested were completely inhibited with garlic and neem extracts with MFC value of 0.390 and 0.781 mg/ml, respectively. It has been proven over and over that garlic is an effective anti-fungal agent. Its compounds are very active against *C. albicans* and NACs isolates (Jafari et al., 2007; Bokaeian et al., 2010). In this study, *A. vera* and *Calendula* extracts were also found effective. In the present study, we confirm the inhibitory effect of Garlic, Neem, *Aloe* and *Calendula* extracts against drug-resistant *Candida* spp. isolateswhich has been described before by Agarry et al. (2005), George et al. (2009), Mahmoud et al. (2011), Doddanna et al. (2013) and Padhye et al. (2013). The observed growth inhibition zones and MICs showed that the isolates exhibited susceptibility. This indicates that garlic, neem, *Aloe* and *Calendula* extracts have broad spectrum of antifungal activity. The MFCs of garlic and neem extract reported in this study are in consonance with previous anticandidal studies on pathogenic and emerging drug-resistant *Candida* species such as *C. albicans*, *C. glabrata* and *C. krusei* (Gautam and Garg, 2013a). Although, *Citrus* peel extract was unable to inhibit the complete growth of any of the isolate tested but its MIC_{50} and MIC_{80} value of 0.390 and 3.125 mg/ml, respectively and intermediate growth inhibition zone diameter showed its anticandidal efficacy against drug-resistant isolates. Mint leaves extract were found comparatively less effective (Doddanna et al., 2013). Tea leaves extract was unable to demonstrate signification reduction in growth of *Candida* isolates as previously reported by Doddanna et al. (2013).

The herbal extracts offer several advantages such as unlimited availability and possibility of minimal problem of drug resistance. The present study showed the presence of tannins, alkaloids and flavanoids as main constituents of the ethanolic herbal extracts. A correlative relationship has been reported between the phytochemicals such as tannins and flavonoids and the free radical scavenging activity and antimicrobial activity (Kaur et al., 2010). Tannins and flavanoids have therapeutic uses due to their anti-inflammatory, anti-fungal, antioxidant and healing properties (Thiago et al., 2008). An added advantage of using herbals to treat yeast infections is that no clinical strains of *C. albicans* have been known to be resistant to herbal therapy.

Conclusion

The high percentage of antifungal resistant against *Candida* isolates in the present study could be due to the fact that many of these patients had received previous treatments with these drugs. Therefore, azole prophylaxis should be considered only in special cases. Our study, suggest to avoid indiscriminate use of antifungal agents, which may ultimately decrease the incidence of candidiasis caused by resistant and non-*albicans Candida* species. The results of this study provided scientific justification for the use of neem, garlic, *Aloe* and *Calendula* extracts in health products and herbal remedies against multidrug-resistant candidiasis. Therefore, complementary and alternative medicine practices with herbal extract including neem, garlic, *Aloe* and *Calendula* as a means of decreasing the burden of

drug resistance and reducing the cost of management of diseases would be of clinical and public health importance in any country.

Conflict of interests

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

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Full Length Research Paper

Parasites in synanthropic rodents in municipality of the Northwest region of the State of Paraná, Brazil

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The aim of this study was to identify the presence of different parasites in synanthropic rodents captured in the urban and peri-urban area in the city of Umuarama, Paraná (PR). The rodents were euthanized for measurement and collection of feces and ectoparasites. Specific identification keys were used for the ectoparasitological analysis and the technique by Hoffman et al. (1934) was used for the coproparasitological analysis. From the 162 rodents captured, 24.70% were found in the urban area and 75.30% in the periurban area in the city. The rodents belonged to the species *Rattus rattus* (96.91%) and *Rattus norvegicus* (3.09%), being 52.47% male and 47.53% female. 62.96% analyzed feces samples contained at least one parasite species. Identified parasites species were *Syphacia* sp., *Aspiculuris* sp., *Strongyloides* sp., *Hymenolepis diminuta* and *Entamoeba* sp. and protozoa eggs. Upon visual inspection, a total of three (1.85%) ectoparasites identified as *Xenopsylla cheopis* were collected. The importance of synanthropic rodents on the maintenance and possible transmission of different parasitic etiological agents with zoonotic potential was demonstrated, making both man and pets susceptible to possible parasitic infections.

Key words: Diagnosis, ectoparasites, enteroparasites, feces, rats.

INTRODUCTION

Rodents belong to the Rodentia order and there are approximately 2,000 rodent species in the world and they live in any terrestrial environment that can provide conditions for their survival (Brasil, 2002) and some species are considered synanthropic to man because they change their environments by their actions (Brasil, 2002; Guimarães et al., 2014).

Among the synanthropic species, the most important ones are the brown rat (*Rattus norvegicus*), the black rat (*Rattus rattus*) and the house mouse (*Mus musculus*)

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which can be found in the urban, peri-urban and rural areas of the cities. Factors such as urbanization and overcrowded cities and inadequate sanitation (water and sewer), have led to increasing numbers of these animals and consequently becoming increasingly common transmitted diseases (Brasil, 2002).

In addition to the diseases brought about by the transmission of different parasitosis, for which the rodents are reservoirs of different etiological agents (Jittapalapong et al., 2011; Ivanova et al., 2012; Sharma et al., 2013), these animals are also responsible for economic losses due to the treatment of different diseases (Guimarães et al., 2014).

Research on the presence of parasites in synanthropic rodents has been done in different countries like Korea, Iran, Spain, Switzerland, Mexico and Poland (Reperante et al., 2009; Yi et al., 2010; Kia et al., 2010; Foronda et al., 2011; Paziewska, 2011; Jiménez et al., 2012; Mowlavi et al., 2013). In Brazil, studies have been done in the states of Paraná (PR) and Sergipe (SE) (Araujo et al., 2010; Guimarães et al., 2014). These studies have shown the importance of synanthropic rodents in the transmission of different parasitic diseases of zoonotic potential chain. Considering the absence of regional data and the importance of rodents as transmitters of parasitic agents with zoonotic potential, the aim of this paper was to identify the presence of different parasite species in synanthropic rodents captured in the urban and peri-urban area in the city of Umuarama, Paraná, Brazil.

MATERIALS AND METHOD

Study setting

The locations chosen for capturing the rodents were selected based on the presence of residues or garbage associated with the proximity to men. The animals used in this work were captured in locations situated in urban and peri-urban areas in the city of Umuarama, located in the northwestern region of the Paraná (PR) State. The determination of the number of samples was based on the number of animals captured during the period of one year (October, 2012 to October, 2013).

Capture of rodents

The capture of rodents was performed using nine galvanized wire traps (Tomahawk), measuring 30 x 14 x 14 cm and 45 x 22 x 22 cm (previously cleaned before each capture), where the triggering mechanism was activated by the presence of the animal in its interior when seeking the bait. The used baits were: raw sweet corn, banana, sausage, cheese and feed developed by the members of the project, using sardines, peanut, corn flour and banana. The traps were assembled in the evening in places presenting traces of rodents (feces, trails and fat stains) and collected the following morning (Araujo et al., 2010). After the capture, the rodents were transported to the Laboratory of Preventive Veterinary Medicine and Public Health at UNIPAR for later conduction of parasitological tests and ectoparasitária identification.

In order to obtain the epidemiological data, a form was filled out for each rodent, containing data related to the species, gender, size, presence of ectoparasites, type of bait used and presence of communicating animals in the capture site. The age of the animals was not possible to identify.

The capture and handling of animals was done following the principles established by National Council for Control of Animal Experimentation (CONCEA) and approved by the Ethics Committee in Animal Experimentation (CEPEEA) in Universidade Paranaense (UNIPAR), under protocol 21822/2012.

Euthanasia, identification and collection of biological samples

The rodents captured were immediately placed in a saturated chamber with halothane vapor for euthanasia (Araújo et al., 2010). Right after they were identified (Brasil, 2002), the following procedures were followed: measurement using conventional measuring tape, visual inspection for the collection of ectoparasites and collection of feces.

After the euthanasia and necropsy of animals, the intestine was exposed for proper collection of feces, which were stored in individual plastic pots containing formalin at 10%, stored at room temperature until the coproparasitological examination.

Laboratory techniques used

In order to identify the ectoparasites, the specimens captured were stored in plastic pots kept in 70% alcohol until they were processed. For identification purposes, they were assembled between blade and coverglass and after, diaphanized with 10% KOH, dehydrated in alcoholic series (100, 90, 85, 80 and 70 for 10 min each stage), and later clarified with xylol. The specific identification was performed with the aid of identification keys proposed by Linardi and Guimarães (2000) using Nikon optical microscope (Eclipse E-200) with a 10x increase.

For the enteroparasitic identification (visualization of eggs, protozoa cysts and helminth larvae), the sedimentation technique described by Hoffman et al. (1934) was performed. The blades were analyzed using a Nikon optical microscope (Eclipse E-200), with an increase of 40x. The microscopic blades were assessed in duplicates for all examinations performed.

Statistical analysis

After the tabulation of epizootiological data, the program Prisma 5.0 for Windows (GraphPad Software, San Diego, CA, USA) was used. In order to verify the association between the variables (gender and animal species) with the presence of enteroparasites, Fisher's Exact Test was used, adopting a 5% significance level and Confidence Interval at 95% (CI).

RESULTS

From the 162 feces samples analyzed, 62.96% (102/162) presented at least one type of parasite. Monoparasitism was detected in 71.57% (73/102) of positive samples.

Different gastrointestinal parasites and parasitic structures were detected: *Syphacia* sp. (54.32%), *Aspiculuris* sp. (0.62%), *Strongyloides* sp. (14.20%), *Hymenolepis diminuta* (1.23%) and *Entamoeba* sp. (10.50%) and protozoa eggs (2.47%) (Table 1).

Table 1. Parasites and parasitic structures detected by the sedimentation method described by Hoffman et al. (1934) in 102 feces of synanthropic rodents captured in the urban and peri-urban area in the city of Umuarama, Paraná, Brazil, 2012-2013.

Phylum	Species	Total	%
Nematode	*Aspiculuris* sp.	01	0.62
	Strongyloide sp.	23	14.20
	Syphacia sp.	88	54.32
Platyhelminthes	*Hymenolepis diminuta*	02	1.23
Rhizopoda	*Entamoeba* sp.	17	10.50
Protozoa	Protozoa eggs	04	2.47

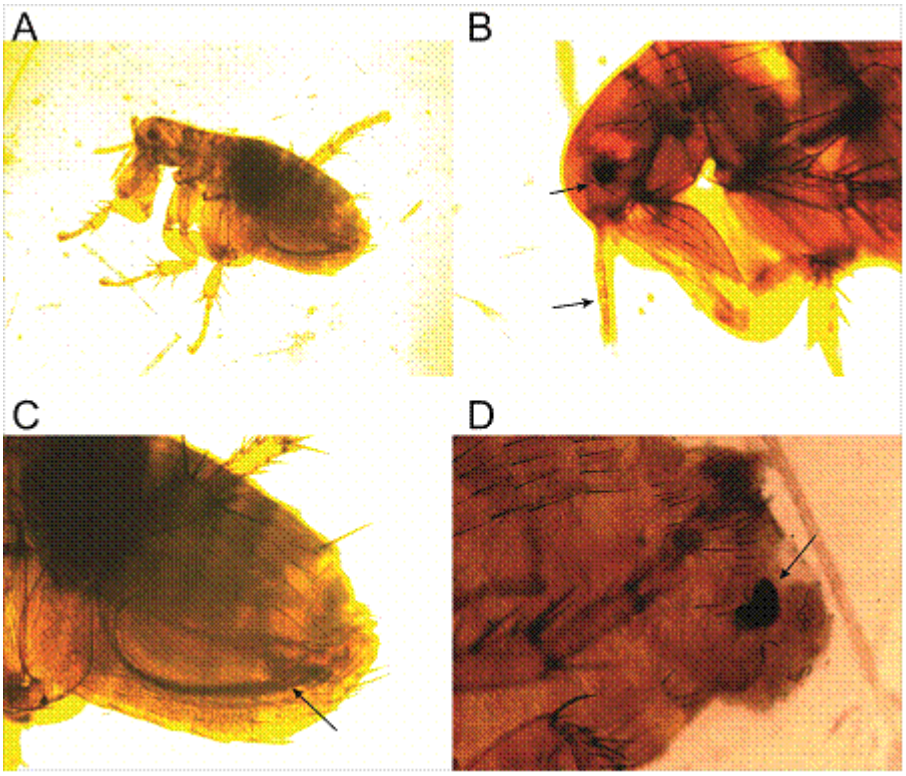

Figure 1. *Xenopsylla cheopis,* collected from *Rattus rattus* located in the peri-urban area in the city of Umuarama, Paraná, Brazil, 2012-2013. A) *Xenopsylla cheopis* - 10x increase; B) Presence of occiput with bristles forming a V shape and a pre-ocular bristle - 100x increase; C) Presence of genal bristle - 100x increase; D) Presence of spermatheca - 100x increase.

Upon visual inspection, we were able to collect only three (1.85% - 03/162) ectoparasites (two males, one female) that were identified as *Xenopsylla cheopis* species (Rothschild, 1903) (Figure 1).

Regarding the two variables analyzed, gender and animal species, there were no statistical difference when associated with the presence of enteroparasites (Table 2).

Regarding the presence of communicating animals in the place of capture of the rodents, in 9.26% there was the presence of birds *(Gallus gallus* and *Coturnix coturnix)* (15/162) and in 1.85% there was the presence of dogs (03/162).

The traps were set 89 times and there was a total of 32.58% (29/89) captures, with more than one animal captured per cage. The best bait used was the feed, with

Table 2. Variables associated with the presence of enteroparasites in 162 synanthropic rodents captured in the urban and peri-urban areas in the city of Umuarama, Paraná, Brazil, 2012-2013.

Variable	Positive sample (%)	p*	CI (95%)
Gender			
Male	59/162 (36.41)	0.2490	0.8-2.9
Female	46/162 (28.39)		
Species			
Rattus rattus	101/162 (62.34)	0.6575	0.04916-4.135
Rattus norvegicus	04/162 (2.46)		

p = Probability; *= Fisher's Exact Test; CI= confidence interval.

efficacy of 75.30% (122/162), followed by raw sweet corn, with 11.11% (18/162), cheese 7.41% (12/162), sausage 3.70% (09/162) and banana 0.62% (01/162).

Regarding the success of capture, traps were set 89 times and there was a total of 32.58% (29/89) captures, with more than one animal captured per cage.

DISCUSSION

The capture of 162 synanthropic rodents was performed throughout a year with possible catches in all months, which indicates that the locations where the traps were assembled provided favorable conditions (shelter, water and food) for the survival of this animal species.

Success in the capture of rodents shows an elevated infestation in the urban and peri-urban area in the city of Umuarama (PR), which can become an important public health problem, with possible transmission of different parasitic and infectious diseases with zoonotic potential (Jittapalapong et al., 2011; Ivanova et al., 2012; Sharma et al., 2013; Guimarães et al., 2014).

The most prevalent rodent species in the city of Umuarama (PR) is *Rattus rattus* and according to Brasil (2002), its action radius tends to be greater than the one for *Rattus norvegicus* (brown rat) due to its ability of scale vertical surfaces and the ease of walking on wires, cables and tree branches, which makes it a species that is more prone to capture. Also relating to public health, this action is of concern, since both direct exposure to the animal or indirect exposure to its waste (urine and feces) makes man and other animal species susceptible to different infectious-parasitic infections (Jittapalapong et al., 2011; Ivanova et al., 2012; Rohela et al., 2012; Sharma et al., 2013; Guimarães et al., 2014).

In the present paper, we observed polyparasitism, which was also documented by other studies (Seong et el., 1995; Franjola et el., 1995; Shintoku et al., 2005; Waugh et al., 2006; Sharma et al., 2013; Guimarães et al., 2014). This phenomenon is explained by the elevated physiological resistance of the animal in surviving a diverse parasitic load, which reflects on the capacity of these hosts in enduring the adversities of the invasion and developing a harmonious parasite-host relationship (Ribeiro et al., 2003; Claveria et al., 2005).

In this study, the conventional technique described by Hoffamm et al. (23) was used. This technique allows for the detection of eggs, protozoa cysts and helminth larvae; however, it is known that in rodents, the intestinal scraping technique (Kamiya, 2008) allows for the detection of other parasites such as protozoa, eggs, cysts, larva and adult helminths, with a better sensitivity (Hofer et al., 2000), which could possibly have influence on the results of this paper in the detection of other parasites.

Rodents present a growing importance in the transmission of different diseases to man (Guimarães et al., 2014), and can transmit parasitic diseases which have zoonotic potential, such as toxoplasmosis, hymenolepiasis, amoebiasis, strongyloidiasis, among others (Araujo, 2009; Araujo et al., 2010; Becker et al., 2011; Guimarães et al., 2014; Zanet et al., 2014); and in this paper, the presence of *Aspiculuris* sp., *Hymenolepis diminuta*, *Entamoeba* sp., *Strongyloide* sp., *Syphacia* sp. and protozoa eggs were detected in the feces samples analyzed. The different prevalences related to each parasite species detected in this work as compared to different research can reflect the environmental conditions, study location, time of the year, presence of hosts and animal reservoirs, and mainly the individual aspects of each parasite in each study region, enabling greater or smaller probabilities of a parasitic infection happening. Regarding the ectoparasites identified as *Xenopsylla cheopis* (Rothschild, 1903), it is possible to state that this is the first report of this Siphonaptera in rodents in the northwestern region in the state of Paraná (PR). This ectoparasite, common in rodents, has also been reported by Linardi et al. (1985) in Belo Horizonte (MG), where the authors identified 1,274 specimens collected from *Rattus norvegicus,* and in a more recent study by Ribeiro et al. (2003) in the city of Capão do Leão (RS), which identified 24 specimens, all collected from

Rattus rattus; results are similar to the one found in the city of Umuarama (PR). The importance of identifying this ectoparasite species is due to the possibility for transmitting *Yersinia pestis, Rickettsia typhi and Bartonella* spp. which are respectively the etiological agents for Bubonic Plague, Murine Typhus and Bartonellosis, which are zoonotic diseases of rodents (Ribeiro et al., 2003; Billeter et al., 2011). It must also be considered that *Xenopsylla cheopis,* which is adapted to the urban environment and to men, can parasite not only rodents but also other animal species, with its parasitism already described in foxes (*Cerdocyon thous)* (LINNAEUS, 1789) (Cerqueira et al., 2000; Ribeiro et al., 2003).

Considering further works with rodents, two epizootiological factors in this paper are worthy of emphasis. The first one is the type of bait used for the captures, since the feed developed by the members of this project was proven more efficient as compared to other foods (raw sweet corn, cheese, sausage and banana) used during the capture period. This shows how selective the rodents are, and in areas with plenty of food, the bait has to be very attractive in order to be successful. The second one is the presence of communicating animals, since both birds and dogs are considered important reservoirs for parasitic diseases when exposed to rodent waste and thus become trans-mitters of different parasitic diseases to men and other animals (Araújo et al., 2010; Jittapalapong et al., 2011; Rohela et al., 2012; Costa et al., 2010; Ferreira et al., 2013).

The results of this paper show the importance of synanthropic rodents on the maintenance and possible transmission of different parasitic etiological agents with zoonotic potential, making both man and pets susceptible to possible parasitic infections. Moreover, further copro-parasitological studies should be done on dwellers and students from the same locations as the capture of rodents would be essential to establish the relation of public health with the parasitosis detected in these rodents. Molecular studies on the ectoparasites collected would be critical for detecting possible etiological agents and consequently establish a possible zoonotic infection.

Conflict of Interests

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

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Full Length Research Paper

Studies on Conidiomata developmental morphology of *Pestalotiopsis disseminata*

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This study shows the conidiomatal development of *Pestalotiopsis disseminata* (Thuen.) Stey., using light and transmission electron microscope. Light microscopic study showed the presence of non-ostiolate pycnidial conidiomata in culture, whereas it is known to produce only acervular conidiomata on leaves. Interestingly, the fungus showed the ultrastructure of the conidial wall in the coloured cells as well as the basal and apical hyaline cells with the appendages. *P. disseminata* was studied for the first time on the development of the conidiomata and conidia by light and transmission electron microscopes.

Key words: Coelomycetes, Conidiomata, conidium ontogeny, appendages.

INTRODUCTION

Traditional classification of coelomycetes was based on morphology and was thus subjective, often resulting in artificial generic and species boundaries (Wijayawardene et al., 2012). Coelomycetes have conidia formed within a cavity lined by fungal or fungal host tissue. The conidia-bearing structure (conidioma) is classified into five types according to exterior morphology: pycnidial, pycnothyrial, acervular, cuplate and eustromatic (Hawksworth et al., 1995). The genus *Pestalotiopsis* steyaert is a hetero-geneous group of coelomycetous consisting of 205 described species that are differentiated primarily on conidial characteristics such as size, septation, pigmen-tation and presence or absence of appendages (Sutton, 1980). These facts suggest that the morphogenesis of conidioma has taxonomic value. In view of the variability and diversity, it is not easy to be classified based on conidiomata, satisfactorily. Pycnidial-type conidiomatal development has been described (Maas et al., 1979; Punithalingam, 1966). Development is divided into three stages: primordia, cavity formation and conidiogenesis with each pycnidial fungus having a determinate mode in each stage.

In describing acervular development, Archer (1926) noted that the pseudo- acervulus in the genus *Pestalotia* (written as *Pestalozzia)* was formed by the breaking open of the pycnidial wall to form a structure similar in appearance to an acervulus. Also, another manner of acervulus development in which the upper cells of a cell aggregation proliferate and produce conidia was noted. Generally, acervuli are formed by the breaking open

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of the wall of a pycnidium-like structure after it has developed as a pycnidium.

Cryptosporiopsis radicicola produces only excipular covering conidioma-like tissue with adhesive amorphous material and setae. Synnematous conidiomata with abundant macroconidia dominate the colony of *Cryptosporiopsis ericae*. *Cryptosporiopsis rhizophila* is different in its globose to subglobose conidiomata, consisting of loosely aggregated vegetative hyphae developing macroconidial conidiophores. *Cryptosporiopsis grisea*, being the only teleomorph-connected species, differs from the others in its distinct columnar surface (Wang, 2011).

Therefore, as more and more data on this effect becomes available, the distinction between Hyphomycetes and Coelomycetes may be abandoned because of the presence of intermediary stages between hyphomycetes, acervular, stromatic and cupulate conidiomata. The present investigation revealed that *P. disseminata* produced pycnidial conidiomata in culture and acervular conidiomata on natural hosts.

The various stages of development of the conidiomata were investi-gated in culture and they also resembled that of a typical pycnidial conidioma which were already studied. The developmental morphology of the conidiomata in *P. disseminata* in culture was described in this study.

MATERIALS AND METHODS

Culture character and identification

The *Pestalotiopsis disseminata* (Thuen.) Stey. was isolated from leaf of *Syzygium amotica* collected in Kodaikanal, India. *P. disseminata* isolates derived from single spores were grown on PDA. Cultures were incubated at 24°C in continuous light, and cultural morphology was examined after seven days. Spore size was determined by measuring the length and width of 30 to 40 arbitrarily selected conidia from a conidial suspension. The isolates were identified initially by comparing morphological and cultural characteristics (size of conidia, color and length of median cells, thickness and length of apical appendages, and length of basal appendage).

Germination study

The initial stages of the development of conidiomatal primordia were studied by slide cultures (Riddell, 1950). For germination studies, conidia were collected aseptically from the teased out conidiomata in 1% glucose solution and allowed to germinate in cavity slides kept at room temperature (28°C) and were examined every 5 h for 36 h to study germination.

Light and transmission electron microscopy (TEM)

Selected conidiomata with agar were trimmed into 2 mm square blocks and fixed in 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 2 h at room temperature (27°C) and 1 h at 4°C and post fixed for 12 h in 1% osmium tetroxide. Specimen were dehydrated through an ascending series of acetone (30-100%) at room temperature, each changes at 30 min intervals, followed by 2-3 changes in fresh spurr in the ratio of 3:1 (acetone : spurr) for 6 h, followed by two changes with absolute spurr mixture for 24 h each lasting for 8 h and polymerised in fresh spurr at 70°C for 8 h. The samples infiltrated with the resin were transferred to a vacuum chamber for 1 h for complete removal of air bubbles. Thin sections (0.5 µm) were cut from these blocks and stained with 1% aqueous toluidine blue to study the development of conidiomata and conidiogenesis under the light microscope. The same specimens as LM were used for TEM. Ultrathin sections were collected on copper girds (400 mesh) and excess water in the grids was removed by filter paper. The sections were picked up on mesh sheets, and then post stained for 40 min on droplets of 0.5% uranyl acetate followed by lead acetate. Transmission elelctron micrographs were taken by using Philips CM 10 at 40 and 60 KV.

RESULTS

Description of the fungus in culture

Conidiomatal acervuli stomatic, is black, 75-250 µm in diameter, conidia 5-celled, elliptic to clavate, fusiform, tapering to the base to slightly curved, 23 x 8 µm, median coloured cells unequally coloured, sometimes two upper cells slightly darker. The basal cells hyaline have a single unbranched appendage, 5 µm. Apical cell hyaline has 3 apical appendages, 22 µm. The apical cell hyaline has 3 apical appendages, 22 µm. The apical and basal cells are conic to cylindric, 5 µm (Figure 1A).

Light microscopic study

Development of conidiomatal initial

In the germination study, conidia became swollen and resulted in the breakage of outer wall and the germ tube emerged. The germ tubes mainly arise from the lower most median cells. One to two germ tubes arises from each cell (Figure 1B). The conidiomatal initials were first evident as small knots of fungal hyphae. Some of the cells became swollen and thick-walled and multiply by repeated divisions to form the knot-like primordial. This type of primordium formation is referred to as "meristogenous type" (Figure 1C). The primordium is also initiated by "symphogenous type" where the cells of adjacent hyphae by continued cross and longitudinal divisions, form the primordium. Also, intertwining of several hyphae resulted in primordia formation. Initially, the cells constituting the primordium are spherical to subspherical and are hyaline which stain deeply when compared with the cells of the surrounding hyphae (Figure 1D).

During further development, the primordium continuously increased in size by continued transverse and transverse and longitudinal divisions of the cells. As the primordial initials increase in size, several layers of the promordium became differentiated into morphologically distinct layers. The outermost one or two layers were

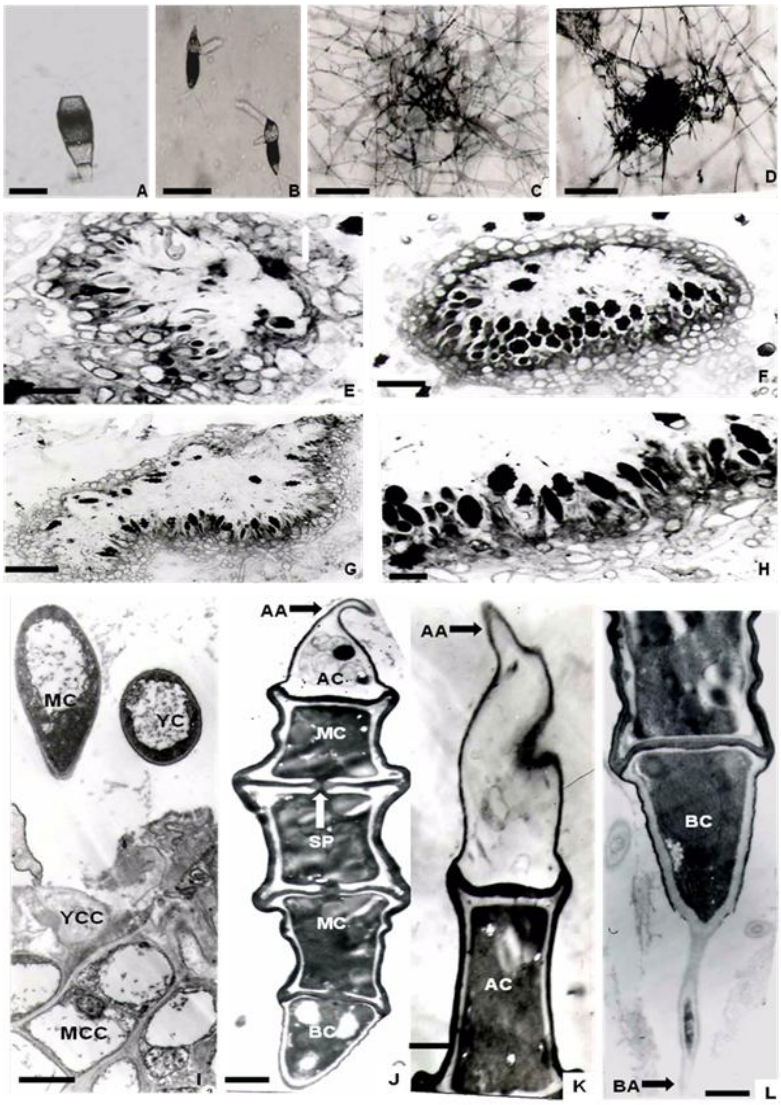

Figure 1. A. Mature conidia, bar = 12.5 µm; B. Germinating conidia producing germ tube from the lowermost cell and basal hyaline cells, bar = 100 µm; C. Early stages of simple symphogenous method of conidiomatal formation, bar = 25 µm; D. Aggregation of hyphal to form the conidiomatal primordium, bar = 50 µm; E. Section through a young primordium showing cavity formation, bar = 100 µm; F. Section of the conidioma showing temporary conidiogenous cells and the conidia, bar = 50 µm; G. Section of conidioma showing permanent conidiogenous cells and conidia, bar = 50 µm; H. Section of the pycnidia showing different stages of development of conidia, bar = 50 µm; I. TEM picture showing young conidiogenous cells with conidia, bar = 1 µm; J. Section through the apical part of the conidia showing the apical appendage. Note the continuation of outer electron dense layer of median cells to the end cells as well as appendage in the form of a thin layer, bar = 1 µm; K. Longitudinal section of a conidium showing the apical cell with the appendage, bar = 1 µm; L. Longitudinal section of a conidium showing the basal cell with the appendage and the upper most coloured cell, bar = 1 µm. CC- Conidiogenous cell, MCC- matured conidiogenous cell, YCC- young conidiogenous cell, YC- young conidium, MC- median cell, SP- septal pore, AC- apical cell, BC- basal cell, AA- apical appendage, BA- basal appendage.

pseudoparenchymatous with thick, lightly pigmented walls, which form the outer wall layer of the conidiomata (Figure 1E). Inside this outer wall, there are four compact layer of cells.

Formation of cavity and sporogenous tissue

The central cells in the primordium showed sign of schizogenous and lysigenous activity to form the central cavity. Further developmental stages showed the formation of the conidium simultaneously with the cavity formation. As the conidia nature, they were released from the conidiogenous cells to fill up the cavity (Figure 1F). The later formed conidiogenous cells were typically cylindrical in shape with one to three annellation (Figure 1G). The conidioma become flattened in shape during the later stages of development. The mature conidioma produces conidiogenous cells which line only the flattened basal region but not the sides and upper region of the condioma (Figure 1H). There was no regularly formed ostiole found in this species. After maturity the upper layers of the conidioma open quite irregularly to release the conidia.

Electron microscopic studies

Conidiogenous cell

The initial of the conidium arises as a small protrusion of the apex of the conidiogenous cell (Figure 1I) and develops holoblastically. Cell organelles migrate into the developing conidium until a delimiting septum was formed more or less near the base of the conidium initial. As the conidium enlarges, the conidium wall forms an electron-opaque outer layer, which starts from the base of the conidium.

The inner transparent layer of the conidium was continuous with the wall of the conidiogenous cell. The conidiogenous cell itself does not develop an electron-opaque outer wall layer. Successive conidia develop just at or below the level at which the preceding conidium was delimited. The conidia were produced from the annellides and more than 3 annellations were observed in some conidiogenous cells.

Mature conidia

As sections through young conidiomata showed, conidia arise from spherical to subspherical conidiogenous cells lining their cavity (Figure 1J). The conidia consist of three thick-walled median cells with thin apical and basal hyaline cells. The wall layers of the three median cells appear granular and pale brown. Prior to septation, the conidium initial has a thin electron transparent wall. During septation, the wall increases in thickness. The first septum was laid normally near the base of the conidium (Figure 1K).

Gradually, the peripheral region of the conidial wall becomes electron dense by the deposition probably of melanin in the wall matrix.

Median coloured cells

During the growth of a membrane across the conidium, wall material is continuously produced through the cell wall developed by each membrane. The septal pores are present between the cells of the conidium (Figure 1J and L). The septal pore is formed as a result of cessation of wall deposition at the junction where the plasma membranes from either side remain fused to form the trans-septal membrane. Simultaneous to the septal formation, wall deposition occurs over the entire inner surface of the conidium. Peripheral walls and the septa of the conidia becomes distinctly electron dense. The cell wall is thicker in the upper two median cells than in the lower median cell. The lower median cell is structurally different from the other two median cells of the conidium in that it showed pronounced wrinkling of the wall (Figure 1J). Probably because of the difference in the nature of the wall, it appears pale brown in colour under the light microscope.

Apical and basal cells with appendages

The apical and basal cells are morphologically indistinguishable from the median cells during the early stages of the development of conidium. At maturity, the end cells showed cytolysis and the cytoplasmic content completely disappears from these cells. The thickness of the electron-dense layer of the apical and basal cells gradually decreases (Figure 1J). The apical appendage originated from the apex of the conidium as a simple elongation of a small bud produced at an early stage of the development of the conidium (Figure 1K). Occasionally, the basal appendage of the developing conidium was observed within the annellation, which proves that the basal appendage is endogenously produced (Figure 1L).

DISCUSSION

The fungus is known to produce only acervular conidiomata on its host whereas in artificial culture media, pycnidial conidiomata were formed. The morphology of conidia, appressoria and cultural characters of the ex-neotype culture was provided (Liua, 2011). The development of the pycnidial primordium (the earliest stage of pycnidial development) was systematized as simple meristogenous, compound meristogenous, symphogenous (Kempton, 1919) and hyphal coiling (Maiello and Peterson, 1976). Primordia grow by aggregation of cells due to hyphae accumulating at the primordial surface. We were unable to observe pycnidial primordia. However, the aggregation of swollen cells in our specimens was observed under the leaf tissue or on the leaf surface, indicating that the cell aggregation

originated from hyphae accumulating in the same way as in pycnidial primordia. As the primordium increases in size, the central cavity was formed because the primordium compactly packed pseudoparenchymatous tissue. The central cells in the primordium showed signs of schizogenous and lysigenous activity to form the central cavity. Sutton (1961) also reported the developmental studies on *Pestalotiopsis* with various stages in spore development in some species.

The remaining portions of the cells often gelatinize and fill up the cavity with the mucilagenous matrix. Probably, the matrix provides nutrition to the developing conidiogenous cells and young conidia. In *Pestalotiopsis guepinii* and *Pestalotiopsis neglecta,* cavity formation was mainly lysigenous. The origin of the cavity was due to cytolysis of the central cells of the cell aggregation. Nag (1981) suggested that the slime which originates through lysis occurring during cavity formation play an important role in conidium dispersal. The slimy matrix also plays an indirect role in the dispersal of the conidia by insects. During the later stages of development of the conidiomata, secondary conidia are formed enteroblastically showing annellidic conidiogenesis. The conidiogenous cells or annellides became cylindrical with 4-5 annellations. This showed that the first formed conidia may be morphologically different from the secondary conidia formed successively from the conidiogenous cell. The present study showed that the disorganized central cells gelatinize to form the slimy matrix in *P. disseminate*. However, initially formed conidiogenous cells after releasing the conidia showed signs of degeneration inside the cavity. The present observation clearly revealed that *Pestalotiopsis* species have mucilagenous matrix arising either through lysis occurring during cavity formation or formed by the degenerating temporary conidiogenous cells which play an important role in the survival of the fungus. The presence of mucilagenous matrix during the formation of the pycnidial conidiomata was also reported in other pycnidial coelomycetes (Murugan and Muthumary, 2003).

The production of temporary and permanent conidiogenous cells were observed in species of *Pestalotiopsis.* The dual conidiation process was reported in various fungi, *Phyllosticta caryota* and *Ascochyta* species in grown culture (Punithalingam, 1979).

The fine structure of the conidiogenous cells and conidia were demonstrated in six coelomycetes species (Griffiths and Swart, 1974a, 1974b). Longitudinal sections through the conidia of *P. disseminata* showed massive and highly pigmented conidial walls. The transverse septa vary in the degree of pigmentation, perhaps, due to the varying sequences of development within the maturing conidium. The conidial wall was characterized by the deposition of electron dense material in the outer layers of the septa. The basal and apical cells have partly pigmented and partly unpigmented walls, which were clearly distinct from the central cells. Externally, the conidia are sheathed in an electron-dense outer wall and an electron-transparent inner wall. The conidial cells showed perforation between individual cells by a simple septal pore (Figure 1K). The conidium in *Pestalotiopsis* has an outer electron-dense layer and an inner electron transparent layer in the wall. The thicknesses of the two wall layers differ in that the median cells have a thick inner wall layer and a thin outer wall layer when compared with the end cells of the conidium. Therefore, the outer zone of the wall is melanized while the inner zone remains unpigmented. The transverse septa arise as outgrowths of the conidial wall and new wall material is deposited outside the invagination of the plasma membrane. In mature conidia, the transconidial septum consists of a thin, central, electron dense layer flanked on either side by a hyaline layer. A distinct pore formed centrally, following cessation of wall material deposition perforates the septum. When all the four septa are formed, the conidium has pigmented peripheral walls and the transconidial septa. The well-pronounced wrinkling observed in the outer-pigmented wall of the lowermost median cell implies that it is structurally different from the rest of the cells.

Conclusion

This study clearly shows the presence of non-ostiolate pycnidial conidiomata in culture whereas the fungus is known to produce only acervular conidiomata on leaves. Ultrastructure of the conidial wall in the coloured cells as well as the basal and apical hyaline cells with the appendages are shown.

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Full Length Research Paper

Determination of morphometric, biochemical and genetic variation in *Sclerotium delphinii* isolates

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Variability among four isolates of *Sclerotium delphinii* isolated from infected diseased Khirni (*Manilkara hexandra*); chafa (*Plumeria rubra acutifolia*) and Jackfruit (*Artocapus heterophyllus*) plants of Western Maharashtra region of India were studied. This is the first report on presence of *S. delphinii* species in India. These isolates varied in colony morphology, mycelial growth rate, sclerotia formation period, sclerotia size and color. On the basis of morphological characters, these isolates were identified as *S. delphinii* and were also confirmed in Indian type culture collection (ITCC), New Delhi. In mycelial compatibility study, isolates of *S. delphinii* did not intermingle with *Sclerotium rolfsii* isolates indicating genetic variation among the species. The isolates of *S. delphinii* also showed distinct differences in their oxalic acid content. The classification and variability was further confirmed using molecular studies. It was observed that only morphological features and MCGs differentiated the *Sclerotium* species rather than molecular characterization because they shared some common bands of DNA at genetic level, but showed distinctness in morphological and MCGs study.

Key words: Variation, cultural; biochemical, *Sclerotium delphinii*, RAPD-PCR.

INTRODUCTION

Sclerotium is a devastating soil borne plant pathogenic fungus which causes diseases in over 500 plant species (Punja, 1985; Harlton et al., 1995; Cilliers et al., 2000; Okabe and Matsumoto, 2000; Okabe et al., 1995) and is prevalent in warm, temperate and subtropical regions of the world. Symptoms of the fungus include crown and root rot, stem rot, stem canker and damping off (Aycok, 1966; Punja, 1995).

The Genus sclerotium includes three species *viz*., *Sclerotium rolfsii, Sclerotium delphinii and Sclerotium coffeicola* which are differentiated from each other on the basis of morphological features (Punja and Damiani,

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1996). *S. delphinii* produces larger and irregular shape sclerotia (Harlton et al., 1995; Okabe et al., 2000) lighter brown to orange in colour (Stevens 1931; Harlton et al., 1995; Punja and Damiani,1996). *S. delphinii* is reported to occur on ornamental and bulb plants. It is not clear whether these differences in host occurrence and morphology of sclerotia are sufficient to warrant a separate species of *S. delphinii* from *S. rolfsii* (Harlton et al., 1995; Punja and Damiani,1996)

Cultures of *S. rolfsii* originating from different geographical areas and host frequently displayed variation in morphological characteristics (Punja and Gregon, 1983). However, research work has not been done to study the variation among the *S. delphinii* isolates from different host in India. Therefore, detailed investigation on variation among the *S. delphinii* isolates from different host with regards to morphological, biochemical, genetical and molecular characters were carried out to differentiate them.

MATERIALS AND METHODS

Collection of different isolates of *S. delphinii*

Hard woody plants like khirni (*Manilkara hexandra*), jackfruit (*Artocapus heterophyllus*) and chafa (*Plumeria rubra acutifolia*) showing symptoms of collar rot/root rot in Western Maharashtra region of India were subjected to isolation of sclerotia on potato dextrose agar (PDA) medium.

Four cultures resembling *S. delphinii* were obtained from these isolations. Mycelial growth obtained was purified, sub cultured and maintained on potato dextrose agar in the form of mycelial and sclerotial bodies. These mycelia were used for morphological, pathological, biochemical and molecular studies (Punja and Sun, 2001).

Identification of *S. delphinii* isolates

The isolated fungal cultures were identified as *S. delphinii* on the basis of their morphological and sclerotia characters and were further confirmed from Indian Type Culture collection (ITCC), New Delhi, with allocated ITCC numbers 7168.68/6426 for khirni, 7167.08/6425 for chafa and 7166.08/6424 for jackfruit.

Morphological variation

Growth characteristics of *Sclerotium* isolates of khirni, jackfruit and chafa were studied on PDA medium. Circular discs (5 mm diameter) of an actively growing fungal colony (3-4 days old) of these isolates were taken out with the help of cork borer and was placed in the centre on PDA medium in the plate with the mycelial side facing downwards under aseptic conditions. The plates were incubated at 28°C for seven days.

Morphological characteristics of the fungus like growth rate, type of colony, growth pattern, radial colony diameter, development of sclerotia and its number, weight and diameter of sclerotial bodies, days required to form the sclerotia, color, type and location of sclerotial bodies for these isolates were recorded up to seven days at 24 h intervals.

Biochemical variation

Oxalic acid estimation

Richard's broth (KNO_3 10 g; KH_2PO_4 5 g; $MgSO_4.7H_2O$ 2.5 g; sucrose 35 g; distilled water 1000 ml) was used for estimation and quantification of oxalic acid. Fifty milliliter Richard's broth (in 250ml flasks) was inoculated with two 5 mm^2 mycelial disc of actively growing colony of *S. delphinii* isolates and was incubated for 14 and 21 days at 28 ± 1°C. All experiments were performed in triplicates. After desired incubation, cell free cultural filtrate was obtained through Whatman filter paper No.1 and subsequently through sortorious filter paper. The filtrate was centrifuged at 10000 rpm for 10 min. The supernatant of each cultural filtrate (10 ml) was used for the quantitative determination of oxalic acid as per the method suggested by Mahadevan and Sridhar (1986). Biomass of each isolate was also measured.

Mycelial compatibility/incompatibility among the isolates

Four isolates of *S. delphinii* and four isolates of *S. rolfsii* were paired against each other on PDA medium to determine mycelial compatibility among them. For this mycelial bits taken from the edge of an actively growing colony (three to four days old) of each isolate was placed approximately 2.5 to 3.5 cm apart on opposite sides of each other on PDA medium in 100×15 mm Petri plates and incubated at 28 ± 2°C.

Three isolates were usually paired on one dish and pairing test was repeated thrice. The pairing was examined under stereo microscope after seven days and up to 15 days for the presence of an antagonistic (barrage or aversion) zone in the region of mycelial contact (Powell, 1995).

Molecular characterization

Procedure for extraction and purification of DNA of fungus S. rolfsii

Genetic similarities and difference among isolates of *S. delphinii* were assessed through randomly amplified polymorphic DNA (RAPD) analysis as described by Punja and Sun (2001) and was also compared with S. *rolfsii* isolates. DNA from each isolate under observation was extracted from the mycelial mat of each isolate grown on PDA plate for seven days at 28°C.

For this, aerial mycelia were collected by scraping it from the agar surface with sterile scalpel blade and the mycelial mat was suspended in sterile distilled water for washing. These mats were further taken out and placed in aluminum foil for drying at 60°C in hot air oven for half an hour.

After drying, the mycelial mat were collected in another aluminum foil and kept in freeze at 4°C. Dried fungal mat (20 mg) grounded to fine powder under liquid nitrogen was used for isolation of DNA with DNeasy mini kit (Qiagene).

Primer selection and DNA amplification

The decamer primers (OPERON) Set B, D and E were initially screened to detect polymorphism among seventeen isolates of *Sclerotium* spp. The primers which indicated the higher degree of polymorphism were selected for the diversity analysis studies. DNA sequences (5"-3") of all primer used for DNA amplification are mentioned in Table 1.

Table 1. RAPD primers and their sequences.

Primer name	Primer sequences (5'- 3')	No. of polymorphic bands	No. of monomorphic bands	Polymorphism (%)
OPB - 11	GTAGACCCGT	6	01	85.71
OPB - 12	CCTTGACGCA	10	-	100
OPB - 16	TTTGCCCGGA	17	-	100
OPB - 20	ACTTCGCCAC	13	-	100
OPD - 7	TTGGCACGGG	09	-	81.82
OPD - 13	AAGCCTCGTC	11	-	100
OPD - 16	AGGGCGTAAG	14	-	100
OPD - 20	ACCCGGTCAC	14	01	93.33
OPE - 12	TTATGGCCCC	04	01	80.00
OPE - 16	GGTGACTGTG	16	-	100
OPE – 20	AACGGTGACC	09	01	90.00

DNA amplification

The RAPD-PCR reaction was carried out in 20 µL volume. The PCR reaction mixture contained 10X Taq DNA polymerase Buffer (GeNei, Banglore), 2 µL dNTP mix (10mM dNTP GeNei, Banglore), 1 µL of respective primers, Taq DNA Polymerase 0.5 µL (5 units μL^{-1} GeNei, Banglore) and 1 µL template DNA (100 ng μL^{-1}). 13.5 µL deionized water was used to make the total reaction volume up to 20 µL. PCR tubes were placed in Thermocycler (Eppendrorp, Germany) for DNA amplification using the following PCR programme: (Cycle, 1 Initial denaturation at 94°C for 5 min, Cycle-2, 45 denaturation at 94°C for 1 min; Primer annealing at 35°C for 1 min, Primer extension at 72°C for 2 min and final extension at 72°C for 10 min).

On completion of PCR amplification, the tubes were removed from the Thermocycler. 1 µg of 100 bp gene ruler DNA (Fermentas) ladder was used as a marker. After adding 4 µL of loading buffer dye (0.1% bromophenol blue, 0.5% xylene cyanol FF 30% Glycerol), the 20 µL of RAPD Product was loaded on 1.2% agarrose gel using Tris acetate EDTA (TAE) buffer (pH -8). Electrophoresis was performed for 3 h at 80 V in an submarine electrophoresis apparatus (Bio Rad No.96). The gel was stained with ethidium bromide (0.1%) and photographed under UV illumination. The fungal isolates were compared with each other by using their RAPD-PCR profiles and bands of DNA fragments were scored as (1) for present and (0) for absent for each of the primer used. The binary data were analyzed under the SIMQUAL module using DICE coefficient (Nei and Lei, 1979). A dendrogram based on the UPGMA clustering method (SAHN) (Sneith and Sokal, 1973) was constructed using NT SYS- PC software (Rohlf, 1998). Principal coordinate analysis (PCO) was performed to estimate the genetic distance between each group of the isolates using NT-SYS software. It involved first transforming similarity value to a scaler product by DCENTER, then analyzing the product matrix by EIGEN to get Eigenvector (which is PCO) and Eigen values and finally getting its 2D/3D scatter plot graph.

RESULTS

Four isolates of *S. delphinii viz*., Jackfruit, chafa, khirni-1 and khirni-2 varied greatly in their morphological characters (Table 2). The isolate of chafa and jackfruit produced filamentous mycelial growth whereas khirni-1 and khirni-2 isolate produced fluffy mycelial growth. Growth rate of khirni-2 and chafa isolate was much faster (30 and 29 mm/day respectively) than khirni-1 (28mm/day) and isolate of jackfruit was slowest in rate (23 mm/day). All isolates produced globose to irregular shape light brown color sclerotia of medium to large size (1.9 to 3 mm) and this is the typical character of *S. delphinii.* However, the size of the sclerotial bodies varied among these isolates (Figure 1). The chafa isolate produced the sclerotia having highest diameter of 3 mm, whereas the rest of the isolates were in the range of 1.9 to 2.1 mm. In general, 9 to 11 days were required for formation of sclerotial bodies. The number of sclerotial bodies per plate was in the range of 60 to 104. Presence of basidial stage was detected in khirni isolate but not in jackfruit and chafa isolate.

Production of oxalic acid and fungal biomass (mycelial mat) varied significantly among four different isolates of *S. delphinii* (Table 3). Maximum oxalic acid (8.86 mg/g of mycelial mat) production was recorded in chafa isolate followed by khirni-1 (6.16 mg/g) and khirni-2 (5.54 mg/g) at 14 days after incubation, whereas isolate of jackfruit produced low oxalic acid (4.94 mg/g). However, in the mycelial compatibility reaction studies of four isolates of *S. delphinii* and *S. rolfsii* making 36 pairings for comparison on PDA medium (Table 4) revealed that all the four isolates of *S. delphinii* were different from *S. rolfsii* and this did not intermingle with *S. rolfsii* isolates. The *S. delphinii* isolates were only compatible with each other except Khirni- 1 isolate, which formed the separate mycelial compatibility group (Figure 2). Among four isolates, in two MCGs were detected three isolates *viz.,* Jackfruit, Chafa and Khirni-2 under the same MCG and Khirni-1 isolate formed the separate MCG. This indicates the extent of diversity amongst *S. delphinii* and *S. rolfsii* isolates.

Table 2. Morphological variation among the isolates of *S. delphinii*.

Sclerotium delphinii isolate of	Colony character	Mycelial growth (mm/day)	Sclerotial formation pattern	Sclerotial Diameter (mm)	Color of sclerotia	No. of days for sclerotia formation	Weight of 100 sclerotia	No. of sclerotia per plate	Basidial stage present/absent
Jackfruit	Filamentous	23	Pattern5	2.1	Light brown	9	0.07	90	_
Chafa	Filamentous	29	Pattern 5	3.0	Light brown	10	0.23	60	_
Khirni-1	Fluffy	28	Pattern 5	2.0	Light brown	11	0.09	96	+
Khirni-2	Fluffy	30	Pattern 4	1.9	Light brown	9	0.08	104	+

Pattern no. 1, Small size (1 to 1.5 mm), round sclerotia, spread all over the plate but more in number preferably at margin side; Pattern no. 2, Medium size (1.5 to 2 mm), globose sclerotia spread all over the plate. Pattern no. 3, medium size (1.5 to 2.5 mm), round sclerotia spread all over the plate. Pattern no. 4, Medium size (1.5 to2.0 mm), globose to irregular sclerotia spread all over the plate. Pattern no. 5, Large size (2 to 3 mm), globose to irregular sclerotia spread all over the plate.

Figure 1. *Sclerotium delphinii* isolates of: a) Chafa b) Jackfruit c) Khirni-1 d) Khirni-2.

To assess the genetic variability within the species, 13 isolates of *S. rolfsii* and four isolates of *S. delphinii* collected from different crop plants were analyzed. RAPD-PCR generated very distinct amplification products with considerable variability between the isolates of *Sclerotium* spp. In total, 129 fragments were generated from 11 RAPD primers (Table 1). The number of RAPD fragments produced per primer varied between 5 and 17 and ranged in size from 250 to 1358 bp. However, OPB 16 was found to be the most useful primer yielding polymorphic bands within and between fungal species (Figure 3). UPGMA cluster analysis of RAPD data separated the isolates of each particular species of *Sclerotium* into unique group based on genetic similarity coefficient. Though all isolates of *Sclerotium* spp. were genetically variable, they share some common bands indicating phylogenetic relationships among themselves. A dendrogram representing the genetic relationships among the isolates was developed and presented in Figures 4 and 5). On this basis the isolates of *Sclerotium* spp. that is, *S. rolfsii and S. delphinii* can be classified into six broad groups. Among These groups, group III consisted of *S. delphinii* isolates consisted again of two subgroup *viz.,* III_a (Khirni- 1 and Khirni- 2 isolates) and III_b (Jackfruit and Chafa isolates) subgroup consisted of four isolates each with 0.75 to 1.0 genetic similarity coefficient.

DISCUSSION

Variability among four Indian isolates of *S. delphinii* isolated from infected diseased Khirni (*Manilkara hexandra*), chafa (*Plumeria rubra*

Table 3. Production of oxalic acid by different isolates of *S. delphinii* at 14 and 21 DAI.

S. rolfsii isolate of	Mycelial mat (gm) produced at days		Amount ((mg/ml)of oxalic acid produced at days	
	14	21	14	21
Jackfruit	0.59	1.22	2.92 (4.94)	3.03 (2.48)
Chafa	0.23	0.25	2.04 (8.86)	2.19 (8.76)
Khirni-1	0.36	0.93	2.22 (6.16)	2.56 (2.75)
Khirni-2	0.37	0.35	2.05 (5.54)	2.24 (6.05)

Figures in parenthesis indicate mg of oxalic acid/g of mycelial mat of respective isolates

Table 4. Mycelial compatibility/incompatibility reaction among different isolates of *S. rolfsii* and *S. delphinii*.

Sclerotium isolate of	1	2	3	4	5	6	7	8
Chickpea	NI	A	NI	NI	A	A	A	A
Soybean		NI	A	A	A	A	A	A
Groundnut			NI	NI	A	A	A	A
Sugarbeet				NI	A	A	A	A
Khirni-1*					NI	A	A	A
Khirni-2*						NI	NI	NI
Jackfruit*							NI	NI
Chafa*								NI

NI = Normal intermingling, A = antagonistic reaction; *indicates *Sclerotium delphinii*.

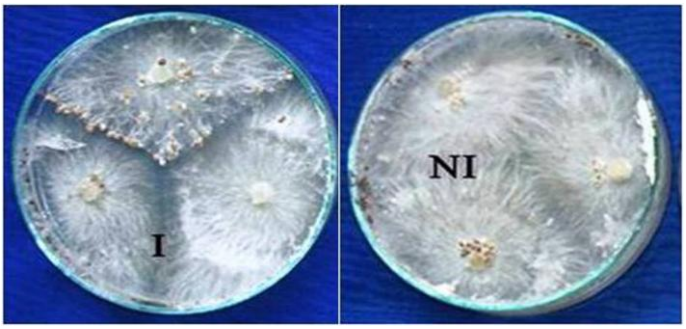

Figure 2. Mycelial compatibility/incompatibility among the isolates (Where I = Inhibition, NI = Normal intermingling).

acutifolia) and Jackfruit (*Artocapus heterophyllus*) plants of Western Maharashtra region of India were studied. These isolates varied in colony morphology, mycelial growth rate, sclerotia formation period, sclerotia size and color. On the basis of morphological characters, these isolates were identified as *S. delphinii.* Punja and Damiani (1996) reported that the sclerotia of *S. delphinii* isolates were lighter brown to orange in colour. Stevens (1931) reported 2.18 mm diameter of sclerotial bodies in *S. delphinii.* Adandonen (2000) reported variation in

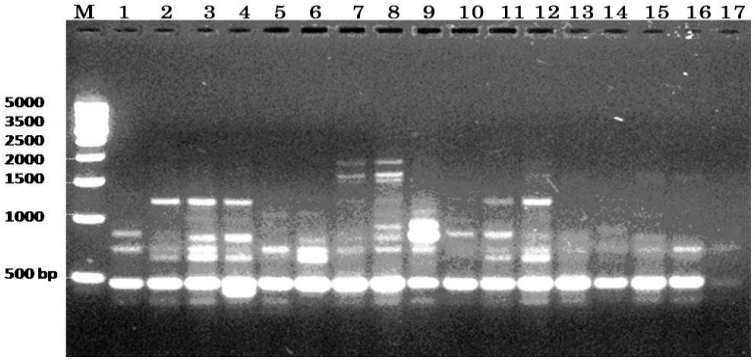

Figure 3. 1.2% agarose gel electrophorosis and amplification profiles of seventeen different isolates of *Sclerotium* species by OPB-16
(Where, M represent DNA (5000 bp) Ladder Lane 1: *Sclerotium* species isolates of Cotton, Lane 2: Sugar beet, Lane 3 :Groundnut, Lane 4: Chickpea, Lane 5: Isabgol, Lane 6: Tomato, Lane 7: Khirni -1*, Lane 8: Khirni-2*, Lane 9: Wheat-2, Lane 10: Wheat-1, Lane 11: Bottle gourd, Lane 12: Mango, Lane 13: Lily, Lane 14: Tuberose, Lane 15: Chafa*, Lane 16: Jackfruit*, Lane 17: Dahalia) *indicates isolate of *Sclerotium delphinii*.

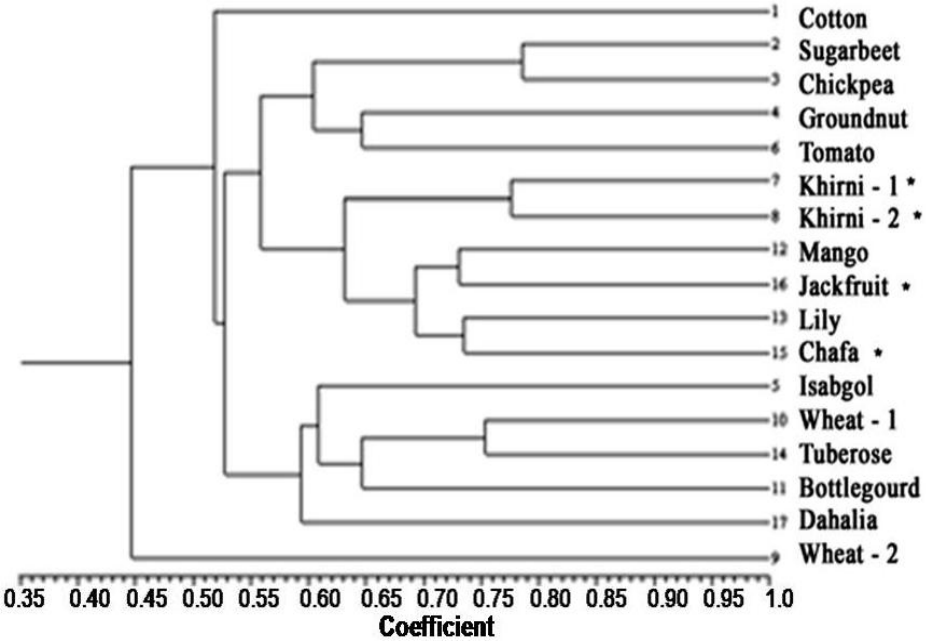

Figure 4. Dendrogram representing the genetic relationship among seventeen different isolates of *Sclerotium* sp.

growth rate, sclerotial numbers and time required for first appearance of sclerotia in isolates of *S. rolfsii* and *S. delphinii* collected from different villages in the Oueme valley. The present findings are in line with the morphological characters of *S. delphinii* species described by above workers and are also confirmed from Indian Type Culture Collection, (ITCC) New Delhi. This is the first report of presence of *S. delphinii* species in India.

Production of oxalic acid and fungal biomass (mycelial mat) varied significantly among four different isolates of *S. delphinii.* Ansari and Agnihotri (2000) characterized 44 isolates of *S. rolfsii* from soybean and classified them into four groups on the basis of quantity of oxalic acid produced. Sharma et al. (2002) recorded maximum production of oxalic acid after 14 days of incubation. Shukla and Pandey (2006) also observed maximum

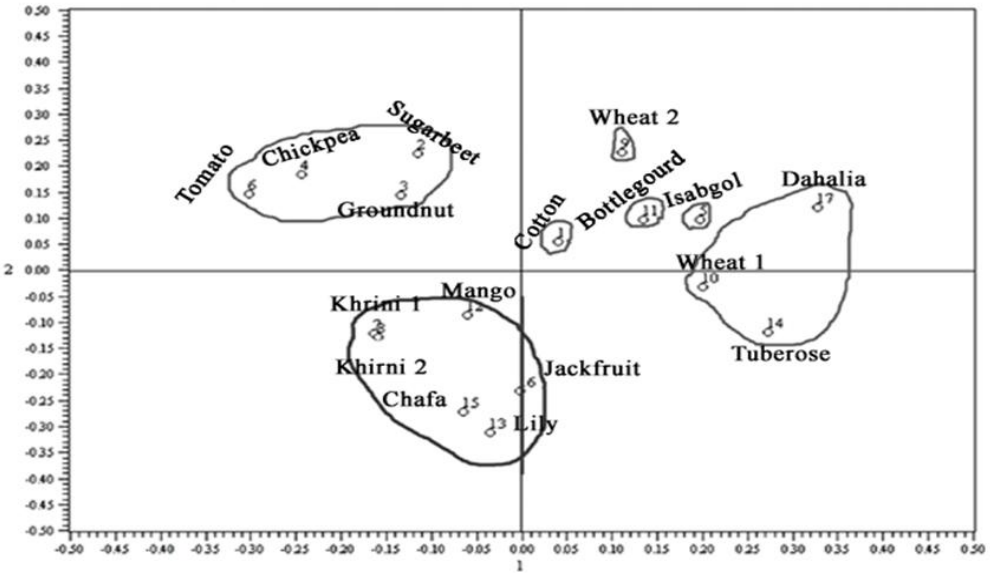

Figure 5. RAPD 2D PCO scatter plot representing the genetic relationship among seventeen different isolates of *Sclerotium* spp.

oxalic acid after incubation for fourteen days. Punja and Jenkins (1984) reported that isolates of *S. rolfsii* varied in oxalic acid production in the culture. Present investigations on oxalic acid production of S. *delphinii* are in agreement with variation in oxalic acid content of *S. rolfsii.*

In mycelial compatibility study, isolates of *S. delphinii* did not intermingle with *S. rolfsii* isolates inflicting genetic variation among *S. delphinii* and *S. rolfsii* species. In the present study, two MCGs were detected among four isolates of *S. delphinii.* The three isolates *viz.,* Jackfruit, Chafa and Khirni-2 is under the same MCG and Khirni-1 isolate formed the separate MCG and in corroboration with the study undertaken by Harlton et al. (1995) where paired 119 isolates of *S. rolfsii*, 11 of *S. delphinii* and 2 of *S. coffeicola* and recorded 49, 3 and 2 MCGs, respectively. Remesal et al. (2013), Twelve MCGs (i–xii) were identified among 459 *S. rolfsii* isolates. MCG iii was the most prevalent group in all countries except Italy. MCG i, the most abundant group (64Æ7% of isolates) was identified in Portugal and Spain. The remaining MCGs were restricted to various regions within one country (ii, vi and ix) or different countries (v), or to specific localities (iv, vii, viii, x, xi, xii).MCGs iv, vii and x each comprised one isolate.

The RAPD analysis was reported to be efficient for studying the phylogenetic relationships among the *Sclerotium* spp. RAPD-PCR analysis had also been used by other workers to study the genetic variability among isolates of *S. rolfsii* collected from different geographical regions and hosts. Punja and Sun (2001) as compared to 128 isolates of *S. rolfsii* from 36 host species and 283 geographic regions by means of RAPD-PCR which confirmed that many isolates from the same host belong to the same MCG. Almeida et al. (2001) studied the variation among 30 isolates of *S. rolfsii* from different hosts and region of Brazil by undertaking analysis of genomic DNA through RAPD-PCR technique (Le et al., 2012). Based on internal transcribed spacer (ITS) ribosomal DNA sequence analyses, three distinct groups were identified among a total of 103 randomly selected *S. rolfsii* field isolates, with the majority of the isolates (*n* = 90) in one ITS group. *S. rolfsii* isolates originating from groundnut, tomato and taro were all pathogenic on groundnut and relatively sensitive to the fungicide tebuconazole but displayed substantial diversity of various genetic and phenotypic traits, including mycelial compatibility, growth rate and sclerotial characteristics. This technique confirmed that the variability among the isolates of *Sclerotium* spp. in relation to the number, size, colour and location of sclerotia on the surface of the medium. It was observed that only morphological features and MCG's differentiated *Sclerotium* species rather than molecular characterization because they shared some common bands of DNA at genetic level, but showed distinctness in morphological and MCG's study. Thus, the data obtained under this experiment confirmed the efficiency and necessity of morphological, genetical and molecular (RAPD-PCR and ISSR) techniques for determination and estimation of genetic similarities and differences among the isolates of *S. rolfsii* and *S. delphinii* collected for the present study. The data obtained under this investigation confirmed the potential of RAPD-PCR technique for determination of genetic

similarity and differences among *Sclerotium* isolates collected for the present study. Therefore, RAPD analysis was found an efficient and informative DNA marker system to assess genetic relatedness and diversity among different isolates of *Sclerotium* spp. It was observed that only morphological features and MCG's differentiated the *Sclerotium* species rather than molecular characterization because they shared some common bands of DNA at genetic level, but showed distinctness in morphological and MCG's study. Thus, the data obtained under this experiment confirmed the efficiency and necessity of morphological, genetical and molecular (RAPD-PCR) techniques for determination and evaluation of genetic similarities and differences among the isolates of *S. rolfsii* and *S. delphinii*.

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Full Length Research Paper

Hypocrea/Trichoderma viridescens ITV43 with potential for biocontrol of *Moniliophthora roreri* Cif & Par, *Phytophthora megasperma* and *Phytophthora capsici*

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A *HypocreaTrichoderma* strain was isolated from cacao (*Theobroma cacao* L.) pods from a cacao plantation in Huimanguillo, Tabasco, Mexico. The strain was identified as *H./T. viridescens* ITV43 by its morphological characteristics and DNA sequence analysis of ITS1-5.8s-ITS2 and was tested against *Moniliophthora* roreri Cif & Par and *Phytophthora* strains by confrontation experiments. The biocontrol of *H./T. viridescens* ITV43 measured as biocontrol index (BCI) at seven days against *M. roreri*(HT-ITV01, HT-ITV20, HT-ITV27), *P. megasperma* (HT-ITV08, HT-ITV15, HT-ITV37) and *P. capsici* (HT-ITV14, HT-ITV16, HT-ITV33) strains ranged from 78.28-81.63, 63.04-69.95 and 69.77-76.31%, respectively. After 31 days of incubation, the BCI values increased to 81.52-86.50 for *M. roreri* and 100% for *Phythopthora* strains. Samples taken from the interaction zone were observed by optical and scanning electron microscopy. The results show the effect of *H./T. viridescens* ITV43 strain on morphological deformations and disorganization of the *M. roreri* and *Phytophthora* cell wall structures. Highest BCI values obtained suggest that the *Hypocrea* strains isolated from *T. cocoa* could have the ability to antagonize more efficiently against fungal pathogens of this crop.

Key words: Antagonism, *Hypocrea*, mycoparasitism, *Moniliophthora*, *Phytophthora, Theobroma.*

INTRODUCTION

Cacao (*Theobroma cacao* L.) is one of the most important tropical crops since the seeds of its fruits are the raw material in the production of chocolate. However, diseases caused by fungi that attack cacao plantation have seriously affected cacao production. Among these, the witches broom caused by *Moniliophthora perniciosa* and the frosty pod rot caused by *Moniliophthora roreri*, are the most devastating. Losses are been estimated between 40 to 90% in several countries of America (Phillips-Mora and Wilkinson, 2007; Sánchez-Mora and Garces-Fiallos, 2012). On the other hand, black pod disease caused by species of *Phytophthora,* which are

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widely distributed in several countries of America, Asia and Africa cause losses in cacao plantation between 45 and 100% (Ploetz, 2007; Djocgoue et al., 2010). The control of these diseases is carried out by the application of fungicides, plant health practices and use of resistant cocoa varieties (Tondje et al., 2007). However, these conventional methods are not enough to completely stop the development of diseases in cocoa producing regions, being still necessary new tools to integrate into a comprehensive management program of cocoa diseases. An alternative strategy is the use of microorganisms isolated from the cacao tree or from its environment. For example, *Trichoderma ovalisporum* isolated from *Theobroma grandiflorum* and from *Banisteriopsis caapi* in Brazil and Ecuador, respectively demonstrated its ability to antagonize and parasitize *M. roreri* (Holmes et al., 2004). On the other hand, *Trichoderma theobromicola* and *Trichoderma paucisporum* isolated from healthy *Theobroma cacao* in Peru and Ecuador, respectively, showed an antibiotic effect against *M. roreri* on cocoa fruit *in situ* and *in vitro* experiments (Samuels et al., 2006). *Trichoderma martiale* isolated in Brazil from *Theobroma cacao* reduced the severity of symptoms caused by *P. palmivora* on cocoa fruit in the field (Hanada et al., 2008, 2009) and *Trichoderma stromaticum* isolated from *Theobroma grandiflorum*, was mycoparasitic on *Phytophthora palmivora* (Hanada et al., 2010) and is the active ingredient of 'Tricovab', a product that is being applied in the field to control black pod disease in Brazil (De Souza et al., 2008). Moreover, *Trichoderma asperellum* isolated from soil in Cameroon was mycoparasitic on *P. capsici*, *P. citrophthora* and *P. palmivora*, other causal agents of black pod worldwide (Tondje et al., 2007).

In a previous study, 128 fungal isolates were isolated from aerial plant tissues of cacao including tree trunks, stems and fruits with visual symptoms of frosty pod rot and black pod disease (Cuervo-Parra et al., 2011a). One of these isolates named ITV43 was identified to the genus level as *Hypocrea*/*Trichoderma*. The objectives of this study were to determine (i) the species of *Hypocrea*/*Trichoderma* strain ITV43 and (ii) its antagonistic activity *in vitro* against the plant pathogens *M. roreri*, *P. megasperma* and *P. capsici.*

MATERIALS AND METHODS

Fungal strains

Hypocrea/Trichoderma strain ITV43 was isolated from aerial plant tissues of cacao tree. *P. megasperma* (HT-ITV08, HT-ITV15, HT-ITV37) and *P. capsici* (HT-ITV14, HT-ITV16 and HT-ITV33) strains were isolated from cocoa fruits with symptoms of black pod from El Caobanal, La Noria and La Hacienda plantations, located in the municipality of Huimanguillo, Tabasco. *M. roreri* HQ231236, *M. roreri* JN241966 and *M. roreri* JN241967 strains were isolated previously from the same plantations (Cuervo-Parra et al., 2011a). The strains were maintained on potato dextrose agar medium (PDA) while the experiments were performed.

Morphological characterization of *Hypocrea/Trichoderma* ITV43

The *Hypocrea/Trichoderma* strain ITV43 was cultured on cornmeal agar with 2% (w/v) dextrose (CMD: cornmeal agar 17 g, dextrose 20 g, 1000 ml distilled water) and on synthetic low-nutrient agar (SNA: KH_2PO_4 1.0 g, KNO_3 1.0 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, KCl 0.5 g, glucose 0.2 g, sucrose 0.2 g, 1000 ml distilled water, agar 20.0 g). Petri dishes (9 cm-diam) were incubated under ambient laboratory conditions of light and dark at 25-28°C for 10 days. The morphology and color of the colony, formation and shape of pustules, diffusing pigment in the agar and odor were recorded. To analyze conidiophores, phialides and conidia, samples of pustules were placed in a drop (3% w/v KOH) and observed with a compound microscope (Leica DM 3000, Leica Microsystems GmbH Wetzlar, Germany) using bright field (BF) and phase contrast (PC). Chlamydospores were observed by examining the reverse of the colony grown on CMD with 20x objective. Thirty measurements for each of the following structures were made: conidium length, width, and length/width ratio (L/W), phialide length, width, length/width ratio (L/W), base of phialide and cells supporting the phialides, chlamydospore length, width, and length/width ratio (L/W). Measurements of the characters were taken from images using the software LAS 3.2.0 version Leica Microsystems (GmbH Wetzlar, Germany) and were reported as extremes in brackets separated by the means plus and minus the standard deviation. Also, the 95% confidence interval (CI) was calculated for each parameter. The ITV43 strain was identified using the key to species of *Hypocrea* with warted conidia (Jaklitsch et al., 2006).

Molecular characterization of *Hypocrea/Trichoderma* strain ITV43, *P. megasperma, P. capsici* strains and phylogram tree

Hypocrea/Trichoderma strain ITV43, *P. megasperma* (HT-ITV08, HT-ITV15, HT-ITV37) and *P. capsici* (HT-ITV14, HT-ITV16, HT-ITV33) strains were grown on potato broth medium (PD) at room temperature and 250 rpm. PD medium composition (expressed in g per liter in 1000 ml of distilled water) was as follows: glycerol, 20; polypeptone, 10 yeast extract, 10, glucose 5, and 100 ml of potato extract. The mycelium was grown for three days recovering and washing twice with TE buffer (TrisHCl 10 mM, pH 8.0, EDTA 1 mM, 2% SDS). The mycelium was ground and DNA was extracted by the method described by Kurzatkowski et al. (1996). A nuclear DNA region, containing the ITS I/5.8s/ITS II sequence, was amplified by PCR using the ITS1 (5′tccgtaggtgaacctgcgg3') and ITS4 (5′tcctccgcttattgatatgc3') primers (White et al., 1990). PCR amplification was performed in a final volume of 50 µl of reaction that contained: 10 µL of stamp 5x Colorless, 200 µMdNTPSs, 0.2 µM of each primer, 2,5 units of *Taq*DNA polimerase and 10-50 ng of template DNA. PCR reaction was placed in a thermocycler (Bio-Rad Gene CyclerTM series model No. 11453 Hercules, California, EE.UU.) under the following parameters: 5 min of initial denaturalization at 95°C, followed by 30 cycles of denaturalization of 1 min at 95°C, 1 min of alignment at 57°C, 1 min of extension at 72°C and a final extension period of 12 min at 72°C. The resulting product was purified with the kit Geneclean® II (Hercules, California, EE.UU.) according to the manufacturer's protocol and DNA was sequenced by the Biotechnology Institute, Cuernavaca, Morelos, México. Fungal DNA sequences were obtained with the program Chromas 1.45 (School of Health Science, Griffith University, Gold Coast Campus, Southport, Queensland, Australia) and a search was conducted with the BLAST (Zhang et al., 2000), for comparison of the homologies with the fungi sequences deposited in the GenBank (www.ncbi.nlm.nih.gov). The DNA sequence was aligned with other sequences through ClustalX (Thompson et al., 1997; Larkin et al., 2007) to identify the variable regions or base sequences. The distance matrixes between all pairs of sequence of multiple alignments were calculated and a tree

was generated by the Neighbor-Joining method developed by Saitou and Nei (1987). Two bootstrap analyses were performed with 1000 interactions with the program ClustalX (Thompson et al., 1997). Two phylogram trees were generated and rooted by importing the calculated values with the PhyloDraw program version.0.8 (Graphics Application Lab, 1999).

Confrontation experiment of *Hypocrea/Trichoderma* against phytopathogenic fungi

The confrontation experiments of *Hypocrea/Trichoderma* strain ITV43 against *M. roreri*, *P. megasperma* and *P. capsici* strains were performed using the technique described by Szekeres et al. (2006). Petri dishes with PDA medium were seeded at equidistant points and were incubated at 25°C in the dark for 31 days. The antagonistic activity was recorded at 7 and 31 days of incubation. Digital images were taken with a camera Cyber-shot DSC-P72, using the same vertical distance from the camera to the Petri dish (18 cm). The percentage growth inhibition of the pathogenic fungi was calculated using the biocontrol index (BCI) according to the formula reported by Szekeres et al. (2006): BCI = [A / B] x 100. Where A is the area of the colony of *Hypocrea/Trichoderma* and B is the total area occupied by colonies of *Hypocrea/Trichoderma* and each pathogen. The software ImageJ (on line: http://rsbweb.nih.gov/) was used to calculate the area of the fungi colonies. From the digital images taken with the digital camera, the area was measured and its outline was drawn using the free tool of the program ImageJ. During the analysis, the scale was set to 28.346 pixels per centimeter and the corresponding unit area was calculated in cm^2. The experiments were made by triplicate and analysis of variance (ANOVA) was performed with the software Statistix9. Means were compared with Tukey test ($P \leq 0.05$). Samples obtained from the interaction area between *Hypocrea/Trichoderma*ITV-43 and the phytopathogenic fungi were observed with an optical and scanning electron microscope (JEOL, JSM-5600 LV).

RESULTS

Morphological characterization of *Hypocrea/-Trichoderma* strain ITV43

The *Hypocrea/Trichoderma* strain ITV43 covered the surface of CMD and SNA in 4-5 days. It produced a coconut odor and a diffusing yellow pigments were detectedon CMD. Pustules were cottony first white and then turned dark green distributed in 3 concentric rings on SNA (Figures 1A and B), and on CMD were scarce and developing around the edge of the colony. The formation of pustules was more quickly on SNA than on CMD medium being visible the long terminally fertile conidiophores at the surface (Figure 1D). Two types of conidiophores were seen depending on the culture medium: on SNA Type 2 and on CMD Type 3 (Figure 1E) as described by Jaklitsch et al. (2006). Intercalary phialides were observed in SNA medium. In the conidiophore Type 3 there were production of Type 2 lageniform straight, some curved or hooked, (3.79-)4.39-9.48(-13.20) µm long, CI = 6.03-7.85, n = 30; (1.54-)1.74-2.18(-2.38) µm wide in the widest point, CI=1.88-2.04, n=30; (0.93-)1.21-1.81(-2.01) µm at the base, CI=1.40-1.61, n=30; L/W=(0.98-)0.98-1.18(-1.50), CI=1.05-1.12, n=30; arising from a cell (1.09-)1.53-1.95(-2.12) µm wide, CI=1.66-1.81, n=30. Intercalary phialides were observed in SNA medium (Figure 1F). In the conidiophore Type 3, there was production of chains of proliferated phialides. Conidia on CMD with ornamentation warts, shape subglobose to ellipsoidal, (3.69-)4.01-4.85(-5.60) µm long, CI=4.28-4.58, n=30; (3.56-)3.84-4.31(-4.66) µm wide, CI=3.99-4.16, n=30; L/W=(0.98-)0.98-1.18(-1.50), CI=1.05-1.12, n=30 (Figure 1G, 1H). Chlamydospores on CMD (Figure 1I) were produced terminally and intercalary in hyphae, smooth, globose to subglobose (6.59-)8.21-11.01(-13.45) µm long, CI=9.11-10.11, n=30, (5.55-)7.47-10.50(-11.86) µm wide, CI=8.44-9.53, n=30, L/W= (0.93-)0.98-1.17(-1.39), CI=1.04-1.11, n=30. Based on the phenotype characteristics and the measurements described in the Table 1, the strain ITV43 was identified as *H./T. viridescens*.

Identification of fungal strain by ITS1-5.8s-ITS2 sequencing and phylogeny

Using ITS1 and ITS4 primers, the ribosomal DNA (rDNA) region containing the ITS1-5.8s-ITS2 sequence was amplified from each fungal strain. The amplified region of *H./T. viridesens* ITV43 had a size of 679 bp and was deposited in the NCBI GenBank (JX144329). The ITS1-5.8s-ITS2 sequence amplified showed the highest similarity of 100 and 94% with DNA *H. viridescens* sequence (GenBank EF534007) and *H. viridescens* (GenBank EU280104, EU280137), respectively. The first sequence was obtained from a strain isolated in the United States of America by Liu et al. (2008) and the other two from strains isolated in Colombia and Peru by Hoyos-Carvajal et al. (2009). Phylogenetic analysis of *H./T. viridescens* ITV43 and 11 representative ITS1-5.8s-ITS2 sequences of isolates of *Trichoderma*/*Hypocrea* generated a tree by the Neighbor Joining method and formed three majors groups (Figure 2). *H./T. viridescens* ITV43 grouped with *H. viridescens* (GU566274). This clade is affiliated with *H. viridescens* (EU280104 and EU280137) and close to *H. viridescens* (HQ833353).

On the other hand, the amplified ITS1-5.8s-ITS2 rDNA of *Phytophthora* strains were sequenced and the strains were identified with the highest similarity of the sequences obtained with the Blast tool. The GenBank accession numbers obtained for the ITS1-5.8S-ITS2 sequences were: *P. megasperma*HT-ITV08 (KC753539), HT-ITV15 (KC753540), HT-ITV37 (KC753541), and *P. capsici*HT-ITV14 (KC753542), HT-ITV16 (KC753543), and HT-ITV33 (KC753544). The Neighbor Joining phylogenetic analysis with nine *Phythopthora* strains ITS1-5.8s-ITS2 sequences yielded a tree (Figure 3). *P. capsici* HT-ITV16 and *P. capsici* HT-ITV33 are affiliated with *P. capsici* AF467083. *P. capsici* HT-ITV14 grouped with *P. capsici* AF467085 and *P. megasperma* HT-ITV37 and HT-ITV15 are affiliated with *P. megasperma* (AF541899, AF54189 and AF541893) and close to *P. cryptogea* EU00073. On the other hand, *P. megasperma* HT-ITV08 is close to *P. megasperma* strains.

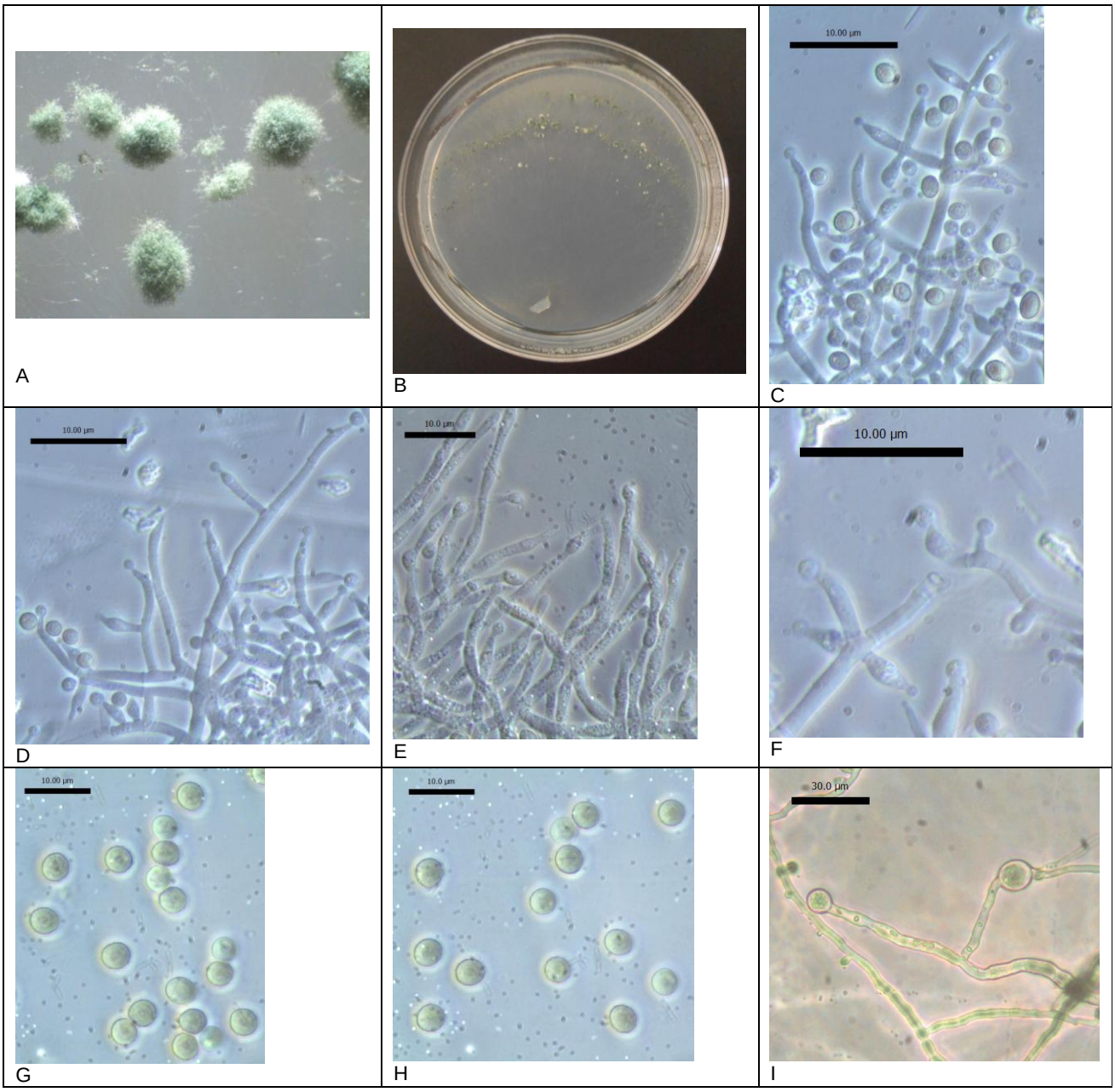


Figure 1. Morphology of *H. viridescens* ITV-43 *strain*. A. Pustules on SNA; B. colony grown on SNA after 10 days; C. Conidiophore Type 2 similar to *Trichoderma* on SNA; D. A long terminally fertile conidiophores; E. Type 3 conidiophore on CMD with chains of percurrently proliferating phialides; F. Intercalary phialide on SNA; G to H. Conidia warted on CMD; I. Chlamydospores on CMD. A. Magnification 25×. Scale bars: C-H = 10 µm, I = 30 µm.

Confrontation experiments

The antagonistic ability of *H./T. viridesens* ITV43 strain was tested against *M. roreri* (HT-ITV01, HT-ITV20, HT-ITV27), *P. megasperma* (HT-ITV08, HT-ITV15, HT-ITV37) and *P. capsici* (HT-ITV14, HT-ITV16, HT-ITV33) strains by confrontation experiments in dual culture. The digital images taken on 7 and 31 days of incubation (Figure 4) were used to calculate the BCIs values. In the seven days of incubation, the BCI values of *H./T. viridescens*ITV43 against *M. roreri*, *P. megasperma*, and *P. capsici* strains ranged between 78.28-81.63, 63.02-69.95 and 69.77-76.31, respectively (Table 2).

According to Tukey´s tests, the highest BCI values were obtained against *M. roreri* HT-ITV20 (81.63), *P. megasperma* HT-ITV15 (69.95) and *P. capsici* HT-ITV16 (76.31). After 7 days, a progressive inhibition zone produced by *H./T. viridescens* ITV43 against all phytopathogenic fungi strains was observed and at 31 days of incubation, 100%

Table 1. Measurements for each structure of *H./T. viridescens* strain ITV43.

	Conidium characters	Phialide characters	Chlamydospore characters
Shape	Subglobose to ellipsoidal	Lageniform, straight, some curved or hooked	Globose to subglobose
Ornamentation	Warted	Smooth	Smooth
Length (µm)	(3.69-)4.01-4.85(-5.60)	(3.79-)4.39-9.48(-13.20)	(6.59-)8.21-11.01(-13.45)
95% CI	4.28-4.58	6.03-7.85	9.11-10.11
N	30	30	30
width (µm)	(3.56-)3.84-4.31(-4.66)	(1.54-)1.74-2.18(-2.38)	(5.55-)7.47-10.50(-11.86)
95% CI	3.99-4.16	1.88-2.04	8.44-9.53
N	30	30	30
L/W	(0.98-)0.98-1.18(-1.50)	(0.98-)0.98-1.18(-1.50)	(0.93-)0.98-1.17(-1.39),
95% CI	1.05-1.12	1.05-1.12	1.04-1.11
N	30	30	30.
Base of phialide (µm)		(0.93-)1.21-1.81(-2.01)	
95% CI		1.40-1.61	
N		30	
Cells supporting the phialides (µm)		(1.09-)1.53-1.95(-2.12)	
95% CI		1.66-1.81	
N		30	

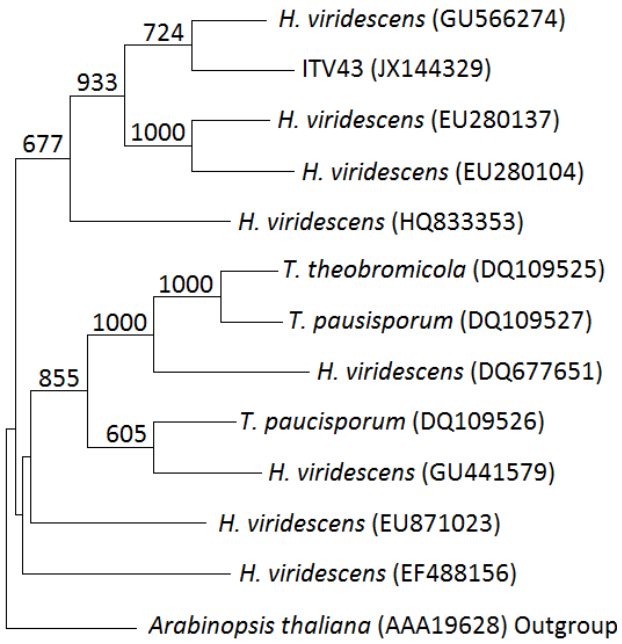


Figure 2. Phylogenetic tree for ITS1-5.8s-ITS2 region of rDNA, of *H. viridescens* ITV43 and the sequences from specimens related, obtained from GenBank (NCBI). The Kimura distance and the Neighbor-Joining method was used. The numbers adjacent to the branch points of ramification are bootstrap values of 1000 iterations.

inhibition was detected on *H./T. viridescens* ITV43-*Phytopthora* plates while *H./T. viridescens* ITV43-*M. roreri* plates produced BCI values between 81.52 and 86.50. The mycoparasitism was analyzed in the areas of interaction of *H./T. viridescens* ITV43-*M. roreri, H./T. viridescens* ITV43-*P. capsici* and *H./T. viridescens* ITV43-

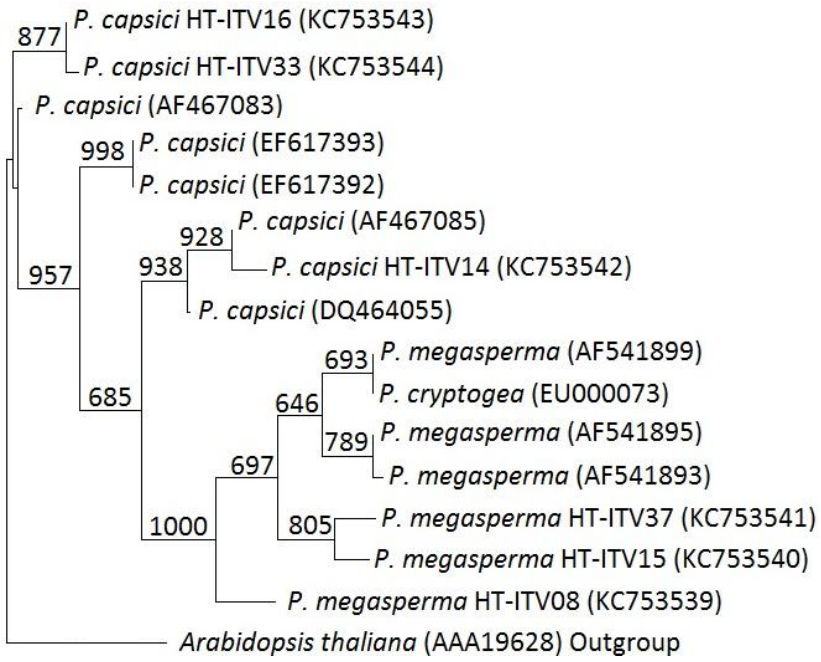


Figure 3. Phylogenetic tree for the ITS1-5.8S-ITS2 region of rDNA, for isolates of *P. megasperma*, *P. capsici* and related sequences of specimens obtained from GenBank (NCBI). The Kimura distance and the neighbor-joining method were used. Numbers adjacent to branch points are bootstrap values for 1000 iterations.

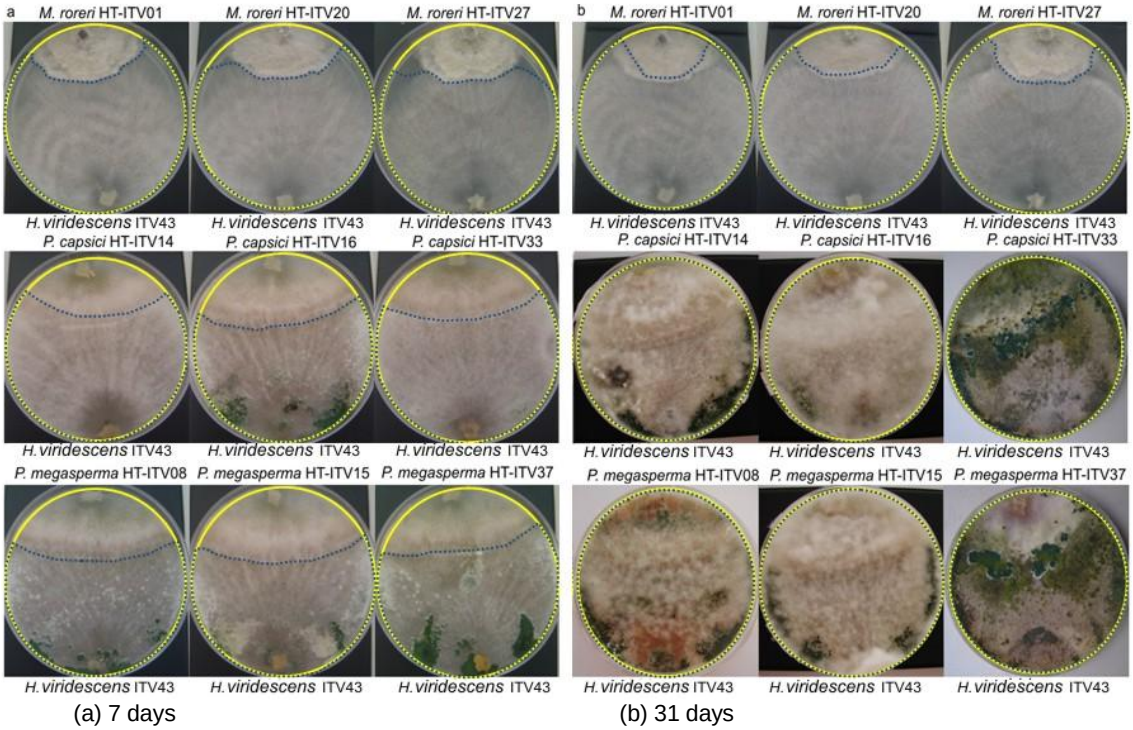
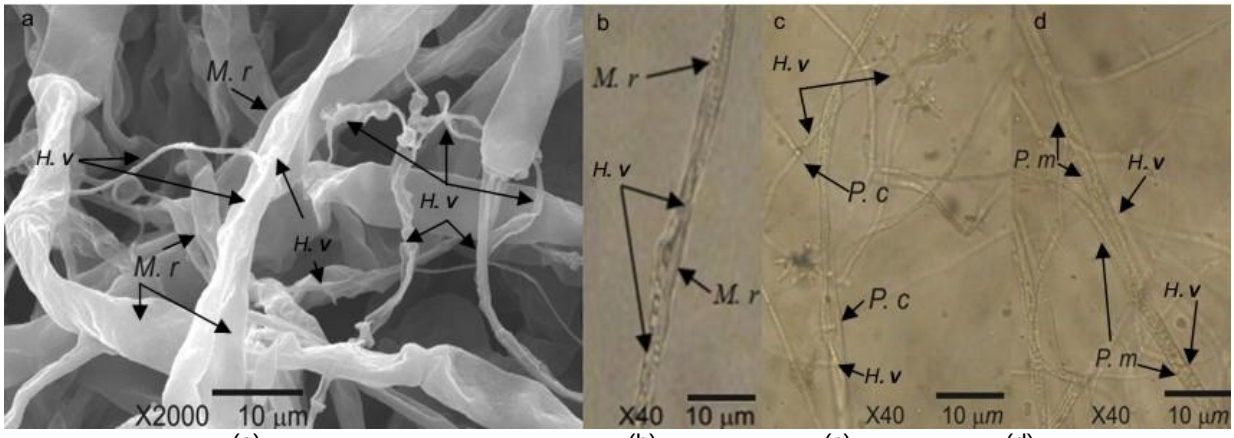


Figure 4. Digital images taken at: (a) 7 and (b) 31 days of confrontation of *H. viridescens* ITV43 against *T. cacao* phytopathogenic fungi (*M. roreri, P. capsici* and *P. megasperma*) in PDA medium. Areas of *H. viridescens* ITV43 colonies (Area T, line blue dots) and the total area occupied by colonies of *H. viridescens* ITV43 and the phytopathogenic fungi (Area T + P, yellow line).

Table 2. Biocontrol index (BCI) of *H. viridescens* ITV43 (inhibition zone) against *T. cacao* phytopathogenic fungi.

Phytopathogenic fungi	BCI ± sd* (7 days)	BCI ± sd* (31 days)
M. roreri HT-ITV01	80.68[a] ± 0.01	84.85[a] ± 0.01
M. roreri HT-ITV20	81.63[b] ± 0.02	86.50[b] ± 0.01
M. roreri HT-ITV27	78.28[c] ± 0.01	81.52[c] ± 0.02
P. megasperma HT-ITV08	65.73[d] ± 0.02	100.00[d] ± 0.00
P. megasperma HT-ITV15	69.95[e] ± 0.01	100.00[d] ± 0.00
P. megasperma HT-ITV37	63.02[f] ± 0.03	100.00[d] ± 0.00
P.capsici HT-ITV14	69.77[g] ± 0.06	100.00[d] ± 0.00
P. capsici HT-ITV16	76.31[h] ± 0.03	100.00[d] ± 0.00
P. capsici HT-ITV33	70.90[i] ± 0.01	100.00[d] ± 0.00

*Standard deviation; Different letter within the column indicate significant differences ($P < 0.05$, ANOVA and Tukey´s tests).



(a) (b) (c) (d)

Figure 5. Images showing the effect of *H. viridescens* ITV43 on phytopathogenic fungi with the scanning electron microscope (a) and light microscope (b, c, d) in PDA medium: (a) *H. viridescens* (*H.v*) parasitizing mycelium of *M. roreri* (*M.r*) and inducing the lysis and deformation of the pathogen hyphae, (b) mycoparasitism by wrapping the hyphae of *M. roreri* by *H. viridescens*, (c) mycoparasitism of hyphae of *P. capsici* (*P.c*) by hyphae of *H. viridescens*, (d) parallel hyphal growth of *H. viridescens* and *P. megasperma* (*P.m*).

P. megasperma by scanning electron and optical microscopy. The effect of *H./T. viridescens* ITV43 on morphological deformations and disorganization in the structure of the *M. roreri* cell walls was observed by scanning electron microscope (Figure 5a) and by optical microscopy for *M. roreri* (Figure 5b), *P. capsici* (Figure 5c) and *P. megasperma* (Figure 5d).

DISCUSSION

Fungi of the genus *Trichoderma* (*Hypocrea*) have been widely studied due to their antagonistic activity on phytopathogenic fungi in agriculture and are an alternative to synthetic chemical products (Mukherjee et al., 2013). Most *Trichoderma* species has been isolated from soil, however there are reports of *Trichoderma* isolates from other sources such as bark, decorticated wood, roots, decaying wood, mushroom compost, leaf litter, as an endophyte from trunks and aerial parts of plants (Harman et al., 2004). In the biological control of cocoa (*T. cacao*) pod diseases, mycoparasites isolated from cocoa fruits, aerial plant parts and rhizospheric soil from plot planted with cocoa trees have shown great promise in the control of *M. roreri* (Villamil et al., 2012), causal agent of black pod rot and *Phytophthora* spp. (Hanada et al., 2009), causal agent of black pod. Only a small number of the vast microbial diversity associated with cacao has been studied and endophytic and epiphytic microbes are candidates for biocontrol of cacao

diseases. In this study, we have isolated a *Hypocrea/Trichoderma* strain from cacao pod with typical symptoms of frosty pod rot and our results show that the strain has strong antagonism against six *Phytophthora* and three *M. roreri* strains. *Phytophthora* and *M. roreri* strains were also isolated from cocoa pod with typical symptoms of frosty pod rot (Cuervo-Parra et al., 2011a). We suggested that *H./T.viridescens* ITV43 could have developed an antagonism against *Phythopthora* and *M. roreri* strains. BCI at 7 days of *H./T. viridescens* was higher on *M. roreri* strains (average 80.2) than on *Phytophthora* strains (average 69.3) and BCI values are higher as compared to other biological control agents. For example, Cuervo-Parra et al. (2011b) tested a *Trichoderma harzianum* strain (VSL291) by confrontation experiments against *M. roreri* and *Phytophthora* strains. *T. harzianum* VSL291 was isolated from soil samples obtained from *Agave tequilana* crop in the State of Jalisco, Mexico (Sánchez and Rebolledo, 2010). In 7 days, the strain inhibited 72.72, 20.77 and 28.60% growth of *M. roreri* HT-ITV01, *P. megasperma* HT-ITV08 and *P. capsici* HT-ITV14, respectively. In contrast to this study, the same strains were tested and the inhibition percentages were 80.68, 65.73 and 69.77%, respectively. BCI was higher: 7.94, 44.96 and 41.17%, respectively. These results strongly suggest that species associated with *T. cacao* can potentially be used as effective biocontrol agents. Although these percentages obtained *in vitro* cannot be translated directly to biocontrol of phytopathogenic fungi in plant, these results are useful to select candidate strains for use as biocontrol agents. Our results show a better inhibition on *Phythophthora* strains as compared to report by Etebarian et al. (2000). In that study, *in vitro* mycelia growth of *P. erythroseptica* was reduced by 49-71 and 49-54% by *T. virens* DAR 74290 and *T. harzianum* T39, respectively. On the other hand, Mejia et al. (2008) isolated fungal endophytes from healthy *Theobroma cacao* tissues and tested their antagonism *in vitro* against *M. roreri, P. palmivora* and *M. perniciosa*. The antagonism percentage obtained by this group was 40, 65 and 27%, respectively.

According to their morphological characteristics, the strain ITV43 was identified as *H./T. viridescens* that match the descriptions reported by Jaklitsch et al. (2006) and we suggest that this identification is reliable. *H./T. viridescens* belongs to Viride clade that includes *Trichoderma viride, Trichoderma atroviride, Trichoderma koningiopsis* and others (Samuels and Ismaiel, 2009). The characteristic of coconut aroma attributed to 6-PAP (6-pentyl-a-pyrone) was detected in *H./T. viridescens* ITV43 and the antifungal compound 6-PAP (Collins and Halim, 1972; Cutler et al., 1986) is a distinctive feature of many members of the Viride clade (Samuels et al., 2006). Analysis by BLAST of ITS1-5.8s-ITS2 DNA sequence of *H./T. viridescens* ITV43 showed high degree similarity (99-100%) with *H. viridescens* strains (EU280104, EU280137) and phylogenetic tree (Figure 2) locate *H./T. viridescens* ITV43 close to *H. viridescens* (GU441579, GU566274) and *T. asperellum* (AY380912) strains and located away from *T. theobromicola* (DQ109525) and *T. paucisporum* (DQ109527, DQ109526) strains. Although ITS1-5.8s-ITS2 DNA sequences are useful for identifying and studying the evolution of microorganisms, do not appear to be useful in identifying some *Trichoderma/Hypocrea* species (Jaklitsch et al., 2006). Druzhinina et al. (2005) developed the Trich OKEY program for the quick molecular identification of *Hypocrea/Trichoderma* at the genus and species levels based on an oligonucleotide barcode for the internal transcribed spacers 1 and 2 ((www.isth.info). To identify *H./T. viridescens* ITV43, we used the ITS1-5.8s-ITS2 DNA sequence and the program was not able to identify the strain. Similar results were obtained by Torres-Palacios (2010) who used the program to identify 16 *Trichoderma* strains isolated near the Nevado de Toluca volcano, Mexico. In that study, 19% of the strains were identify entirely, 19% were identify as new species and 62% were ambiguous mainly *viridescens-konongii*. Other cases reported by Druzhinina et al. (2005) in which the software initiate ambiguities in *Trichoderma/Hypocrea* strains are: *cerinum=tomentosum, crassum=longipile* and *orientalis=cerebriformis=longibrachiatum*.

In conclusion, the strain ITV43 was identified as *H./T.viridescens* and was mycoparasitic on *M. roreri*, *P. megasperma* and *P. capsici.*

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Effect of citron by-product fermented with beneficial bacteria as a functional feed additive for Korean native steers

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This study was conducted to develop a functional feed additive, citron (*Citrus junos* Sieb. ex Tanaka) probiotics (CPB) for beef cattle, using citrus junos by-product (CJ) with probiotics. A two-step fermentation process was developed for the production of CPB and the effects of CPB on growth performance, immune status, carcass characteristics and fatty acid profile in Korean native (Hanwoo) steers were investigated. Twenty (20) Hanwoo steers (22 months old; 619.00±10 kg BW) were randomly assigned to two dietary treatments in a completely randomized design: control (basal diet) and 1.0% CPB (basal diet + CPB 1.0% DM basis). At the end of the trial, steers were slaughtered and carcasses were evaluated. Dietary CPB supplementation significantly increased the average daily gain (ADG) and feed efficiency (kg gain/kg DMI) of Hanwoo steers as compared to the control ($P < 0.05$). Additionally, serum IgG and carcass weight of Hanwoo steers were increased in response to CPB dietary supplementation ($P < 0.05$). No difference ($P > 0.05$) was observed in muscle composition while cholesterol concentration reduced in CPB supplementation group ($P < 0.05$). Overall, the concentrations of n-3 fatty acids were increased, while the ratio of n-6/n-3 decreased in the CPB dietary group ($P < 0.05$). In conclusion, dietary CPB improved growth, immunity and carcass weight of Hanwoo steers while reducing muscle cholesterol concentration with an elevated n-3 fatty acids concentration, indicating that CPB can be used as a functional feed additive for beef cattle.

Key words: Citron probiotics, Hanwoo steer, growth performance, immunity, carcass characteristics, fatty acid profile.

INTRODUCTION

Consumers prefer high-quality beef, which is primarily determined by the appearance, freshness, nutritional value and eating quality. Despite their slow growth rate, Korean Hanwoo cattle consistently produce highly palatable and well-marbled beef (Cho et al., 2010). However, it is widely known that the incidence of cardiovascular diseases (CVD) is closely related to the dietary intake of cholesterol and saturated fatty acid (SFA) contents. Accordingly, the meat industry is looking for dietary strategies to modulate cholesterol and SFA while enriching meat with bioactive compounds such as antioxidants to improve product quality and protect consumers from oxidant-mediated diseases.

Citron (*Citrus junos* Sieb. ex Tanaka), which is also known

as Yuza in Korea, is a citrus fruit of the Rutaceae family commonly used as preserved tea or in tablet form as an herbal antioxidant. Citrus junos by-product (CJ) is the residues of the juice processing industries in Korea. About 10-15% of Yuza fruit is generated as by-product from Yuza juice extraction (Lee et al., 1987) and approximately 1800 tons per year have been produced (Kim et al., 2010a). These residues are however discarded as processing by-products, causing environmental and economic problems for waste disposal. CJ are mainly composed of peel, pulp and seed. Especially, peel includes valuable compounds such as terpenoid, pectin and flavonoid. Fresh or dehydrated citrus pulp is primarily used in ruminant feeding (Fegeros et al., 1995). Fermentation of feed silage for ruminants using beneficial bacteria has been practiced for many years. Citrus by-products have been reported to have characteristics required for use as a substrate for the growth of probiotics during fermentation (Contreras Esquivel et al., 1999).

Ruminant feeding systems based on locally available by-product feed stuffs are often a practical alternative as the rumen microbial ecosystem can utilize food by-products, which often contain high levels of structural fiber, to meet their nutrient requirements for maintenance, growth, production and reproduction (Bampidis and Robinson, 2006). Probiotics also have pseudo-antibiotic properties that improve feed efficiency, growth performance and health of animals (Hossain et al., 2012). In ruminants, dietary probiotics supplementation has been found to reduce acidosis and improve energy utilization in the rumen, while maintaining the intestinal microbial balance and improve animal performance (Krehbiel et al., 2003). They also reported increased daily gain and feed efficiency in feedlot cattle, enhanced milk production in dairy cows and improved immune response in stressed calves. Dietary yeast cultures (*Saccharomyces cerevisiae*) supplementation increased weight gain and feed conversion efficiency of bulls (Mutsvangwa et al., 1992); increased ADG and feed to gain ratio in Awassi lambs (Haddad and Goussous, 2005). However, the combined effects of CJ and probiotics in ruminant feeding systems have not been evaluated to date. Hence, this study was conducted to verify the collective effect of CJ fermented with beneficial probiotics strain on growth performance, immune status, carcass characteristics and fatty acid profile of Hanwoo steers.

MATERIALS AND METHODS

Steers were handled in accordance with the guidelines of animal care and use of animals in research (Korean Ministry for Food Agriculture Forestry and Fisheries, 2008). All experimental procedures used in this study were approved by the Animal Care and Use Committee of Sunchon National University.

Preparation of CPB

Candidate probiotics strains consisting of 14 strains, *Lactobacillus acidophilus* (3111, 3146, 3150), *Lactobacillus plantarum* (KCTC 3104, 3107), *Enterococcus faecium* (KCTC 2022, 3078, 3080), *Bacillus subtilis* (KCTC 1022, 1103, 3239) and *S. cerevisiae* (KCTC 7107, 7915, 7928), were obtained from the Korean collection for type cultures (KCTC). The KCTC microbial strains were collected from the Korea Research Institute of Bioscience and Biotechnology, Daejeon, Korea. From the above strains, *L. acidophilus* KCTC 3111, *B. subtilis* KCTC 3239, and *S. cerevisiae* KCTC 7915 were selected as starter cultures based on acid, bile and heat tolerance level according to Hossain et al. (2012).

CJ (30%), defatted rice bran (58%), ground barley stone (10%) and molasses (2%) were used as the fermented solid media. A two-step fermentation procedure was conducted using a commercial fermenter (W-1000; Wonbalhyo Industry Co., Icheon, South Korea). First, 0.5% *L. acidophilus* KCTC 3111 was added to solid substrate media and fermented at 40°C for two days under repeating cycles of 5 h of anaerobic and 3 h of aerobic conditions. The second fermentation was performed with 0.5% *B. subtilis* KCTC 3239 and 0.5% *S. cerevisiae* KCTC 7915 strains for 2 days at 40°C under aerobic conditions. The formulated probiotics mixtures were then dried for 2 days until the moisture level was less than 15% using a dry oven (DooriTech FA, Co., Ltd, Korea). To determine the number of cells, 1 g of CPB was diluted with sterilized distilled water (10 ml) at room temperature. Approximately 10 min later, 1 ml of this mixture was serially diluted 10-fold in 0.85% NaCl solution and was cultured in agar media. The culture plate was then incubated at 37°C for 24-48 h, after which the number of colonies was counted. The chemical compositions of CJ and CPB were determined by the method described by the Association of Official Analytical Chemists (AOAC, 2000). The microflora concentration and chemical composition of CJ and CPB are shown in Table 1.

Animals, experimental design and diet

The study was conducted at the experimental farm of Sunchon

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Abbreviations: ADG, Average daily gain; **AOAC,** Association of Official Analytical Chemists; **BW,** body weight; **CJ,** citrus junos by-product; **CPB,** citron (*Citrus junos* Sieb. ex Tanaka) probiotics; **CVD,** cardiovascular diseases; **DFM,** direct fed microbial; **DM,** dry matter; **DMI,** dry matter intake; **GLM,** general linear model; **IgG,** immunoglobulin G; **IgM,** immunoglobulin M; **KAPE,** Korea Institute for Animal Products Quality Evaluation; **KCTC,** Korean collection for type culture; **MUFA,** monounsaturated fatty acids; **NRLSI,** National Rural Living Science Institute; **PUFA,** polyunsaturated fatty acid; **SAS,** statistical analysis system; **SFA,** saturated fatty acid; **UFA,** unsaturated fatty acid.

Table 1. Microflora concentration and chemical composition of *Citrus junos* by-product (CJ) and citron probiotics (*Citrus junos* Sieb. ex Tanaka) (CPB).

Item	CJ	CPB
Microbial strains in CPB (cfu/g)		
Lactobacillus acidophilus KCTC 3111		2.0×10^7
Bacillus subtilis KCTC 3239		1.0×10^7
Saccharomyces cerevisiae KCTC 7915		2.0×10^7
Chemical composition (% DM)		
Moisture	76.02	13.09
Crude protein	3.53	14.84
Crude fat	0.45	1.64
Crude fiber	3.73	8.18
Crude ash	0.83	17.21

Korean collection for type culture (KCTC) strains obtained from the Korean Research Institute of Bioscience and Biotechnology.
DM = Dry matter.

Steers were weighed using a platform balance and body weights were measured before feeding and watering at the onset and end of the experiment. Feed bunks were cleaned and residues were collected daily and weighed at intervals corresponding to weigh dates. Feed efficiency was calculated as the ratio between body weight gain and average feed intake.

Immunoglobulin (IgG and IgM) determinations

To determine the IgG and IgM levels, approximately 5.0 ml of blood was collected from the jugular vein. Samples were then centrifuged at 1,610 x*g* for 15 min in a cold chamber (4°C), after which the serum was collected. The separated serum was then carefully removed to plastic vials and stored at -20°C for further analysis. Serum Ig concentrations were assayed using bovine IgG (Cat. No. E10-118) and IgM (Cat. No. E10-101) ELISA Quantification Kits (BETHYL Laboratories Inc., USA) according to the manufacturer's instructions. Tests were performed in duplicate and results were presented as the mean value of three replications.

Carcass grading and meat quality

At the end of the trial, steers were slaughtered in a commercial slaughterhouse in Suncheon, South Korea. Carcasses were graded for quality and yield factors by a trained carcass evaluator in accordance with Korean beef carcass grading standards (KAPE, 2012). The beef carcasses were graded as 1++, 1+, 1, 2, or 3, which were principally based on marbling scores and then adjusted according to other carcass traits such as meat color, fat color, texture of lean meat and maturity. One of three yield grades (A, B or C) was determined by assessing the live weight, carcass weight and back fat thickness in the rib eye area.

Muscle composition and cholesterol determination

Within approximately 2 h of slaughter, samples were stored at -20°C until required for analysis. To investigate the meat chemical compositions, longissimus muscles from the loin area were selected and ground using a meat grinder. The moisture, crude protein, crude fat and crude ash contents were determined using the AOAC methods (AOAC, 2000). The cholesterol concentration was determined according to the slightly modified method of King et al. (1998). One gram of ground meat, 100 µg of 5α-cholestane and 15 ml water were homogenized with 200 ml of Folch solution (chloroform : methanol, 2:1 v/v) and filtered. The filtrate was added to 50 ml of 0.5% sodium hydroxide. The sample was saponified at 85°C for 60 min with 10 ml of 2 M ethanolic potassium hydroxide solution (Adams et al., 1986). After cooling to room temperature, cholesterol was extracted with 1 ml of hexane. The process was repeated four times. The hexane layers were transferred to a round-bottomed flask and dried under vacuum. The extract was redissolved in 1 ml of hexane and was stored at -20°C until analysis. Cholesterol content was determined by gas chromatography (DS 6200, Donam Co., Seongnam, Gyeonggi-do, Korea) as shown in Table 3.

Fatty acid composition

Meat fatty acids were determined by the methyl ester extraction method described by Yang et al. (2003). One gram of ground meat sample was dissolved separately into 100 ml of Folch solution (chloroform : methanol 2:1 v/v) for 15 min. The samples were then flushed with nitrogen gas for 30 min in an evaporator and filtered through a Buchner funnel. The filtrate was subsequently dissolved in 70 ml of double distilled water and kept at 5°C in a refrigerator until the fat layer separated. After phase separation, the bottom layer was evaporated at 35°C with nitrogen gas and dissolved in 3 ml of 5% sulfuric acid methanol. The tubes were then heated in a water bath at 95°C for 45 min, after which they were allowed to cool at room temperature. Next, fatty acid methyl ester was extracted three times with 3 ml of petroleum ether and dried with nitrogen gas and the fatty acid profile of meat was analyzed by gas chromatography (DS 6200, Donam Co., Korea). Samples were loaded onto the column via 1 µl splitless injections under the conditions described in Table 3. Fatty acids were identified by matching their retention times with those of their relative standards (polyunsaturated fatty acid (PUFA)-2; Supelco, USA) and the Food Composition Table (NRLSI, 2002).

Statistical analysis

Data were analyzed by a randomized block design, using the GLM procedure of SAS (SAS, 2003) based on the statistical model:

$Yij = \mu + Ti + eij$

Where, Yij = dependent variable of the jth animal on the ith treatment; µ = overall mean; Ti = the fixed effect of ith treatment effect (i = 1, 2); Eij = random residual (error) associated with the dependent variable from the jth animal on the ith treatment.

For growth performance parameters, a group of two steers served as the experimental unit. For carcass characteristics, meat composition, cholesterol concentration and fatty acid profile of meat, an individual steer served as the experimental unit. Variability in data was expressed as the pooled S.E. The α-level for analysis was < 0.05, with P-values > 0.05 and < 0.10 considered as tendencies.

RESULTS

Growth performance and immunity

The effects of dietary CPB supplementation on the growth

Table 2. Ingredients and chemical composition of total mixed ration feed.

Ingredient/chemical composition	**Experimental diet**
Ingredients (% fed basis)	
Concentrated feed	17.03
Corn, ground	18.44
Corn gluten feed	7.74
Wheat bran	11.36
Brewers grain	8.32
Whole crop barley silage	15.90
Italian ryegrass	11.36
Tall fescue	6.81
Salt	2.00
Vitamin-mineral mix[1]	0.23
Limestone	0.81
Calculated chemical composition (% DM)	
Moisture	24.28
Crude protein	8.48
Crude fat	1.55
Crude fiber	13.89
Crude ash	5.36
Acid detergent fiber	17.95
Neutral detergent fiber	41.90
Non-fibrous carbohydrate	42.71
Total digestible nutrients	72.30

[1]Premix provided the following nutrients per kg of diet: Vitamin (Vit) A, 9,000,000 IU; Vit. D3, 2,100,000 IU; Vit. E, 15,000 IU; Vit. K, 2,000 mg; Vit. B1, 1,500 mg; Vit. B2, 4,000 mg; Vit. B6, 3,000 mg; Vit. B12, 15 mg; Pan-Acid-Ca, 8500 mg; Niacin, 20,000 mg; Biotin, 110 mg; folic-acid, 600 mg; Co, 300 mg; Cu, 3,500 mg; Mn, 55,000 mg; Zn, 40,000 mg; I, 600 mg; Se, 130 mg.

performance of Hanwoo steers is shown in Table 4. The ADG and feed efficiency of Hanwoo steers were significantly increased in the CPB dietary group relative to the control ($P < 0.05$). Dietary CPB supplementation significantly increased the serum IgG level, whereas the IgM level remained unaffected relative to the control group ($P < 0.05$) (Table 5).

Carcass characteristics and muscle composition

As shown in Table 6, the carcass weight of Hanwoo steers increased significantly in response to CPB supplementation relative to the control group ($P < 0.05$). No significant variation was observed in dressing out percentage, meat quality grade and carcass yield grade between groups. The moisture, crude protein, crude fat and crude ash content of the longissimus muscle of Hanwoo steers was unaffected by CPB supplementation (Table 7). A significant reduction of meat cholesterol concentration was observed in the CPB dietary group as compared to the control group ($P = 0.01$) (Table 7).

Fatty acid profile

The fatty acid profiles of the longissimus muscle of Hanwoo beef are shown in Table 8. An increased concentration of stearic acid (C18:0) in CPB dietary group as compared to the control ($P < 0.05$) was seen.

Among the monounsaturated fatty acids (MUFA), myristoleic acid (C14:1) increased and eicosenoic acid (C20:ln-9) decreased in response to CPB dietary supplementation relative to the control ($P < 0.05$). Supplementation of CPB increased ω-3 (α-linolenic acid, C18:3n-3 and eicosatrienoic acid, C20:3n-3) and ω-6 (arachidonic acid, C20:4n-6) fatty acids ($P < 0.05$). No significant variation was observed in total SFA and unsatu-

Table 3. Conditions applied for gas chromatography analysis of cholesterol and fatty acid profile.

Item	Condition	
	Cholesterol	**Fatty acid**
Instrument	DS 6200 (Korea)	DS 6200 (Korea)
Detector	FID 270°C	FID 270°C
Injector	Capillary ING 250°C	Capillary ING 250°C
Column	HP-5 (J&W, 30 m × 0.32 mm, 0.25 µm film thickness)	HP-5 (J&W, 30 m × 0.32 mm, 0.25 µm film thickness)
Carrier gas flow	Nitrogen (1.0 mL/min)	Nitrogen (1.0 mL/min)
Make up gas flow	H_2 (3.0 mL/min)	H_2 (3.0 mL/min)
Oven temperature	250°C	140°C
Detector temperature	280°C	270°C
Injector temperature	280°C	250°C
Temperature program	250°C (2 min) - 15°C/min - 290°C (10min) - 10°C/min - 310°C (10 min)	140°C (1 min) -10°C /min - 220°C (2min) -2°C /min - 240°C (9 min)
Split ratio	50:1	50:1
Injection volume	2 µL	1 µL

Table 4. Effect of citron (*Citrus junos* Sieb. ex Tanaka) probiotics (CPB) on growth performance of Hanwoo steers over 180 days[1].

Parameter	Treatment		Pooled S.E.[2]	P-value
	Control	**CPB**		
Initial body weight (kg/steer)	619.18	621.73	6.45	0.79
Final body weight (kg/steer)	750.30	762.52	5.32	0.13
ADG (kg/steer)	0.73	0.78	0.02	0.04
ADFI (kg DM/steer)	11.34	11.42	0.16	0.74
Feed efficiency (kg gain/kg DMI)	0.06	0.07	0.002	0.03

Significant difference ($P < 0.05$) or tend to differ ($P < 0.1$). [1]Data presented as the mean value of five replicate groups with two steers per replication (n=10). ADG = average daily gain; ADFI = average daily feed intake; [2]Pooled standard error.

Table 5. Effect of citron (*Citrus junos* Sieb. ex Tanaka) probiotics (CPB) on serum immunoglobulin level of Hanwoo steers[1].

Parameter (ng/ml)	Treatment		Pooled S.E.[2]	P-value
	Control	**CPB**		
IgM	3.55	3.35	0.23	0.54
IgG	184.82	243.11	4.34	<0.0001

Significant difference ($P < 0.05$) or tend to differ ($P < 0.1$). [1]Data presented as the mean value of five replicate steers for each treatment (n=5); IgM = immunoglobulin M; IgG = immunoglobulin G; [2]Pooled standard error.

rated fatty acid (UFA) concentration between treatments. Dietary CPB supplementation elevated the total concentration of n-3 PUFA ($P = 0.01$), while it reduced the ratio of n-6/n-3 fatty acids ($P < 0.05$).

Table 6. Effect of citron (*Citrus junos* Sieb. ex Tanaka) probiotics (CPB) on carcass characteristics and meat quality of Hanwoo steers[1].

Parameter	Treatment		Pooled S.E.[4]	P-value
	Control	CPB		
Carcass weight (kg)	454.10	476.18	6.68	0.04
Dressing out percent	60.56	62.46	1.02	0.21
Meat quality grade[2]	3.75	2.88	0.36	0.11
Carcass yield grade[3]	1.25	1.38	0.17	0.62

Significant difference ($P < 0.05$) or tend to differ ($P < 0.1$). [1]Data presented as the mean value of five replicate steers for each treatment (n=5); [2]Meat quality grades: 5 = 1++; 4 = 1+; 3 = 1; 2 = 2; 3 = 1 (According to KAPE, 2012); [3]Carcass yield grades: 3 = A; 2 = B; 1 = C grade; [4]Pooled standard error.

Table 7. Effect of citron (*Citrus junos* Sieb. ex Tanaka) probiotics (CPB) on longissimus muscle chemical composition (g/100 g) and cholesterol content of Hanwoo carcass[1].

Parameter	Treatment		Pooled S.E.[2]	P-value
	Control	CPB		
Moisture	61.94	60.67	1.87	0.65
Crude protein	20.91	19.18	0.93	0.25
Crude fat	16.39	19.27	2.10	0.37
Crude ash	0.90	0.88	0.04	0.80
Cholesterol (mg/100 g meat)	66.06	52.00	2.66	0.01

Significant difference ($P < 0.05$) or tend to differ ($P < 0.1$). [1]Data presented as the mean value of five replicate steers for each treatment (n=5); [2]Pooled standard error.

DISCUSSION

Growth performance and immunity

The use of feed additives of natural origin has been encouraged in human and animal nutrition over the last decade. The new approach of feeding Hanwoo steers with a combination of CJ fermented with beneficial probiotics strains (*L. acidophilus* KCTC 3111*, B. subtilis* KCTC 3239, S. cerevisiae KCTC 7915) is believed to adapt adapt the rumen to the presence of large quantities of lactic acid, either directly by feeding lactate-utilizing bacteria, or indirectly by feeding lactate-producing bacteria, which in turn stimulates the growth of lactate-utilizers (Krehbiel et al., 2003).

In the present study, dietary CPB supplementation significantly increased the ADG and feed efficiency of Hanwoo steers as compared to the control (Table 4). These findings are in agreement with those of Bueno et al. (2002), who reported the increased daily gain, dry matter intake and feed conversion efficiency in response to supplementation of the diets of growing kids with dehydrated citrus pulp. Supplementation of lactate-producing and/or lactate-utilizing bacteria has been shown to improve daily gain and feed efficiency of feedlot cattle (Swinney-Floyd et al., 1999; Galyean et al., 2000). During the fermentation process, lactic acid bacteria act on water-soluble carbohydrates in feed particles to produce a number of products, primarily lactic acid. This results in a rapid reduction of pH, protecting the microbes and preserving the maximum level of nutrients in the products (Merry and Davies, 1999).

The yeasts *S. cerevisiae* (Kim et al., 2010b) and *B. subtilis* (Nimker et al., 2010) can produce extracellular enzymes such as α-amylase, which stimulate starch hydrolysis and the growth of lactic acid bacteria. Direct fed microbial (DFM) has been reported to alter the efficiency of production as well as proportional concentrations of volatile fatty acids in the rumen (Kmet et al., 1993). If propionate production is both energetically and proportionately increased by DFM, then it is likely that the energy available to the animal also increase (Elam et al.,

Table 8. Effect of citron (*Citrus junos* Sieb. ex Tanaka) probiotics (CPB) on longissimus muscle fatty acid compositions in Hanwoo steers[1].

Fatty acid (% total fatty acid)	Treatment		Pooled S.E.[2]	P-value
	Control	CPB		
Myristic acid (C14:0)	3.02	3.65	0.26	0.14
Palmitic acid (C16:0)	27.21	26.32	1.72	0.72
Stearic acid (C18:0)	10.68	14.7	1.12	0.05
Myristoleic acid (C14:1)	0.29	1.14	0.04	<.0001
Palmitoleic acid (C16:ln7)	4.45	4.32	0.14	0.60
Oleic acid (C18:l n-9)	51.27	46.73	2.30	0.22
Eicosenoic acid (C20:l n-9)	0.51	0.27	0.06	0.03
Linoleic acid (C18:2 n-6)	2.32	2.41	0.14	0.69
α-Linolenic acid (C18:3 n-3)	0.04	0.09	0.01	0.04
Linolenic acid (C18:3 n-3)	0.10	0.16	0.03	0.24
Eicosatrienoic acid (C20:3 n-3)	0.06	0.11	0.01	0.05
Arachidonic acid (C20:4 n-6)	0.05	0.10	0.01	0.01
ΣSFA	40.92	44.67	2.71	0.36
ΣUFA	59.09	55.34	2.10	0.26
UFA/SFA	1.46	1.27	0.12	0.31
ΣMUFA	56.23	51.33	2.22	0.17
ΣPUFA	2.57	2.88	0.14	0.19
Σn-3	0.20	0.36	0.02	0.01
Σn-6	2.37	2.51	0.15	0.54
n-6/n-3	12.25	7.11	1.28	0.04
MUFA/SFA	1.38	1.17	0.12	0.26
PUFA/SFA	0.06	0.06	0.00	0.85

Significant difference ($P < 0.05$) or tend to differ ($P < 0.1$). [1]Data presented as the means of five replicate steers for each treatment (n=5). ΣSFA = total saturated fatty acid; ΣUFA = total unsaturated fatty acids; ΣMUFA = total monounsaturated fatty acids; ΣPUFA = total poly unsaturated fatty acids; Σn-3 = total n-3 PUFA; Σn-6 = total n-6 PUFA. [2]Pooled standard error.

2003). Accordingly, the growth performance observed in the present study may have been due to the synergistic effects of CJ components and probiotics metabolites (Hossain et al., 2012). The increased feed efficiency might be the result of increased ADG in CPB dietary group.

The increased IgG concentration in the CPB dietary group relative to the control (Table 5) might have been due to the combined effects of CJ immunomodulatory compounds and fermentation output with beneficial probiotics strains. Flavonoids, a major bioactive component of citrus fruits can act on immune responses via different mechanisms such as protein binding, active site interference or antioxidant effects (Provenza and Villalba, 2010). Besides, DFM have been shown to affect the innate, humoral and cellular arms of the host immune system (Krehbiel et al., 2003). A bi-directional communication pathway between the immune and endocrine system supports the health and optimal growth of animals (Carroll, 2008). Feed fermented with probiotics showed increased levels of lactic acid and other organic acids, stimulating the specific and non-specific immune function and modulating the composition of the intestinal microbial population against harmful organisms (Van der Wielen et al., 2000).

Supplementation of the diets with CPB resulted in an increased carcass weight of Hanwoo steers as compared to the control (Table 6). The heavier carcass weight in the CPB dietary group was likely due to the increased slaughter weight of animals, since carcass measurements are significantly affected by the slaughter weight (Mohammed et al., 2007). No significant effect of dietary CPB was observed on dressing out percent, meat quality grade and carcass yield grade in the present study (Table 6), which is consistent with the findings of Henrique et al. (2006). Caparra et al. (2007) also reported that lambs fed diet containing solar-dried citrus pulp had similar carcass weight and meat quality grade as the control.

Supplementation of CPB had no significant effect on proximate components of longissimus muscle in the present study (Table 7). The results of our study are in agreement with the findings of Henrique et al. (2006) who also found no significant variation in beef chemical composition when supplemented with dehydrated citrus pulp pellets in finisher bull. In the present study, CPB supplementation significantly reduced the meat cholesterol concentration as compared to the control (Table 7), which might have been due to the combined effect of flavonoids in CJ and multiple probiotics strains activity. Paengkoum et al. (2011) observed a reduced meat cholesterol concentration when *Lactobacillus* and *Saccharomyces* probiotics fermented feed were supplemented in broilers and goats.

Fatty acid profile

Tissue fatty acid composition is affected by the amount and structure of dietary fat, *de novo* fatty acid synthesis, rate of conversion to other fatty acids and metabolites, and the proportion of oxidation used for energy consumption (Nuernberg et al., 2005). Meat fatty acid profile and cholesterol concentration are considered as the pivotal role in human diet as they have hypercholesterolemic properties, which are associated with coronary heart diseases (Daley et al., 2010), while dietary n-3 fatty acids including α-linolenic acid and its metabolites reduce the risk of heart disease (De Lorgeril et al., 1994). The increased concentrations of SFA (stearic acid) and UFA (myristoleic acid, α-linolenic acid, eicosatrienoic acid, arachidonic acid) in CPB dietary group (Table 8) supported the findings of Mourão et al. (2008). They reported palmitic and stearic acids as the predominant fatty acids found in chicken meat as SFA, whereas linoleic and arachidonic acid as PUFA when supplied citrus by-product in broiler diet.

The increased concentration of stearic acid of our study may have little effect on human health. Hu et al. (1999) reported that the major SFA within beef (myristic acid, palmitic acid and stearic acid) is significantly associated with coronary heart disease, however others argue that a distinction should be made for stearic acid which has been found to have little cholesterol-raising effects in humans as compared to myristic and palmitic acid (Grande et al., 1970; Kelly et al., 2002; Mensink et al., 2003). Zock and Katan (1992) also reported that stearic and linoleic acids to be more or less equivalent with respect to their cholesterolemic effect. The increased concentration of total n-3 PUFA and the decreased ratio of n-6/n-3 in CPB dietary supplementation might have been due to the synergism of probiotic strains and CJ (Table 8). Supplementation of dietary citrus pulp increased meat PUFA concentration in broiler (Mourão et al., 2008).

Paengkoum et al. (2011) found a reduced ratio of n-6/n-3 fatty acid in meat when supplemented with *Lactobacillus* and *Saccharomyces* fermented feed in broilers and goats. Dietary probiotics such as *Lactobacillus* and *Saccharomyces* can reduce the SFA concentration and increase the linolenic and total PUFA contents in pig meat through a positive effect on the intestinal flora (Ross et al., 2012).

Conclusion

Based on the results of this trial, the use of CPB significantly improved the daily gain and feed efficiency of Korean native steers. Dietary CPB increased body immunity and carcass weight of the Hanwoo steers, and significantly reduced the meat cholesterol concentration without any significant variation in total SFA or UFA concentration between treatments. The increased content of n-3 PUFA and a reduced ratio of n-6/n-3 PUFA will build up the confidence of beef consumers because it will minimize the risk of cardiovascular diseases. Therefore, CPB can be used as a functional feed additive for Hanwoo steers to improve growth performance and immunity, as well as to produce functional beef for human consumption.

Conflict of Interests

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

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Full Length Research Paper

Massive mortality associated with *Streptococcus iniae* infection in cage-cultured red drum (*Sciaenops ocellatus*) in Eastern China

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In August, 2011, an enzootic disease characterized by hemorrhage throughout body surface, enlarged spleen and kidney occurred in cage farmed red drum fish, in Dongtou, Zhejiang of China. The diseased fish weighed between 100 to 200 g, and the cumulative mortality within 60 days was higher than 70%. Several bacterial isolates that exhibited the same phenotypic traits and biochemical characteristics were isolated from the lesions of diseased fish. According to the results obtained from biochemical tests and sequence analysis of the 16S rDNA, the disease's pathogen (strain WZMH110819) was identified as *Streptococcus iniae*. In the challenge trials, the LD_{50} value of the clinical bacterial isolate WZMH110819 was 9.65×10^6 CFU per fish. Moreover, bath exposure or oral administration by *Streptococcus iniae* also caused a considerable number of deaths in fish. Antibiotic susceptibility tests showed that strain WZMH110819 was sensitive to most of the antibiotics including ampicillin, erythromycin and gentamicin *in vitro*. This finding has provided a basis for the control and prevention of further outbreaks of this enzootic disease in red drum farms.

Key words: *Streptococcus iniae*, *Sciaenops ocellatus*, biochemical characterization, 16S rDNA analysis.

INTRODUCTION

Streptococcal infections in fish have become an increasingly important health problem in modern intensive aquaculture (Eldar et al., 1995a). The main pathogenic species responsible for these streptococcal infections include *Streptococcus iniae*, *Streptococcus agalactiae*, *Streptococcus parauberis* and *Streptococcus dysgalactiae* (Eldar et al., 1995a; Domenech et al., 1996; Bercovier et al., 1997; Nomoto et al., 2004). *S. iniae* was firstly isolated from the subcutaneous abscesses in Amazon freshwater dolphins *Inia geoffrensis* (L.) in aquariums (Pier and Madin, 1976), and then large epidemic diseases in fish associated with this pathogen occurred. The most significant clinical signs of *S. iniae* infections in fish are septicemia and meningoencephalitis which are quite similar to signs caused by other streptococcal pathogens of fish (Eldar et al., 1994,

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1995a, 1995b; Eldar and Ghittino, 1999). More importantly, humans can also be infected by *S. iniae* and a number of human cases have been reported in North America and Asia (Weinstein et al., 1997; Lau et al., 2003; Koh et al., 2004; Lau et al., 2006).

The red drum (*Sciaenops ocellatus*) is one of the most important economic fish species and it was introduced in China in 1991 (Shen et al., 2005). In a few decades, bacterial infections in red drum have become an increasingly important health problem because of the modern intensive aquaculture. *Streptococcosis* is one of the most common bacterial diseases in cultured red drum worldwide (Eldar et al., 1999; Colorni et al., 2002; Shen et al., 2005). Recently, an infectious disease outbreak with high mortality occurred in cage farmed red drum, causing severe economic impact on fisheries in Dongtou, Zhejiang province, China.

The aim of the present study was to isolate, identify and characterize the pathogenic bacteria of enzootic disease in red drum as well as to determine some potential drug candidates. The results obtained upon completion of this research can be taken from the laboratory into the field as a guideline for assisting red drum cage-cultured fish farmers for the control and the prevention of further streptococcal disease outbreak.

MATERIALS AND METHODS

Bacterial isolation and biochemical characterization

In August, 2011, an infectious fish disease occurred among the red drum cage farmed fish in Dongtou, Zhejiang province China. More than 80 moribund fish with an approximate weight of 100 to 200 g were subjected to bacteriological examination. Several pure colonies of bacterial isolates (Strain WZMH110819) from liver, spleen and head kidney tissues of the diseased fish were recovered on Brain Heart Infusion Agar (BHIA; HuanKai, China) supplemented with 50% distilled sterile seawater. The sampling was conducted over a conservative two days period of high mortality rate. For the isolation of the etiological pathogen, tissues of liver, spleen and kidney from affected fish were streaked across the Brain Heart Infusion Agar (BHIA; HuanKai, China) supplemented with 50% distilled sterile seawater. The inoculated plates were incubated at 28°C for 24 h. Single colonies from plates with virtually pure culture growth were re-streaked onto the same media to obtain pure isolates. All pure isolates were cultured in liquid BHI and then add 20% glycerol for preservation at -80°C.

Bacterial identification was conducted using a number of biochemical and phenotypical tests by the API20 STREP kits (BioMerieux, France), according to the manufacturer's instructions. For the detection of hemolysis, the bacterial isolate was cultured overnight on trypticase soy agar supplemented with 5% defibrinated sheep blood (Toocle co., Hangzhou, China) at 28°C. The destruction of red blood cells (zones of beta-hemolysis) in the blood agar was detected prior incubation. The gram staining procedure for the bacterium followed the standard protocol.

16S rDNA amplification and sequence analysis

Total genomic DNA was extracted using a genomic DNA extraction Kit (Takara). The conserved primers used for the amplification of 16S rDNA were reported previously (Edward and Ewing, 1986): 8F 5'-AGAGTTTGATCCTGGCTCAG-3' and 1542R 5'-AAGGAGGTGATCCAGCCGCA-3'. The PCR mixture contained bacterial DNA, PCR buffer (10 mM Tris-HCl, pH 8.8, 50 mM KCl, 2 mM $MgCl_2$, 0.08% Nonidet P40), a 200 µM concentration of each deoxynucleoside triphosphate, 0.1 µM of each primer and 1.5 U of Taq polymerase (Takara). The thermocycling parameter used for this conserved primer set was 30 cycles of 94°C for 1 min, 56°C for 1 min and 72°C for 2 min, and a final extension at 72°C for 5 min in an automated thermal cycler (PTC-100, Bio-RadTM, USA).

The amplification products were gel-purified using PCR purification kit (Omega) and sequenced using the same primers as for PCR. Sequence was aligned using Clustal X version 2.1 followed by refinement by eye. Phylogenetic analysis of the 16S rDNA sequence was performed with Molecular Evolutionary Genetics Analysis (MEGA) software version 5.05 with the two-parameter of Kimura model for DNA based on the Neighbour Joining (N to J).

Experimental infection trials

Healthy red drum fish (102.3±15.5 g) were divided into 10 groups (n=10 for each group) and then were acclimatized in tanks (400 l) for 7 days at 30 ± 2°C. Before experimental infection, three fish selected randomly were subjected to bacteriological examination for confirming the fish not to be infected with bacteria. To determine the virulence of the bacterial isolate WZMH110819, four groups were injected intraperitoneally with the bacterium suspension at the doses of 1.93×10^6, 1.93×10^7, 1.93×10^8 and 1.93×10^9 CFU per fish respectively and one group of fish was injected intraperitoneally with PBS for negative control. The fish mortalities were recorded daily for 14 days after the challenge, and the LD_{50} value was calculated by using the Reed-Muench method (Reed and Muench, 1938).

To investigate the infection routes of this bacterial pathogen, fish were also exposed to bacterium through oral or bath exposure. For oral administration, two group of normal fish (n=10) were challenged by oral admission of 1 ml of bacterial suspension at the doses of 1.93×10^6 and 1.93×10^8 CFU per fish, respectively. For bath exposure, normal fish or fish undergoing epidermal scarification were removed from their tanks and placed into a container of seawater containing 1.93×10^7 CFU/ml bacteria. After an exposure period of 30 min, the fish were removed and placed into their respective tank. Controls used for every method of challenge were subjected to identical handling procedures without being exposed to the isolated bacterium. Bacterial concentrations administered in the artificial experimental trials were evaluated using the indirect viable cell counts method as previously described (Todar, 2009). Briefly, a bacterial suspension was made from bacterial isolate grown on Brain Heart Infusion Agar (BHIA; HuanKai, China) for 24 h at 28°C were serially tenfold diluted with sterile phosphate-buffered saline to 10^8. Each dilution was then plated to BHIA to determine cells in each dilution. Each viable unit grows and forms of a colony were counted prior incubation.

Antimicrobial susceptibility testing

The antimicrobial susceptibility of the bacterial isolate WZMH110819 was determined using the standard Kirby-Bauer method (Bauer et al., 1966). Briefly, a suspension (0.1 ml) of the bacterial strain WZMH110819 (diluted to a turbidity equivalent of a Macfarland No. 0.5 standard solution) was spread onto Mueller-Hinton agar and antimicrobial agent paper discs (Hangzhou Tianhe Microorganism Reagent) were then added to the surface of the agar.

Figure 1. Diseased red drum fish showing (A) enlarged kidney and spleen, (B) cornea opacity or POP eye, (C) hemorrhages on the base of fins and (D) dorsal and tail fin erosion.

RESULTS

Bacterial isolation from diseased fish

Diseased fish exhibited emaciation, lethargy, abnormal swimming, exophthalmia and hemorrhages on the base of fins. Internally, congestive kidney and spleen were observed in diseased fish (Figure 1). Multiple small bacterial colonies (strain WZMH110819) were isolated from different lesions of the diseased red drum.

Phenotypic traits and biochemical characteristics of the bacterial isolates

These bacterial isolates formed whitish colonies that were smooth around the edges across the BHIA plates and all strains were positive for β-hemolysis on blood agar plate. These isolates were all Gram-positive streptococcus-shaped bacteria in optical microscope. Moreover, all bacterial isolates and reference *S. iniae* showed the same biochemical characteristics by using the API 20 STREP kits (Table 1).

16S rDNA sequence analysis

The universal 16S rDNA primers set used in this study yielded the expected 1541 bp amplicon for bacterial strain WZMH110819. Sequence analysis showed that bacterial strain WZMH110819 showed 100% similarity with the reference *S. iniae* strain ATCC 29178^T, but showed 98% similarity with *S. parasuis* and 97% similarity with *S. agalactiae* and *S. dysgalactiae,* respectively. The phylogenetic analysis based on the 16S rDNA sequence (accession numbers: KF815728) also showed that the bacterial strain WZMH110819 clustered most clearly with *S. iniae* strain ATCC 29178^T (Figure 2).

Result of experimental infections

In challenge trails, the bacterial isolate WZMH110819 exhibited moderate virulence to the red drum with an intra-peritoneal LD_{50} value of 9.65×10^6 CFU per fish. Depending on the amount of doses challenged, mortality rate ranged from 20 to 100% was recorded in our study (Figure 3). The cumulative mortality of each intraperitoneal

Table 1. Biochemical Profile of strain WZMH110819 and references *S.iniae*

API 20 STREP test	Strain *WZMH110819*	*S. iniae*ATCC 29178[a]	*S. iniae*SO-2[b]
Oxidase	-	-	N.A
Vogus-proskauer	-	-	-
H_2S production	-	-	N.A
Indole production	-	-	N.A
Citrate utilization	-	-	N.A
β-Galactosidase	+	+	N.A
Arginine dihydrolase	+	+	+
Lysine decarboxylase	-	-	N.A
Ornithine Decarboxylase	-	-	N.A
Tryptophane deaminase	+	+	N.A
Urase	-	-	-
Gelatinase	-	-	N.A
Glucose	+	+	+
Mannitol	+	+	+
Inositol	-	-	N.A
D-sorbitol	-	-	N.A
Rhamnose	-	-	N.A
Sucrose	+	-	+
Melibiose	-	-	-
Amygdalin	-	-	N.A
L-arabinose	-	-	N.A

+, Positive; -, negative; [a]data was from Pier and Madin 1976; [b]data was from Shen et al., 2005; N.A, Not available.

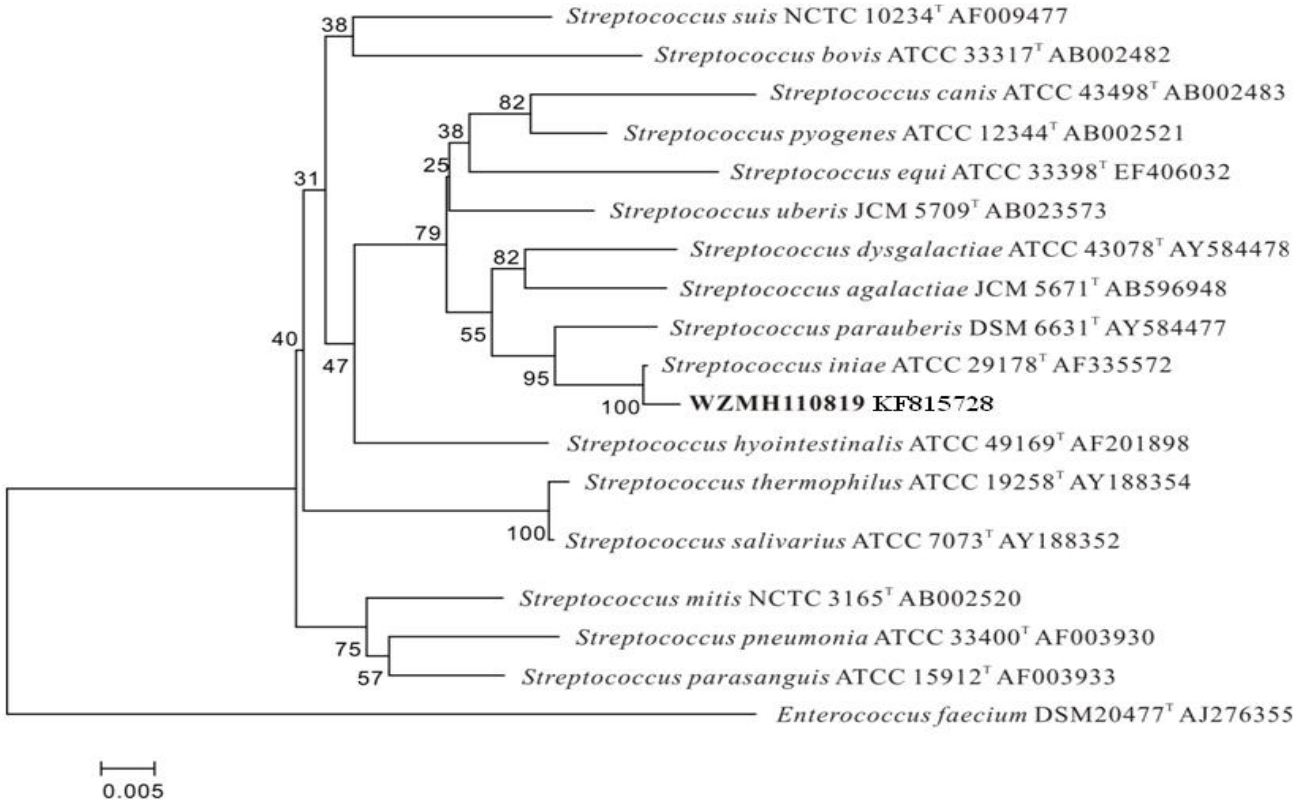

Figure 2. Neighbor-joining phylogenetic tree of 16S rDNA sequences of bacterial isolate WZMH110819, reference *S. iniae* and the most closely related species of streptococci.

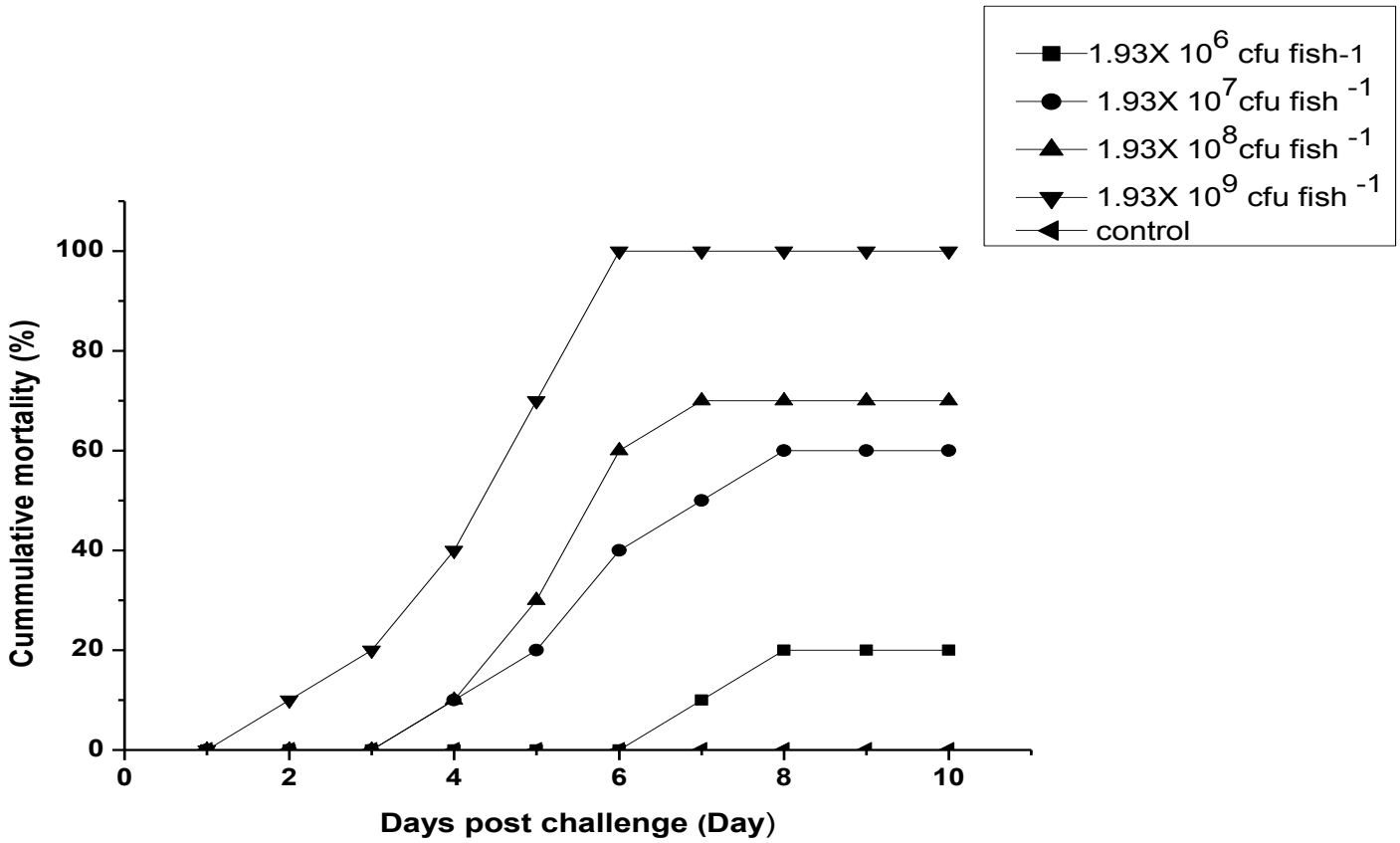

Figure 3. Mortality rate of red drum intraperitoneally injected with various concentration of S. iniae isloate WZMH110819.

challenge group or negative control is showed in Figure 4. Bath exposure with the bacteria isolate WZMH110819 after skin trauma resulted in 40% mortality for 1.93×10^7 CFU per fish, in contrast to 0.0 % mortality for 1.93×10^7 CFU per fish with normal pathogen exposure. For oral administration, the mortality was 10.0% for 1.93×10^6 CFU per fish and 40% for 1.93×10^8 CFU per fish (Figure 4).

The typical clinical signs of the moribund fish in experimental trails were hemorrhages on the base of fins, lethargy, abnormal swimming and enlarged and congestive visceral organs which were similar to naturally infected fish.

Antimicrobial susceptibility results

Antimicrobial susceptibility results showed that the bacterial isolate WZMH110819 was susceptible to 19 of 20 antibiotics tested in present study, including ampicillin, chlorophenicol, erythromycin, cefazolin, piperacilin, cefoperazone, ceftzaidime, sultamicillin, cefepime, streptomycin, kanamycin, amikacin, gentamicin, tetracycline, doxycycline, norfloxacin, ciprofloxacin, imipenem and trobramycin, but showed resistance to SMZ+TMP (Table 2).

DISCUSSION

Red drum is one of most important economic fish species mainly farmed in Zhejiang and Fujian provinces, China. During 2011 to 2012, massive mortality in cage cultured red drum occurred in Dongtou fish farms, Wenzhou, Zhejiang province. The outbreak of the disease occurred in breeding fish which were almost one year of age (approximately 100 to 300 g) when the water temperature was up to 29°C. However, the large yellow croaker, another important economic native fish cultured in the same coastal region, was not affected by this epidemic disease. The diseased or moribund red drum exhibited skin lesions, exophthalmia, disorientation and hemorrhage around the tail and anal fin. Moreover, there were some gram-positive streptococcus-like bacteria observed in the tissue smears.

The bacterial strain isolated from the diseased fish was G. positive, cocci-chain and oxidase-negative. On the basis of these results and those of the API20 STREP kits (BioMerieux, France) listed in Table 1, the isolate was identified as *S. iniae*. The bioche-mical characters of the *S. iniae* strain WZMH110819 were similar to those results reported previously by other researchers (Pier and Madin, 1976; Shen et al., 2005; Zhou et al., 2008).

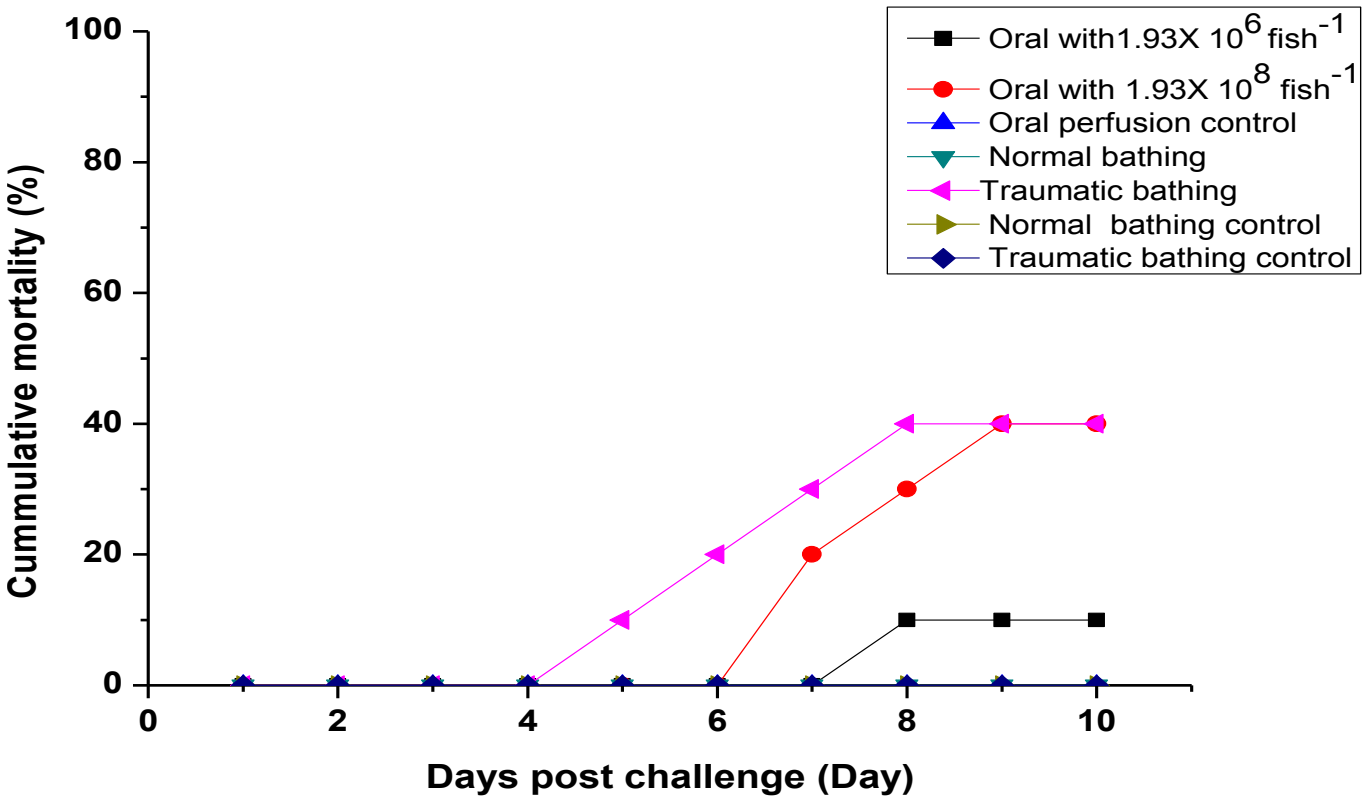

Figure 4. Mortality rate of Red drum exposed to bacterial strain WZMH110819 in deploys of natural infection trials via bath treatment or oral administration.

Table 2. Results of disc diffusion drugs susceptibility testing

Name of antibiotic	Paper content (μg/disc)	Inhibitory zone diameter (mm)	Sensitivity
Ampicillin	10	30	+
Chloramphenicol	30	25	+
Erythromycin	15	30	+
Cefazolin	30	23	+
Piperacilin	100	22	+
Cefoperazone	75	25	+
Ceftzaidime	30	23	+
Sultamicillin	10/10	25	+
Cefepime	30	31	+
Streptomycin	10	24	+
Kanamycin	30	28	+
Amikacin	30	25	+
Gentamicin	10	25	+
Tetracycline	30	26	+
SMZ+TMP	23.75	10	-
Doxycycline	30	26	+
Norfloxacin	10	28	+
Ciprofloxacin	5	32	+
Imipenem	10	43	+
Trobramycin	10	19	+

+, Sensitive (S); -, resistance (R).

Meanwhile, this bacterium had ability hydrolyze arginine, suggesting that it may share the same serotype (serotype I) that have been previously reported by other researchers (Pier and Madin, 1976; Shen et al., 2005; Zhou et al., 2008).

Infections associated with *S. iniae* in red drum in Zhejiang province has been reported early in 2005 (Shen et al., 2005). However, the epidemic reported by Shen occurred from September to December and the cumulative mortality was 20 to30%. Moreover, the *S. iniae* isolates seemed of low or moderate virulence with the intra-peritoneal LD_{50} value of 4.18×10^8CFU per fish and 1.19×10^7CFU per fish (100-120 g), respectively, according to the report (Shen et al., 2005). In the present study, the epidemic broke out from July to September, and the cumulative mortality was up to 70%. Experimental infection trials showed that the clinical isolate WZMH110819 exhibited moderate virulence to the red drum with an intra-peritoneal LD_{50} value of 9.65×10^6 CFU per fish (102.3±15.5 g) in the current study. This result suggested that the *S. iniae* strain WZMH110819 might be more virulent than the strains isolated by Shen et al. (2005).

Previously, the study reported that epidermal scarification did not appeared to enhance the ability of *S. iniae* to enter the barramundi and increase mortalities over traumatized fish (Bromage and Owens, 2002). However, it showed that the mortalities of the red drum were up to 40% in bath exposure group undergoing epidermal scarification in the current study, compared to normal bath exposure or oral exposure.

Oral administration and bath exposure experiments, which indicated that the red drum were more likely to be infected with *S. iniae* via skin trauma. The average stocking density of red drum fish in Dongtou cage farms were about 20 to 25 fish per m^3. High stocking density or close contact between the fish themselves may damage the integrity of their epidermal and protective mucosal layers, consequently predispose them to *S. iniae* infection (Shoemaker et al., 2001). Thus, the frequent outbreaks of this enzootic disease in this area may be due to high stocking rates and feeding the affected fish meal (Zhou et al., 2008).

Several investigators have reported that some *S. iniae* strains exhibited resistance to several commonly used antimicrobial drugs. Shen et al. (2005) has reported that the *S. iniae* isolates from red drum fish developed some resistance to gentamycin, lincomycin and norfloxacin. Similarly, Tran et al. (2013) has reported that *S. iniae* isolated from in diseased barramundi (*Lates calcarifer*) cultured in Vietnam was sensitive to several antibiotics including norfloxacin, ciprofloxacin, sulphamethoxazol / trimethoprim, ampicillin, erythromycin and doxycycline but was resistant to gentamicin, and streptomycin. In present study, the bacterial isolate WZMH110819 was sensitive to most of antibiotics tested including gentamicin and norfloxacin *in vitro*, but showed resistance to sulfamethoxazole compound (SMZ+TMP). This result suggests that antibiotics therapy would be effective in treating fish affected by this pathogen.

The results of the present study demonstrates that the massive mortality in red drum in Dongtou fish farms was due to infection with *S. iniae*, and the epidermal scarification might enhance the pathogens ability to enter the fish and increase mortalities. This important finding would suggest that reduced handling and decreased the stocking density might be an important means of preventing problems caused by this pathogen.

Conflict of Interests

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

ACKNOWLEDGEMENTS

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Full Length Research Paper

Associations of cytomegalovirus with type I diabetes mellitus among children in Khartoum State

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Cytomegalovirus is one of the most common microorganisms that cause opportunistic infection that complicate the clinical care and progress of immunecompromised patients. The virus can cause severe diseases with multiple complications including type I diabetes mellitus. The present study is a case control study aimed at determining cytomegalovirus among type I diabetic mellitus in Sudanese children. Sera of eighty one (81) children were collected, 27 (33.3%) from diabetic which represent the study group and 54 (66.7%) from apparently healthy children (control group). The samples were tested for IgG anti-cytomegalovirus using enzyme-linked immunosorbent assay (ELISA) technique. 18 (22.2%) of the total population of study were sero-positive for cytomegalovirus IgG, most of them, 10 (55.6%) were diabetic patients, the results indicate significant association (*P* value 0.025) of cytomegalovirus IgG antibodies with type I diabetes mellitus in children. The study reveals significant relation (*P* value 0.003) of cytomegalovirus IgG antibodies with type I diabetes mellitus in age group (5-9 years).

Key words: Cytomegalovirus, type I diabetes mellitus, children.

INTRODUCTION

The incidence of diabetes mellitus is rising continuously all over the world, and this may be due many reasons that can collectively or separately lead to the disease. These factors include genetic factors, obesity, autoimmunity disorder and infection (Brickell et al., 2007). Certain viruses can infect human and may cause diabetes mellitus through different mechanism such as pancreatitis or hepatitis and their subsequent complications (WHO, 1999; Elhawary et al., 2011). Cytomegalovirus (CMV) is one of the most important factors that is thought to be associated with type I diabetes mellitus owing to its ability to induce immunological beta cells (β-cells) damage (Aarnisalo et al., 2008). CMV is an ubiquitous herpes group virus causing chronic life-long infection in affected participants. It is a widely distributed virus, belonging to betaherpesvirinae, subfamily of herpesviridae. The virus is enveloped and double stranded deoxyribonucleic acid (DNA) with icosahedral

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coat (Aarnisalo et al., 2008; Jawetz et al., 2007).

Molecular mimicry is one of the principal immunological mechanisms that lead to destruction of the pancreatic β-cells. This mimicry could be involved in cytomegalovirus-induced diabetes by inducing islet cell autoantibodies. The loss of T-cell tolerance to self (GAD65) may be due to processing and presentation of molecularly mimic cytomegalovirus protein pUL57 by dendritic cells (Hiemstra et al., 2001).

Interferon (INF) and natural killer cells (NK) play an important role in defense and clearance of cytomegalovirus during primary infection. The virus possesses several proteins called unique long protein (UL) such as UL16, UL40, UL140, UL141 and UL142; all of them play roles in NK cells down modulation (Knipe and Howley, 2007; Tomasec et al., 2005). Moreover, cytomegalovirus encodes others genes for glycoprotein called unique short protein (US) such as US2, US3, US6 and US11 that down regulate cell surface expression of major histocompatibility antigens class I (MHC class I), also US2 and US3 have the same impact on MHC class II expression.

Besides these genes, the virus can produce two types of interleukin 10 (IL-10); cytomegalovirus interleukin-10 (cmvIL-10) that during replication, and cytomegalovirus interleukin-10 that during latency (LAcmvIL-10), and both have the ability to down regulate the T-cells function (Jenkins et al., 2004). However, nucleoside analogue with a modified pentose such as foscarnet, a pyrophosphate analogue and ganciclovir have anti-cytomegalovirus activity and are used in clinical care for both prophylaxis in transplantations and in suppressive treatment in active infections (Jawetz et al., 2007).

In previous studies, results on the association of CMV infection with type 1 diabetes have been contradictory; Nicoletti et al. (1990) reported a significant association between high titer of anti-cytomegalovirus and anti-islet cell antibodies. Hjelmesaeth et al. (2004) showed that asymptomatic cytomegalovirus infection is associated with increased risk of new-onset type I diabetes and impaired insulin release after renal transplantation, whereas Chen et al. (2012) found that the CMV seropositivity is significantly associated with various indicators of glucose regulation and therefore CMV infection might be a risk factor for the development of type 2 diabetes in the elderly.

In contrast to these findings, Hiltunen et al. (1995) did not find any correlation between the presence of anti-cytomegalovirus IgG antibodies and anti islet cell antibodies in children with newly diagnosed type I diabetes mellitus. Several unpublished studies were carried in Sudan to determine Cytomegalovirus among different groups, these studies indicated high rate of immunoglobulin class G (IgG). It was 72.2% in pregnant women in western Sudan (Hamdan et al., 2011), while Redwan et al. (2011) conducted study in Jeddah city, Saudi Arabia to determine the prevalence of cytomegalovirus (CMV) infection among foreign manpower and it was 87.8% among Sudanese. However, the present study aimed to determine the associations of Cytomegalovirus infection with type I Diabetes mellitus among children in Khartoum state, Sudan.

MATERIALS AND METHODS

This was an analytical biomedical case control study, the sample size was calculated by Open Epi statistical program for case control study, and it was found to be 81 candidates, 27 diabetic patients representing the study group and 54 healthy people representing control group.

The samples of study group were collected from the candidates diagnosed with diabetes mellitus type I, aged between five and fifteen years, regardless of the history of diabetes mellitus of their families, whereas the samples of control group were collected from the apparently healthy children of the same age group, regardless of the family history of diabetes mellitus. The candidate who had a history of cytomegalovirus infection after being diabetic was excluded; the study population was asymptomatic.

Using sterile disposable vacationer container, about 5 ml of blood were drawn from the anticubital vein under aseptic conditions. The blood samples were poured in sterile plain containers, and left to clot at room temperature. The clotted samples were centrifuged at 1500 rpm for 5 min in order to separate the sera, the obtained sera samples were kept frozen at -20°C until used. ELISA technique (EUROIMMUN™) was applied in this study to detect the IgG anti-cytomegalovirus.

The information related to the study such as age, gender and family history of diabetes mellitus were collected via direct interview. Chi squire test and frequencies was used to analyze the data by statistical package for social studies (SPSS) program.

The ethical considerations and conformity to individuals were considered by agreement and signature of children's parent and the study was approved by the Ethics Committee, Faculty of Graduate Studies, Alzaeim Alazhari University, Sudan.

RESULTS

Eighty one (81) children participated in this study, 27 (33.3%) represented study group (diabetic children) and 54 (66.7%) were apparently health children representing the control group, 32 (39.5%) of study population were male and 49 (60.5%) were female, 42 (51.9%) of the them were categorized in age group of 5-9 years, while the rest 39 (48.1) were sorted in group of 10-15 years (Table 1).

The IgG antibodies against cytomegalovirus were detected in 18 (22.2%) of the study population; the seropositive of cytomegalovirus was slightly higher in female, 11 (22.4%) than male 7 (21.9%). However, the majority of positive cases 10(55.6%) were diabetic patients, that is, belonging to study group. The positive rate of IgG against cytomegalovirus in the study group (diabetic patients) was 37% while it was 14.8% among control group, this result indicated statistically significant association between IgG antibodies of cytomegalovirus and diabetes mellitus type I (*P* value 0.025; Table 1). Also significant association (*P* value of 0.003) was

Table 1. showed statistical relation of study population (study group and control group), gender, age groups, and family history of Diabetes Mellitus against sero-diagnosis IgG of anti-cytomegalovirus.

Variable		Frequency	CMV (IgG)		P value	Odd ratio	CI (95%)	
			Positive	Negative			Lower	upper
Study population	Study group	27(33.3%)	10(37%)	17(63%)	0.025	3.382	1.145	9.994
	Control group	54(66.7%)	8(14.8%)	46(85.2%)				
Gender	Male	32(39.5%)	7(21.9%)	25(78.1%)	0.588	0.976	0.331	2.830
	Female	49(60.5%)	11(22.4%)	38(77.6%)				
Age group (year)	5-9	42(51.9%)	7(16.7%)	35(83.3%)	0.163	0.509	0.175	1.484
	10-15	39(48.1%)	11(28.2%)	28(71.8)				
Family history of DM	Yes	3(3.7%)	2(66.7%)	1(33.3%)	0.123	7.750	0.660	90.945
	No	78(96.3%)	16(20.5%)	62(79.5%)				
CMV (IgG)	Positive	18(22.2%)	-	-	-	-	-	-
	Negative	63(77.8%)	-	-				

The median age was 7.1 and 7.9 years for study and control groups, respectively. Chi squire test and frequencies was used to analyze the data; P value <0.05 considers significant. Odd ratio >1 indicates strong association. CI 95% means confidence interval at level of 95%.

Table 2. Statistical relation of study group, gender, age groups, and family history of diabetes mellitus against sero-diagnosis IgG of anti-cytomegalovirus.

Variable		CMV IgG		Total	P value	Odd ratio	CI (95%)	
		Positive	Negative				Lower	Upper
Age group 5-9 years	Study group (diabetic)	5(11.9%)	4(9.5%)	9(21.4%)	0.003	19.375	2.777	135.163
	Control group	2(4.8%)	31(73.8%)	33(78.6)				
Total		7(16.7)	35(83.3%)	42(100%)				
Age group 10-15 years	Study group (diabetic)	5(12.8%)	13(33.3%)	18(46.2%)	0.620	0.962	0.237	3.899
	Control group	6(15.4%)	15(38.5%)	21(53.8%)				
Total		11(28.2%)	28(71.8%)	39(100%)				
Gender	Male	2(7.4%)	7(25.9%)	9(33.3%)	0.244	0.357	0.058	2.217
	Female	8(29.6%)	10(37%)	18(66.7)				
Total		10(37%)	17(63%)	27(100%)				
Family history of DM	With family history	2(7.4%)	1(3.7%)	3(11.1%)	0.303	4	0.314	51.027
	Without family history	8(29.6%)	16(59.3%)	24(88.9%)				
Total		10(37%)	17(63%)	27(100%)				

Chi square test and frequencies was used to analyze the data; P value <0.05 is considered significant. Odd ratio >1 indicates strong association. CI 95% means confidence interval at level of 95%.

founded between sero-diagnosis of cytomegalovirus of diabetic children and younger age group (Table 2).

The present study showed no association between seropositive IgG anti-cytomegalovirus among diabetes mellitus type I and gender (*P* value 0.244) and family history of diabetes mellitus (*P* value 0.303).

DISCUSSION

Cytomegalovirus is one of the most common viruses in the world, the virus can cause severe disease with multiple complications. A chronic stressor for the immune system is the common herpes virus cytomegalovirus (CMV) which establishes persistent, life-long infections and can become reactivated periodically (Hiemstra et al., 2001; Hamdan et al., 2011). However, type 1 diabetes is an autoimmune disease resulting from a complex interplay between genetic and environmental factors. CMV infection is one of the environmental factors implicated in the development of type 1 diabetes, although the association remains unproven (Aarnisalo et al., 2008). The present study aimed to determine the pos-

sible correlation between CMV infections and type 1 diabetes among children in Khartoum State, Sudan.

The study showed that the positive rate of IgG against cytomegalovirus in diabetic (37%) was higher than that of normal individuals (14.8%). This result is similar to that reported by Guo and Jia (1998) who studied cytomegalovirus infection in patients with diabetes mellitus and concluded that the positive rate of IgG against cytomegalovirus in diabetic is higher than that of normal individuals.

Our results reveal significant correlation between IgG of cytomegalovirus and type I diabetes mellitus in children (*P* value 0.025). This finding support the hyposis of association between cytomegalovirus and diabetes mellitus type I, and agreed with results of Hjelmesaeth et al. (2004), who found significant association between a symptomatic cytomegalovirus infection and increase risk of new onset diabetes mellitus, Nicoletti et al. (1990) also found a significant association between high titers of anti-cytomegalovirus IgG antibodies and anti islet cell antibodies (ICA), whereas Pak et al. (1988) reported strong correlation between cytomegalovirus genome and islet cell autoantibodies.

CMV might be involved in accelerating pancreatic failure to compensate for insulin resistance via at least two possible mechanisms. First, it could influence the pancreatic cells directly; secondly, it might act indirectly by influencing the immune system which in turn affects the pancreas. This is consistent with the first possibility which reported that CMV may infect and reside in pancreatic cells without causing cytopathic effects but nonetheless influencing insulin production directly after repeated reactivations (Reeves et al., 2005). Additionally, infection of human pancreatic β-cells with CMV induced the release of proinflammatory cytokines and increased cellular immunogenicity (Boppana et al., 1992).

The indirect effects of CMV could be exerted via infected monocyte production of IL-1β which induces TNF-α production in human pancreatic duct cells, driving cells into apoptosis and thus compromising β-cell function (Reeves et al., 2005). CMV seropositivity is associated with accumulations of potentially senescent late differentiated T-cells and elevated numbers of CD^{4+} and CD^{8+} effector cells (Doyle et al., 1996) which are more likely to produce pro-inflammatory cytokines (Aarnisalo et al., 2008). However, recently it was shown that human pancreatic β-cells are susceptible to CMV infection (Chen et al., 2012)

The present study displayed insignificant relation (*P* value 0.660) between cytomegalovirus IgG and family history to diabetes mellitus type I. Several studies were carried out to find the relation between cytomegalovirus and genetic susceptibility. Santos et al. (2000) did not get evidence of statistical interaction between cytomegalovirus antibodies and the DQB10201 allele or the DQB10302 allele. Nicoletti et al. (1990) reported insignificant relation between the presence of any HLA-A-B-C, DR antigens and the prevalence of anti-cytomegalovirus IgM and IgG antibodies and/or ICA.

Contrary to our finding, De Mattia et al. (1991) found significant increase of IgG anti-cytomegalovirus in female, where we reported slightly increased IgG anti-cytomegalovirus in female (22.4%) than male (21.9%) which was similar to findings of Kinpe et al. (2007). At the level of study population (study and control groups), the present study found increased seropositive in 10-15 years age group; similar observation was reported by Kinpe et al. (2007) and De Mattia et al. (1991).

The findings of the present study supported the hypothesis of association between CMV infection and diabetic mellitus type 1, however the number of studies on the possible connection between CMV and type 1 diabetes still remains limited and the results are controversial.

Conflict of Interests

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

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Modeling of ultraviolet (UV) radiation under a large pilot-scale designed for wastewater disinfection and inactivation of selected bacteria of *Pseudomonas aeruginosa* in a laboratory UV device

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The aim of this paper was to propose a modeling system of water ultraviolet (UV) disinfection. Results reveal that application of the model of Chick-Watson in its original form or modified are not representative of the kinetics of disinfection. For this reason, the application of a new kinetic model of Collins-Selleck in UV inactivation of *Pseudomonas aeruginosa* in secondary wastewater appeared to be the best applied model. The modeling of the reactivation process at range of 7.5-50°C temperature was shown. First-order saturation does not fit the obtained data in photoreactivation; a modification of the model is proposed coinciding with the classical logistic equation. To better explain the process of inactivation, we have assumed that the action of disinfectant on the survival of lonely microorganisms is faster than its action on suspended solids protected or agglomerated to each other. For this reason, the application of a new kinetic model by introducing a third factor reflecting the influence of suspended solids in water on disinfection kinetics appeared to be determinant for modeling UV inactivation of *P. aeruginosa* in secondary treated wastewater.

Key words: Secondary wastewater, ultraviolet (UV) disinfection, modeling, photoreactivation. *Pseudomonas aeruginosa*, suspended solids.

INTRODUCTION

Many pathogens are responsible for waterborne diseases. Currently, despite the development of molecular methods, most studies in this area were mainly focused on the concentration of fecal indicator bacteria to

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Abbreviations: FLW, Fermented liquid whey; **LAB,** lactic acid bacteria; **MDA,** malondialdihyde, **MUFA,** monounsaturated fatty acid; **PUFA,** polyunsaturated fatty acid; **SFA,** saturated fatty acid; **TBARS,** thiobarbituric acid reactive substance.

estimate the population of pathogens. Recent studies showed that the species of *P. aeruginosa* are valid indicator of the water sanitary quality (Xuexiang et al., 2012; Brahmi et al., 2010). This parameter is actually used as a criterion in the regulation of wading and swimming pools. Likewise, the absence of P. *aeruginosa* is important not only in terms of its role as an indicator, but also because it is an opportunistic pathogen whose transmission is often associated with water. Its use for evaluating the effectiveness of a treatment of UV disinfection seems therefore reliable.

Ultraviolet (UV)-C (short-wavelength ultraviolet) radiation has been suggested as one of the successful disinfection practices for water treatment. Therefore, UV-disinfection has become a practical solution for the safe disinfection of water.

Actually, UV-disinfection has gained widespread use for municipal wastewater and more recently, interest in using UV for water reuse applications has also increased (Kamani et al., 2006). It accepts the following inherent advantages over all other disinfection methods: no chemical consumption, thereby wiping out large scale storage; no transit, handling and potential safety risks; low contact time; no contact basin is necessary and space demands are thus brought down; no harmful byproducts are formed; a minimum of, or no, moving sections; and high reliability and low energy requirements (USEPA, 2003b).

UV_{254} radiations interact with nucleic acids and other cellular compounds, such as proteins and lipids (USEPA, 2003b). The knowledge acquired in this study indicates the use of UVc for disinfection is a quick, effective, secure and cost-effective (Meiting et al., 2009). It has been practiced for many years in several countries to disinfect water (USEPA, 2003b). However, micro-organisms have evolved repair and can reactivate, once their DNA is partially denatured. Visible light and time may have a positive influence on this process known as reactivation (Eccleston, 1998). UV-disinfection of water employs low-pressure mercury lamps. The lamps generate short-wave UV radiation at 253.7 nm, which is lethal to micro-organisms, including bacteria, protozoa, viruses, molds, yeasts, fungi, nematode eggs and algae.

The mechanism of micro-organisms destruction is currently believed to be that in which UV causes molecular rearrangements in DNA and RNA, which in turn blocks replication (Brahmi and Hassen, 2012). The adoption of UV-disinfection at wastewater plants treating in excess of one billion gallons daily is proof that UV is no longer an emerging technology, but quite an accepted technology to be applied routinely by engineers to safeguard human health and alleviate environmental pressures. Wastewater reuse has been drilled in several configurations for decades, with the United States leading the way in reuse research. It is nowadays a major topic in the U.S., where large areas of the Western and Southern states experience chronic water shortages (Min et al., 2006).

UV water purification lamps produce UV-C or germicidal UV, with radiation of much greater intensity than sunlight. Virtually, entirely a UV lamp's output is concentrated in a 254 nm region in order to get total advantage of the germicidal properties of this wavelength. Most UV purification systems are combined with various forms of filtration, as UV light is only capable of killing micro-organisms such as bacteria, viruses, molds, algae, yeast and oocysts such as *Cryptosporidium* and *Giardia*. UV light generally has no impact on chlorine, volatile organic compounds (VOCs), heavy metals and other chemical contaminants (Oparaku et al., 2011).

The models are based on a mathematical representation of the mechanisms that govern natural phenomena to explain a procedure by identifying some variables or the main factors prevailing to suggest a representation or a simulation that will be interpretable and reproducible (Sterman, 2002). For disinfection, modeling helps to accept a mathematical expression of the disinfection kinetics needed for predicting performance of a reactor operating under conditions similar to that experience. However, exposed micro-organisms may repair damage and can be reactivated. Storing of microbial irradiated cells under visible light condition may have a positive influence on the reactivation, and this is commonly known as photoreactivation. Only UV lamps with low or medium pressure are capable of destroying cellular components such as proteins and enzymes, by preventing cell reactivation. This is justified when the water to be treated must meet certain conditions to get the best effect of UV radiation. The different compounds of water may weaken the transmission rate and deposits can also smear the UV reactor and clog quartz tubes protecting the UV lamp (Oparaku et al., 2011; Sellami et al., 2003). The regular replacement of UV lamps and cleaning their ducts provide good permeability to UV and so increase the UV treatment effectiveness.

Several parameters can influence the rate of inactivation of micro-organisms such as the physical-chemical parameters (pH, temperature, etc), the UV dose applied, the UV-water contact time, and the number and the type of microorganisms existing in the water. The relationship between these parameters can be evaluated using analytical measurements in the laboratory.

This research was aimed at first to understand and evaluate the germicidal UV water disinfection, secondly to establish the influence of UV doses on the kinetics of disinfection, to study UV-resistant strains of *Pseudomonas aeruginosa*, to study the phenomenon of photoreactivation and thirdly to establish and to diagnose a mathematical model for simulation and improvement of the UV_C water disinfection.

Meanwhile, another important target of this work was to study the influence of suspended solids on the kinetics of

disinfection and to exploit the results for proposing a formulation of the UV disinfection kinetic of secondary treated wastewater.

MATERIALS AND METHODS

Main characteristics of treated wastewater

The wastewater sampled in this study was collected at the outflow of trickling filter of the pilot wastewater treatment plant (WWTP) belonging to the Water Research and Technology Center, Tunisia. The WWTP is connected to the sewage network of the city of Tunis and it has a processing capacity of 150 m^3 per day. The pilot plant is composed of four treatment lines running in parallel: trickling filter. It is composed of four treatment lines operating in parallel: trickling filter, rotating biological discs, soil and lagoon optional filter. The values fluctuated between 47 to 49% for UV transmission, 15 to 27 mg L^{-1} for total suspended solids (TSS), 20 to 29 mg L^{-1} for BOD_5 and 90 to 102 mg L^{-1} for COD.

UV processing

The laboratory UV device used in this study was previously described by Hassen et al. (2000). A collection of 22 strains of *Pseudomonas aeruginosa* was used in this work. This collection includes 20 strains of clinical origin (hospital of La Rabta, Service of Bacteriology, Dr C. Fendri, Tunis, Tunisia). Strains 21 and 22 were isolated from raw wastewater of the pilot plant. All these strains were grown in the laboratory for long periods on a nutrient broth (Institute Pasteur production). These 22 strains were referenced from S1 to S22, respectively.

All bacterial strains were cultivated to mid-log phase at 37°C in 20 mL of nutrient broth. Each culture was centrifuged at 5000 xg min^{-1} for 15 min and the pellet was washed twice with sterilized distilled water. The washed pellet was then suspended in 10 mL of sterilized distilled water. Test organisms were afterwards seeded separately into 20 mL of sterilized wastewater having a UV transmittance of 50%, to give a viable cell count of approximately 10^5 to 10^6 mL^{-1}, the same mean count as in the secondary-treated wastewater.

The suspension was exposed to UV light for periods varying from 2 to 90 s. All irradiation experiments were performed at laboratory temperature of 25 ± 5°C. Petri dishes of 90 mm diameter, containing 20 mL of seeded wastewater, were shaken carefully with a mechanical shaker (Edmond Bühler) for at least 15 min in order to remove all bacterial aggregates. Seeded wastewater served for counting bacteria, before (N_0) and after exposure (N) to a definite UV dose. For bacteria counting, a volume of 100 µL collected from decimal dilution of each sample was placed on the surface. Petri dishes contain nutrient agar (Pasteur Institute Production, Paris). After incubation at 37°C for 24 h, colonies were counted and the results were expressed by colony-forming units, CFU/mL.

The layer of water crossed with UV rays was 3-mm depth and each experiment was repeated at least four times. Measurements of incident intensity at the liquid surface, at 254 nm, were made with a Vilbert-Lourmat digital radiometer.

Irradiation dose ($mW.s.cm^{-2}$) was determined as the average incident intensity over exposure time and regulated by controlling the exposure time. A low-pressure mercury vapor discharge lamp that emits short-wave ultraviolet radiation with a radiation peak at 253.7 nm (UV-C) for germicidal action UV-C was used. This lamp emitted an average intensity of about 7 $mW.cm^2$. To avoid the phenomenon of photoreactivation, bacterial counting was performed immediately by standards decimal dilution method using the nutrient agar medium.

Photoreactivation study

To confirm the DNA repair after a visible light exposure of bacteria previously irradiated by UV_{254}, only 3 strains of *P. aeruginosa* S1, S2 and S5, respectively, were chosen arbitrary. Similarly, only one strain S5 was used arbitrarily to verify the possible application of Kashimada penny model on its original form and to show if this model is representative or not, mainly, when an induction period appeared.

All strains were cultivated to mid-log phase (at 37°C) in nutrient broth and cell suspensions were treated as previously described. Test organisms were then seeded separately into 20 mL of sterile distilled water on the basis of 10^5 - 10^6 bacteria/ml and exposed to the UV light during 50 s with a relatively fixed UV intensity of 7 mW cm^{-2} which corresponds to approximately UV_{254} dose of 80 mW. $s.cm^{-2}$. The UV dose supplied was calculated as a product of the average UV intensity rate into the reactor (mW. cm^{-2}) and the irradiation time (s). Irradiation was performed at room temperature, between 25 and 30°C.

Each suspension of bacteria was collected and divided before and after exposure to the UV illumination in two sterile flasks for microbiological assays. Three of the six sterile flasks were exposed to laboratory light for 0, 4, 8 and 12 h, and the enumeration of cultivable bacteria was then carried out as previously summarized. The other three sterile flasks were covered immediately with aluminum foil and incubated at room temperature for 12 h (dark repair).

All reactivation experiments (photoreactivation and dark repair) were repeated at least five times and data were subjected to analysis of variance, and means were separated by the least-significant-difference, according to the Student Newman-Keuls test (SPSS for Windows, SPSS, 17 June, 1993).

To further confirm the process of photoreactivation according to the Student Newman-Keuls test (SPSS for Windows, SPSS, 17 June, 1993) and to better visualize the influence of temperature on the process, the same protocol of preparation of strains was suggested as previously described. After UV-C radiation, each suspension of bacteria was transferred into sterile flasks for microbiological assays (95% transparent for 360 nm light). The three sterile flasks were thermostated in a controlled-environment incubator (refrigerated incubator, model no. FOC 225E; VELP Scientifica), which was equipped with one fluorescent lamp (3.7 W; Philips TLD) at six different temperatures: 7.5; 12.5; 17.5; 22.5; 27.5; 37 and 50°C (photoreactivation). Irradiation periods were in the range of 60 to 480 min.

The count of bacteria was made every 60 min by standards decimal dilution method using the nutrient agar medium. This second data photoreactivation experiments were replicated at least five times. All data photoreactivation experiments were subjected, firstly for analysis of variance, and means were separated by the least significant difference, according to the Student Newman-Keuls test (SPSS for Windows, SPSS, 17 June, 1993); secondly, to adapt the kinetic model (Kashimada et al., 1996) as proposed.

Effects of bacterial density associated with particulate matter

Bacteria in wastewater are often bonded together as a floc, or associated with particulate matter (suspended solids). The bacterial density associated with particulate matter, *Np*, can be achieved by the UV process, and is determined as a function of suspended solid (SS) concentration (a regression analysis of the log of the effluent (after exposure) *P. aeruginosa* density as a function of the log of the effluent SS concentration showed the relationship to be linear). The linear regression analysis of the combined data yielded the expression: Np= a (Sheible, 1987).

Table 1. The kinetic characteristics of all the disinfection models studied during UV irradiation.

Kinetic model	Chick-Watson			Amended Chick-Watson				Collin-selleck			
Parameters	**ε**	**K1**	**R_1^2**	**ε**	**K2**	**R_2^2**	**A**	**ε**	**n**	**τ**	**R_3^2**
Strain											
S1	0.2480	0.023	0.64	0.0035	0.0072	0.75	0.0022	0.0016	1.85	2.78	0.91
S2	0.3185	0.017	0.71	0.0013	0.0086	0.92	0.0088	0.0028	1.55	3.36	1.55
S3	0.4514	0.012	0.60	0.0078	0.0033	0.0033	0.0205	0.045	0..88	0.91	0.76
S4	0.7357	0.023	0.57	0.0022	0.0063	0.81	0.0015	0.00059	1.40	0.62	0.75
S5	0.3412	0.012	0.64	0.0035	0.0061	0.75	0.0097	0.0092	1.43	2.74	0.78
S6	0.3402	0.017	0.45	0.0013	0.002	0.30	0.0017	0.0002	0.52	0.0004	0.38
S7	0.3548	0.017	0.54	0.0059	0.0042	0.64	0.0057	0.0026	1.11	0.75	0.87
S8	0.5133	0.017	0.44	0.0660	0.0003	0.52	0.5602	0.0628	0.07	0.03	0.71
S9	0.2905	0.019	0.59	0.0018	0.0051	0.77	0.0027	0.00054	1.41	1.26	0.92
S10	0.2647	0.021	0.63	0.0023	0.0067	0.74	0.0029	0.0004	1.75	2.75	0.85
S11	0.1953	0.027	0.53	0.00021	0.0057	0.70	0.0003	0.00075	1.46	0.27	0.84

[a]*A, K1, K2 and K3*: Characteristics of the models; R_1^2, R_2^2 *and* R_3^2: Coefficients of determination; *τ*, *m* and *n*: Parameters of adjustment of the model; *ε*: Difference between calculated and measured or experimental values $= \sqrt{\sum \left[(N/N_0)_{cal} - (N/N_0)_{\exp} \right]^2}$.

The kinetic models used for UV-C inactivation

The design and the management of the disinfection systems require knowledge of the removal kinetics of pathogenic microorganisms to control the influence of UV dose on disinfection kinetics (Brahmi et al., 2010). To determine the best combination of contact time-UV dose and to predict the yield of UV disinfection, we used more or less empirical approaches. The simulation models, from the simplest, model of Chick-Watson (Chick, 1908; Watson, 1908) reduced to first-order kinetics, to complex model such as Collin Selleck (1972) model. The model of Chick-Watson is primarily used to express the kinetics of disinfection with chemical disinfectants (Hart and Votgiatzis, 1982; Roustan et al., 1991). The first-order kinetics is expressed as follows:

$$\frac{dN}{dt} = -K \times C^n \times N \quad (1)$$

The integration of this expression gives:

$$\frac{N}{N_0} = e^{-KC^n t} \quad (2)$$

C: Concentration of disinfectant in the environment; *K*: A coefficient reflecting the specific case of disinfecting lethality potential; *n*: Coefficient of dilution, which is a function of disinfectant and pH of the medium (the value of *n* is usually close to unit) and *t*: Exposure time to disinfect.

In the case of UV disinfection, an amendment to this model in replacing the concentration of chemical disinfectant (C) by the intensity of UV radiation is done as proposed by Haas (1990). The disinfection kinetics could be rewritten as follows:

$$dN/dt = -K \times I^n \times N \quad (3)$$

The integration of this expression gives:

$$N/N_0 = e^{-KIt} \quad (4)$$

The change in logarithmic form and using a linear regression, the kinetic parameters (*K* and *n*) of the latter expression could be determined as follows:

$$\ln\left[\ln\left(N/N_0\right)\right] = \ln(K) + n.\ln(I) + \ln(t) \quad (5)$$

When, *n* < 1, the disinfection process is more controlled by the contact time than the UV dose. When *n* > 1, the UV dose takes precedence over the contact time in the control of the process (Leahy et al., 1987).

RESULTS AND DISCUSSION

Modeling of the kinetics of disinfection by UV irradiation

In this study, the determination of *ε*, a representative parameter of the difference between the experimental values $(N/N_0)_{mes}$ and values calculated by the model $(N/N_0)_{cal}$, appears important for all strains (Tables 1 and 2). Therefore, the model of Chick-Watson, reduced to a first-order kinetic with *n* = 1, showing its limits, and that the inactivation process is most often non-uniform, and does not necessarily comply, as implies a first-order kinetics, with an exponential law (Shayeb et al., 1998; Nicholson and Galeano, 2003). In addition, the adopted experimental protocol showed a very noticeable reduction

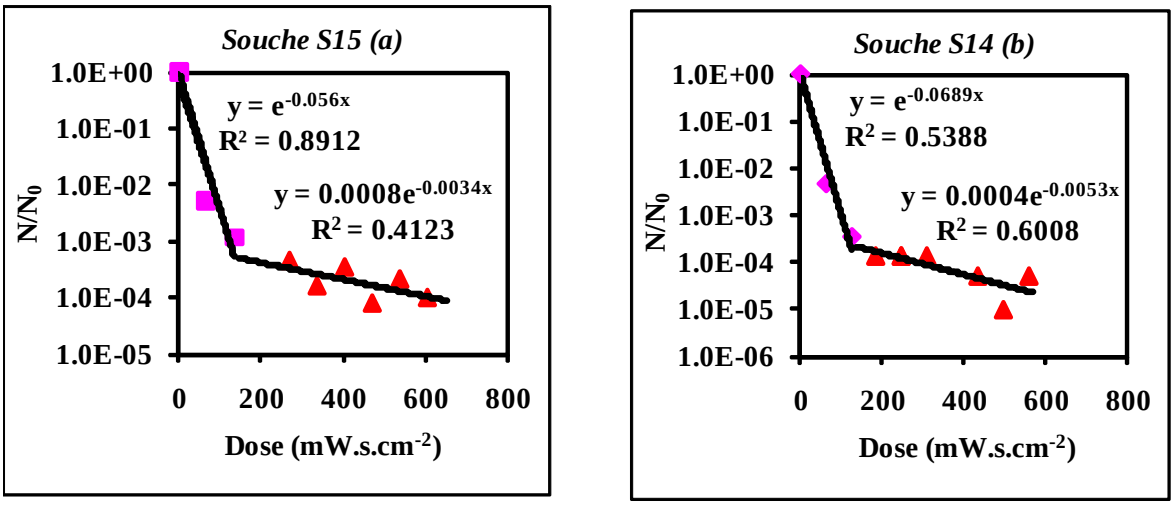

Figure 1. Determination of the variation in the kinetics of disinfection of *P. aeruginosa* as a function of contact time with the approach of Chick-Watson. y: Reduction = N/N_0 with N; number of micro-organisms at the instant T; N_0; Number of micro-organisms at the instant T= 0; R^2: Coefficient of determination; dose (mW. s. cm^{-2}) = X= IT= UV Intensity (mW. cm^{-2}).Time of contact (seconds).

rate for low doses of irradiation.

The importance of UV radiation intensity of the lamp allows achieving a yield rate of 2 log-units after only 2 s of exposure. A reduction of additional log-units could not be reached, even after an exposure time of 90 s. To improve the representativeness of the model of Chick-Watson, taking into account the decrease in speed during the disinfection process, the existence of two stages, each with different kinetics is admitted (Figure 1). Fast inactivation kinetics with doses varying between 0 and 200 (mW. s. cm^{-2}) and a coefficient of lethality ranging between -0.0259, -0.0689 and -0.056 for strains S3, S14 and S15, respectively, were taken as examples. Slow kinetics with doses ranging between 200 and 600 (mW. s. cm^{-2}), and a coefficient of relative low lethality situated between -0.0012 and -0.0034 have been reported by several authors (Mamane-Gravetz and Linden, 2005; Manas and Pagan, 2005). It is therefore necessary to assume the existence of at least two stages during the inactivation process of which only the second was explored during the tests.

The application of a first order kinetic during the second stage needs to adjust the model by introducing a dimensionless coefficient A, to reflect the decline achieved during the first fast kinetics stage (Mamane-Gravetz and Linden, 2004). The expression of bacterial inactivation model becomes as follows:

$$N/N_0 = A.e^{-KIt} \quad (6)$$

With A representing the initial decline or decrease in the number of bacteria. The parameters to identify in this case are, K and A.

In the same way, passing to the logarithm scale, the expression becomes:

$$Ln(N/N_0) = \ln(A) - KI \quad (7)$$

The kinetic equations and the coefficient of reliability of the model for each strain studied were seen utilizing a linear regression. The kinetic parameters of this modified model (A, K, R^2 and ε) are listed in Tables 1 and 2. Results showed a remarkable similarity between the values of the kinetic constant K for some strains, despite the divergence observed for the values of the initial abatement A. This result showed that these strains therefore follow the same kinetics of disinfection.

By calculating the difference $\varepsilon = \sqrt{\left((N/N_0)_{cal} - (N/N_0)_{exp}\right)^2}$ for these two models, the values obtained using the model of Chick-Watson in its modified form appeared smaller than those calculated using the same model in its initial form.

In the same way, the coefficients of determination, R^2 obtained using the amended model of Chick-Watson were generally higher than those obtained using the same model in its original form. Thus, the adjustment of the same model but considering an initial decline describes quite well the kinetics of disinfection for most of the studied strains.

$$Ln(N/N_0) = A.e^{-K.In.t} \quad (8)$$

A key feature of kinetic modelling is not only to simplify,

but also to idealize a complex phenomenon of the disinfection systems. Observation and mathematical modelling of microbial inactivation provides indirect information on the physiological mechanism of inactivetion and, conversely, the mechanisms of resistance. Various models have been suggested to explain the kinetics of inactivation resulting in the existence of latency period following the contact of water and disinfectant (Fair et al., 1948; Shayeb et al., 1998). During this period, the decrease rate of number of bacteria is not measurable and quantifiable. This fact was observed for *Escherichia coli* in the presence of chlorine dioxide taken as disinfectant (Kerwick et al., 2005). The latency period may also be due to the probability of contact between the disinfectant molecules and microorganisms present in water as conglomerates of different sizes (Mounaouer and Hassen, 2011). The existence of many species of microorganisms and their varying sensitivities to the product used for disinfection may also explain the latency period, which is detected through a comprehensive measure giving an apparent rate of inactivation (Berney et al., 2006).

In UV disinfection, several models, for instance, the model of Collins-Selleck (1992), Series event model (Isaac et al., 2007) and the multi-shock model (Kowalski and Witham, 2001) have been built up to identify the initial plateau observed when microorganisms are exposed to a sublethal UV dose. In this case, bacterial inactivation is not significant and the decline is of low amplitude (Pruitt and Kamau, 1993; Kowalski et al., 2000). This latency phase of inactivation for certain strains of *P. aeruginosa*, using UV low doses, has been observed (Figure 1) (Brahmi et al., 2010) and put into evidence by the model proposed by Collins and Selleck (1972) cited by Li et al. (2002).

On the other hand, a stage of initial delay was sometimes found in the majority of bacterial strains used in this experiment (Brahmi et al., 2010). The use of the proposed model of Collins and Selleck (1972) was justified in this situation (Li et al., 2002). In fact, besides the slowdown in the inactivation rate for high doses of UV radiation (Shayeb et al., 1998), this model admits the existence of a period of initial latency. The two following relations expressed this model:

$$\begin{cases} N/N_0 = 1 & \text{for } It \le \tau \quad (9) \\ N/N_0 = (\tau/It)^n & \text{for } It \ge \tau \quad (10) \end{cases}$$

τ is the least dose of radiation to be reached to start the process of micro-organism inactivation, *n*: a constant, *I*: the radiation intensity and *t* is the exposure time. Accordingly, the parameters τ and *n* could be determined by the transition to the logarithmic form and using a linear fit showed, for instance, the position of experimental points as compared to the curve of adjustment for the studied strains. The obtained values seemed to be valid for all examined strains, below the UV dose of 5.5 ($mW.s.cm^{-2}$). In the same way, the determination of ε, a parameter representing the difference between the measured values $(N/N_0)_{mes}$ and the calculated ones by the model $(N/N_0)_{cal}$, appeared very low for all strains as compared to the values calculated using the model of Chick-Watson in its original or modified form (Tables 2 and 3). Consequently, the model of Collins and Selleck was likely to be the most efficient in terms of changing kinetics during the disinfection process.

For overall approach (Figure 2), and for all regression models, the correlation coefficient (R^2) varies from 0.17 for the original Chick-Watson, 0.33 for the amended Chick-Watson model and 0.69 for Collin- Selleck, respectively. Agreeing to this criterion, a sufficient part of the variability of response may be due the explanatory variables. Still, even an R^2 close to 1 is not always a sufficient criterion to validate the quality of a regression model (Thomas et al., 2010). Consequently, other criteria must be analyzed for a better description and better understanding of phenomena involved in the kinetics of inactivation.

In this regard, the determination of ε by the comprehensive approach seems to be important. This parameter is variable, 0.35, 0013 and 0.0071 for the original Chick-Watson, amended Chick-Watson and Collin-Selleck, respectively. As compared to all existing models and based on these two parameters (R^2 and ε), the model of Collin-Selleck gave the best results for describing the inactivation curves of *P. aeruginosa*.
(Figure 2)

Impact of suspended solids content on UV disinfection

The turbidity of water makes the UV ray transmission difficult and therefore reduces their effectiveness. Scheible (1987) found that the number of fecal coliform associated with suspended particles was dependent on water solid content. He proposes to subdivide the whole micro-organism load in water into two categories: micro-organisms isolated and so vulnerable, and micro-organisms associated with suspended particles and so invulnerable. This subdivision of microorganisms in two groups led to the following expression of inactivation kinetics:

$$N' = N_0 e^{(-KIt)} + N_p \quad (11)$$

Where, N_0 is the number of micro-organisms isolated per unit volume of water ($N'_0 = N_0 - Np$); Np is the number of micro-organisms per unit volume of water inaccessible to UV radiation; and N' is the number of micro-organisms remaining after water treatment with UV radiation D ($N' = N - Np$). Agreeing to this model, it was accepted that the

Table 2. The kinetic characteristics of all the disinfection models studied during UV irradiation[a].

Kinetic model	Chick-Watson			Amended Chick-Watson				Collin-selleck			
Parameter	**ε**	**K1**	**R_1^2**	**ε**	**K2**	**R_2^2**	**A**	**ε**	**n**	**τ**	**R_3^2**
Strain											
S12	0.2245	0.028	0.77	0.0568	0.0183	0.75	0.0216	0.0252	2.82	20.08	0.93
S13	0.3635	0.016	0.71	0.0086	0.0063	0.81	0.0195	0.0034	1.54	5.53	0.85
S14	0.2213	0.024	0.66	0.0035	0.0084	0.73	0.0018	0.0019	2.13	3.85	0.85
S15	0.2658	0.02	0.63	0.0034	0.0061	0.73	0.0027	0.0015	1.7	2.55	0.88
S16	0.3234	0.016	0.39	0.0024	0.0018	0.22	0.0014	0.0022	0.49	0.0001	0.22
S17	0.8539	0.019	0.52	0.0030	0.0039	0.72	0.0018	0.00060	1.08	0.24	0.88
S18	0.2114	0.023	0.56	0.0002	0.0053	0.79	0.0005	0.00002	1.33	0.23	0.73
S19	0.2276	0.024	0.50	0.0088	0.0063	0.35	0.001	0.0069	1.80	1.90	0.53
S20	0.3733	0.014	0.55	0.0037	0.0034	0.69	0.0067	0.0005	0.91	0.33	0.66
S21	0.3018	0.018	0.71	0.0014	0.0066	0.88	0.0073	0.0038	1.69	4.18	0.87
S22	0.3191	0.016	0.73	0.0072	0.0071	0.77	0.0188	0.0063	1.72	7	0.71

[a]*K1, K2 and K3*: Characteristics of the models; R_1^2, R_2^2 *and* R_3^2: Coefficients of determination; *τ*, *m* and *n*: Parameters of adjustment of the model; *ε*: Difference between calculated and measured or experimental values $= \sqrt{\sum \left[(N/N_0)_{cal} - (N/N_0)_{\exp} \right]^2}$.

Table 3. Kinetic parameters of the logistic model applied to photoreactivation experiments.

Temperature (°C)	Strain					
	S5		S1		S2	
	Sm (% survival)	*k*2 [(% min) $^{-1}$]	Sm (% survival)	*k*2 [(% min) $^{-1}$]	Sm (% survival)	*k*2 [(% min) $^{-1}$]
7.5	0.018	2.28	0.018	1.110	0.017	6.15
12.5	0.04	0.810	0.028	1.2	0.03	2.41
17.5	0.069	0.388	0.043	0.618	0.037	1.18
22.5	0.201	0.135	0.106	0.172	0.048	1.127
27.5	0.499	0.07	0.264	0.070	0.042	0.867
37	0.831	0.055	0.328	0.223	0.08	0.801
50	0.012	2.01	0.011	1.98	0.01	2.09

*k*2: The new growth, second-order reactivation rate constant [(% min) $^{-1}$]; S_m: the maximum limit of the survival ratio of the microorganisms by reactivation (S_m = 100 x (N_m/N_o)); where, N_m is the maximum concentration of microorganisms reached by reactivation and N_o is the concentration of microorganisms before disinfection (% survival).

isolated micro-organisms were inactivated according to first-order kinetics. It was also recognized that a residual number of micro-organisms (*Np*) persist in the water, whatever the radiation dose applied. Under these adopted operating conditions, it was not possible to fall below a level of microorganisms of 10^3 per 100 mL for most of the tested strains. During the experimental study, the physico-chemical characteristics of treated waste-water by trickling filter did not greatly change.

Values fluctuated between 47 and 49% for UV transmission and 15 and 27 mg/L for total suspended solids (TSS) on average. This concentration of suspended particles was unlikely to be the only reason for hindering the process of inactivation at this high level. Other factors related to the operating conditions or to the type of micro-organisms could have been responsible for this observation. The research work of Scheible (1987) on the installation of Port Richmond in France has led to propose a power function expressed as follows:

$$Np = aCss^b \quad (12)$$

Where, *Css* is the concentration of suspended solids in water; *a*, *b* are two constants. For fecal coliform, *a* and *b* were 0.26 and 1.96, respectively (Scheible, 1987). In the case of treated wastewater by trickling filter and for all studied strains of *P. aeruginosa*, these constants were

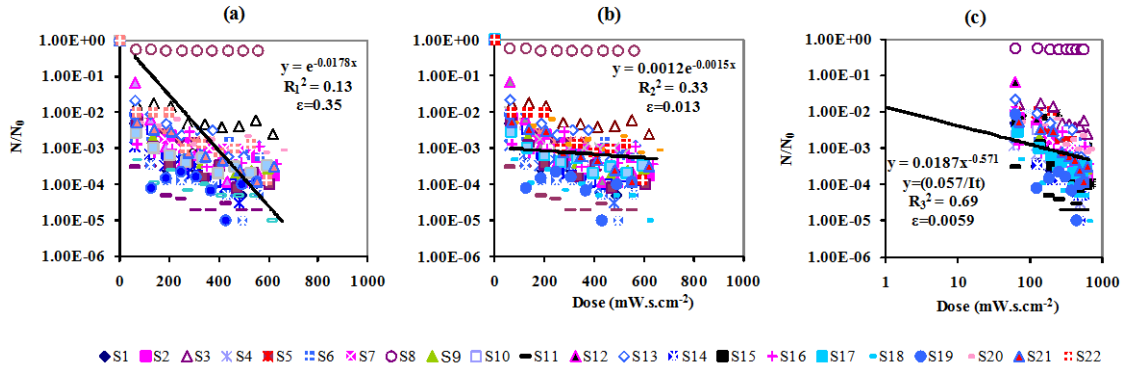

Figure 2. Kinetics of bacterial inactivation according to the models of Chick-Watson (a), Amended chick-Watson (b) and Collin-Selleck (c), respectively. y: reduction = N/N_0 with N; Number of bacteria at the instant T; N_0; Number of bacteria at the instant T= 0; R_1^2, R_2^2 and R_3^2: Coefficients of determination; m: Kinetic characteristic of the model; Dose (mW.s.cm^{-2})= $I \times T$= UV Intensity (mW.cm^{-2})×Time of contact (seconds).

0.28 and 4.97, respectively thus:

$$N_p = 0.28Css^{4.97} \quad (13)$$

As mentioned above, we assumed that the microorganisms were inactivated according to first-order kinetics and that the experimental exploration only concerned with the second stage of inactivation process. The number of microorganisms that are still viable in water having received a D dose of UV radiation is thus given by:

$$N' = AN_0' e^{-KD} + aCss^b \quad (14)$$

The product AN'_0 represents the number of microorganisms still viable at the end of the first and fastest stage of UV inactivation process. In the case of *P. aeruginosa* and the treated wastewater used, this relationship was:

$$N' = 0.0555N_0' e^{-0.00007D} + 0.28Css^{4.97} \quad (15)$$

This equation could be written as the form below based on the experimental results illustrated in Figure 3.

$$N' = 0.0555N_0 e^{-0.00007D} + 0.28\left(1 - 0.0555e^{-0.00007D}\right)Css^{4.97} \quad (16)$$

We have reported the first-order kinetics frequently observed during the inactivation of different types of abatement). Beyond this limit, a slowdown in inactivation microorganisms with a certain limit (nearly 2 U-log of rate was often noticed.

This decrease in radiation efficiency, usually observed when increasing the radiation dose, was often attributed at first to the formation of microorganism aggregates, and secondly to the association of these microorganisms with suspended particles in water. Aggregate-borne microorganisms are protected against the action of UV rays. As previously underlined. Scheible, (1987) divided all microorganisms into two types: free or isolated microorganisms and those associated with suspended solid particles in water. It is assumed that only the first type was available to UV radiation, and therefore vulnerable. By slightly deviating from this hypothesis, we assume that two categories of microbiological population existed in water:

1. Microorganisms which, for one reason or another, were readily exposed to radiation. The rate of inactivation of these microorganisms is rapid.
2. Microorganisms less accessible to radiation, therefore inactivated according to a slower kinetic.

By assuming that these two concomitant mechanisms of the first order were independent, we can express the rate of inactivation of the total number of microorganisms by applying the multi kinetics model of first order, expressed by the relationship as follows:

$$N_0 = pe^{-K_1 D} + (1-p)e^{-K_2 D} \quad (17)$$

Where, P is a fraction of microorganisms which is more susceptible to UV radiation; D is UV dose in (mW.s cm^{-2}); K_1 and K_2: Kinetic constants for each class of microorganisms.

In this study, the applicability of this model was tested to characterize the inactivation of *P. aeruginosa* in treated wastewater by determining the values of the constants *p*,

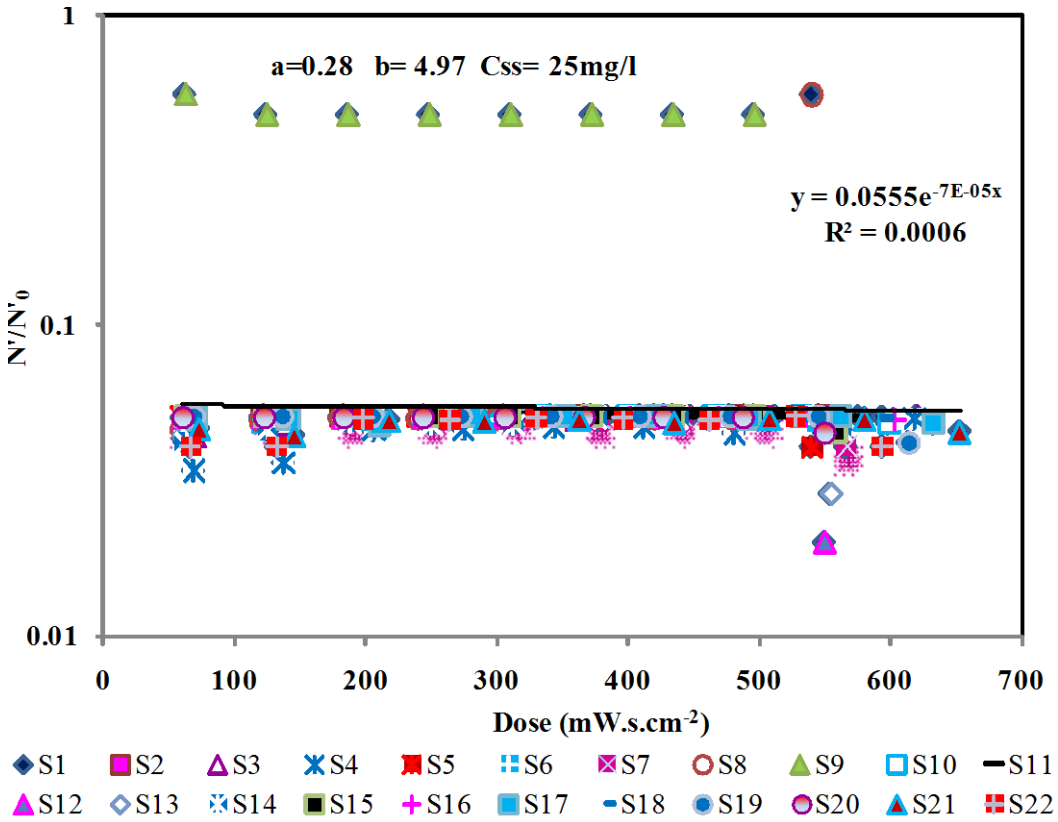

Figure 3. Kinetics of UV inactivation of *P. aeruginosa* according to amended Chick Watson model and taking into consideration the impact of suspended solid content of water. *a* and *b*: characteristics of the model; *Css*: concentration of suspended solids.

K_1 and K_2 (Figure 5). We were able to express the turnover rate by the following equation:

$$N/N_0 = 0.9981e^{-0.071D} + 0.0019e^{-0.006D} \quad (18)$$

This model assumed that it was yet possible to reach the complete inactivation of all microorganis ms if we applied enough UV dose. We noted, however, that during UV disinfection experiments, it was practically not possible to lead down under a sure degree of abatement. Shawn et al. (2011) attributed this fact to the high concentration of suspended solids in water. To integrate this concept, we assumed that the fraction of the vulnerable micro-biological population in water does not exhibit the same sensitivity to UV radiation. In the case of homogeneous populations, the non-uniformity of radiation throughout the reactor's area (irradiation room) or the heterogeneity of the environment might explain the non-uniformity of radiation efficacy. The status of an organism with regard to radiation source and the nature of the environment penetrated by rays have a substantial influence on the kinetics of inactivation. Taking into consideration the experimental results shown in Tables 1 and 2, three main stages could be identified as follows:

1. A first point during which the density of microorganisms is important, therefore, radiation efficiency is the superlative. During this stage, the most vulnerable microorganisms are inactivated.
2. A second stage concerning lonely organisms, but less accessible to the radiation, hence showing a slower rate of inactivation.
3. A third stage related to organisms which are inaccessible to radiation because they are associated with suspended solid particles. During this period, the number of viable microorganisms remain stable. This result corresponds to a null inactivation kinetic. Taking into consideration this approach, the number of micro-organisms remaining viable in water and irradiated with a *D* dose was given by the following expression:

$$N' = N'_0 \left[pe^{-K_1 D} + (1-p)e^{-K_2 D} \right] + Np \quad (19)$$

The characteristic kinetic parameters of *P. aeruginosa*

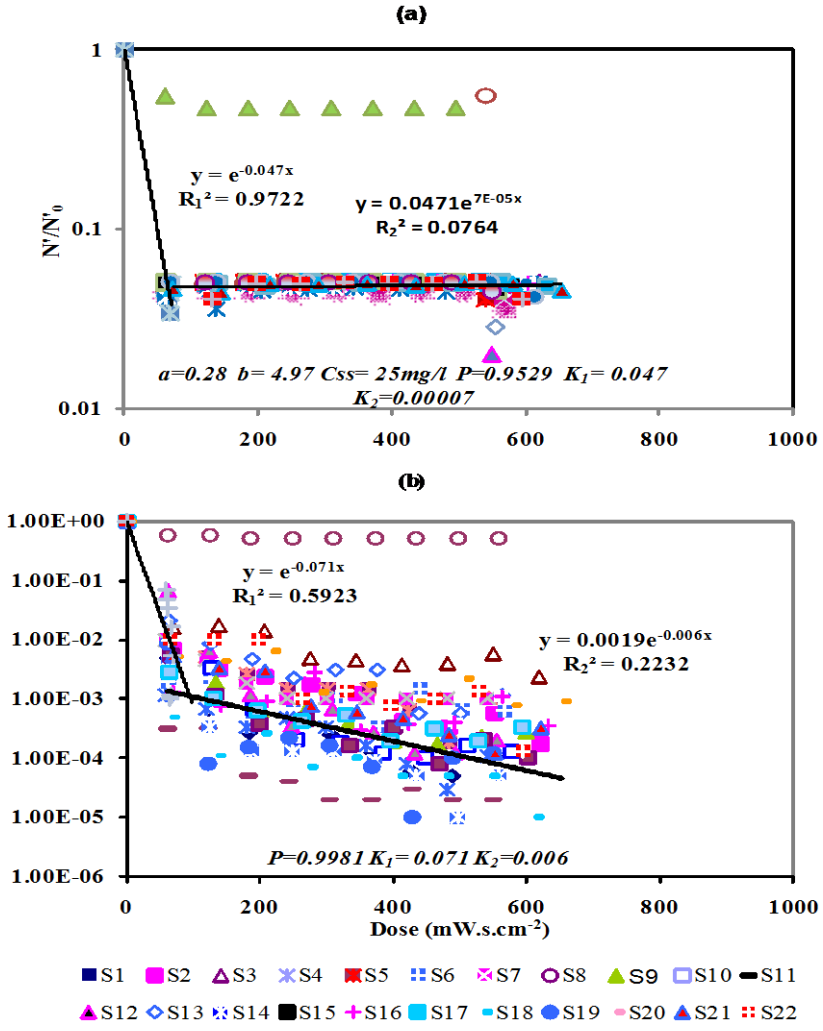

Figure 4. Kinetics of UV inactivation of selected *P. aeruginosa* according to a multi-action of first order with (a) and without suspended solid content (b). *Css*: Concentration of suspended solids; *a*, *b*, *P*, K_1 and K_2: kinetic characteristics of the model; R_1^2 and R_2^2: coefficients of determination; with n=1, *X* (dose UV) = *D* = *I*×*t* = UV Intensity (mW/cm^2) × Time of contact (seconds).

inactivation in secondary treated wastewater used in this study were 0.9529 for *p*, 0.047 for K_1 and 0.00007 for K_2, respectively; for this reason, the expression became:

$$N' = \left(N_0 - 0.28Css^{4.97}\right)\left(0.9529e^{-0.047D} + 0.0471e^{-0.00007D}\right) + 0.28Css^{4.97} \quad (20)$$

An illustration of this model is shown in Figure 4.

Impact of temperature on the photoreactivation repair

Reactivation is frequently expressed as a function of the survival ratio with respect to the initial microorganism concentration existing before the inactivation treatment. Therefore, survival values were calculated using the following equation:

$$S = \frac{N_r}{N_0}.100 \quad (21)$$

Where, *S* is the survival ratio at time *t*, N_o is the concentration of microorganisms before disinfection (bacteria/100 ml), and N_r is the concentration at time *t* after the beginning of the reactivation phase bacteria/100 ml). A typical inactivation-reactivation curve as a function of time is shown by Salcedo et al. (2007). In the figure, it is possible to separate the various phases of (the

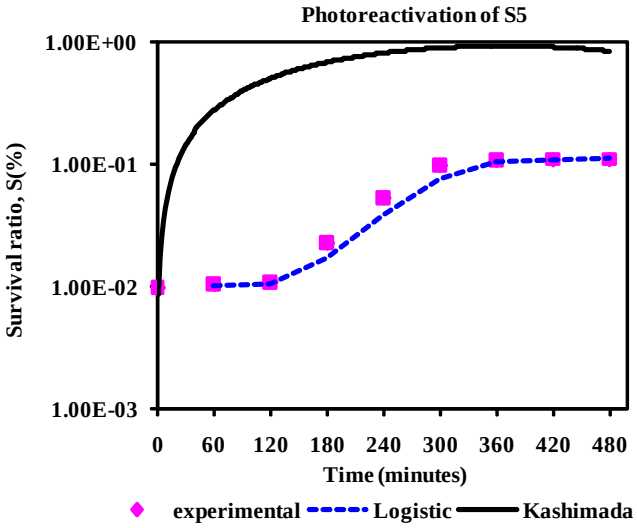

Figure 5. Photoreactivation curves obtained for strain S5 according to typical photoreactivation curve, the model proposed by Kashimada et al. (2006) and the logistic model. Near-optimum growth temperature= 37°C.

process: exponential UV inactivation and the reactivation process, which includes an induction period, the growth phase, the stabilization phase and the decay period. To study the photoreactivation kinetic, Kashimada et al. (2006) proposed an asymptotic model, simulating that the photoreactivation phenomenon follows a saturation-type first-order reaction, as expressed by the following equation:

$$\frac{dS}{dt} = K_1(Sm - S) \quad (22)$$

Where S_m is the maximum survival ratio ($N_m/N_o \cdot 100$ [N_m is the maximum concentration of microorganisms {bacteria/100 ml}]) and k_1 is the first-order reactivation rate constant. As the survival ratio, S is achieving its upper limit value (Sm), the process decelerates, showing an asymptotic tendency (Figure 5).

After application of the model of Kashimada et al. (2006), results showed that the model did not fit the data correctly, mainly at the beginning of the curve, when an induction period is observed (Figure 5). Hence, by utilizing the new model represented by Equation 3, the relationship became a combination of the second-order equation and the driving force concept employed by Kashimada et al. (2006).

$$\frac{dS}{dt} = K_2(Sm - S) \cdot S \quad (23)$$

Where k_2 is the new growth, second-order reactivation rate constant. The equation is really not new because it coincides in its mathematical form with the logistic equation proposed by Verhulst (1883) to interpret the biological population growth. Nevertheless, the originality of our work acted in the innovative application of the equation to microorganism reactivation prediction. The model has the advantage that both kinetic parameters, S_m and k_2, have clear physical significance. On one hand, S_m is the maximum limit of the survival of the microorganisms by reactivation and, on the other hand, k_2 represents the rate at which that value is reached. It can be visualized in Figure 5 that this proposed model fits correctly the experimental data.

By the integration of Equation 23, the following equation is obtained:

$$\ln\frac{S(S_0 - S_m)}{S_0(S - S_m)} = K_2 \cdot S_m \cdot t \quad (24)$$

Where S_o is the survival immediately after UV disinfection ($N_d/N_o \cdot 100$ [N_d is the concentration of microorganisms after disinfection {before reactivation} {bacteria/100 ml}]).

From equation 24, it is possible to express the variable S as a function of the kinetic parameter k_2, S_m, S_o and time (equation 24) and, as a result, both parameters S_m and k_2 were related by nonlinear regression:

$$S = \frac{S_m}{1 + \frac{S_m}{S_0} \cdot e^{K_2 \cdot S_m \cdot t}} \quad (25)$$

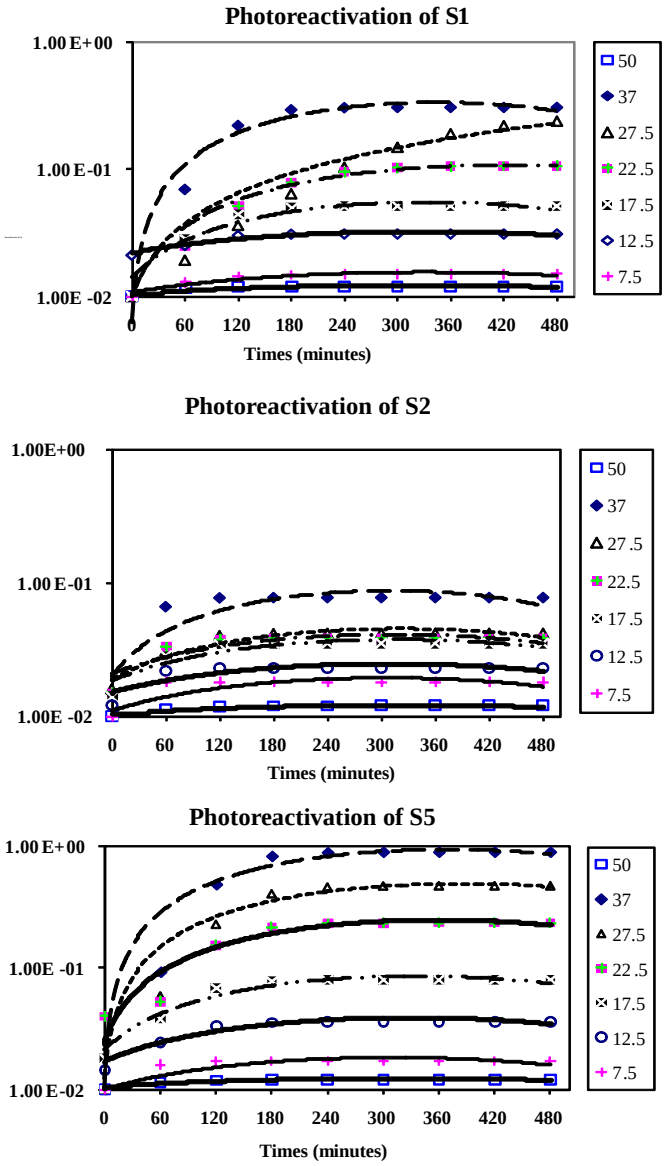

Figure 6. Survival ratio (logarithmic scale) versus time of exposure to photo reactivating light according to different reactivation temperatures (7.5 to 50°C). Experimental data and prediction by the logistic model are shown.

Equation 13 allows the photoreactivation curve to be simulated over time (Figure 6).

As stated, reference strains were exposed to an inactivating UV-C dose of 80 mW.s.cm^{-2} and then to six reactivation temperatures (7, 5; 12, 5; 17, 5; 22, 5; 27, 5; 37 and 50°C). Figure 6 represent the survival ratio (on a logarithmic scale) versus time for S1, S2, and S5, and the asymptotic shape of the curves can be seen, including an induction period, an exponential growth phase, and finally a stabilization phase. The model described in Equation 15 was applied to experimental data using nonlinear regression. This study showed similar behavior with regard to the reactivation temperature for the three reference strains. However, if a number greater than or equal to 10^2 organisms/100 ml of *P. aeruginosa* in treated wastewater was considered, this will pose a potential risk to the environment (Kristina and Charles, 2009). In which case S1, S2, and S5 all showed an expected result: the higher the temperature, the greater the maximum reactivation in which case S1, S2, and S5 all showed an anticipated result: the higher the temperature, the larger the maximum reactivation observed. Inspite of this

apparently low percentage, for a N_o of 10^6 organisms/100 ml, a reactivation of 10^4 organisms/100 ml would be produced; this could cause serious health and environmental problems (Brahmi et al., 2010).

Contrary to the hypothesis advanced by Salcedo et al. (2007), that chemical and biochemical rates are supposed to increase by increasing temperature, according to the Arrhenius relationship; however, in the studied case the opposite was observed (Table 3). In fact, k_2 is not a pure reaction rate constant, but rather is a model parameter that is adjusted to predict the experimental data. Its physical meaning is related to the time required to reach the maximum survival ratio and then the stabilization phase: high k_2 values signify short induction and growth phases. The trend of k_2 versus temperature found in these experiments could be explained as follows: since an elevated temperature provides a higher maximum of reactivation (S_m), reaching the maximum, needs certainly more time to be realized.

At the same time, another quantification review of photoreactivation was applied to the same strains and data were subjected to analysis of variance, and means were separated by the least significant difference according to the Student Newman-Keuls test (SPSS for Windows, SPSS, 17 June, 1993). The examination of cell number obtained after different periods of exposure to visible light showed that only strain S5 had a major revival after UV irradiation exposition and an exposure of 0, 4, 8 and 12 h to visible light at room temperature (25± 5°C) and in a non-nutrient suspension. Concerning the results obtained for S1 and S2, the statistical analysis did not show significant difference in the results after 12 h of exposure to visible light exposure (Table 4). These results obtained are inconsistent with those mentioned by Logistic model, especially for strains S1 and S2. The results raise nevertheless questions about the effectiveness of UV to inactivate microorganisms. These irradiated or inactivated' microorganisms did not appear surely in the growth media (uncultivable) but they could be still metabolically active and virulent in the precise case of pathogenic. Conversely, higher photoreactivation rates and levels were observed with increasing fluorescent light intensities. When exposed to near-optimum growth temperatures (23-37°C), photoreactivation levels were higher than those high (50°C) or low (7.5°C) temperatures. Since UV irradiation occurs at room temperature (20 to 25°C), the reactivation experiences at the most extreme temperatures (7.5 and 50°C) could cause a temperature shock to the bacteria and therefore alter the reactivation process.

Results shown in Table 5 indicate that in the case of *P. aeruginosa* S1, S2 and S5, statistical analysis did not show any significant differences in bacterial reduction in conditions of darkness after different periods of exposure to laboratory light, suggesting that reactivation was not perceptible. These results could be corroborated by the work advanced by Hassen et al. (2000).

In general, variation in the bacterial photoreactivation is seriously discussed in the literature; and it seems that certain micro-organisms are able to repair some of the damage caused by UV light when exposed to light in the near-UV or violet-blue spectral range (Hassen et al., 2000). Different mechanisms are proposed to explain the process of photoreactivation. Dimers of pyrimidine, resulting from the UV alteration, are reduced *in situ* to monomers by an active enzyme through the action of visible light in the near-UV or violet-blue spectral range (310 ± 480 nm). The second mechanism is the substitution of damaged nucleotides. The best known example is cutting-repairing: a sequence of low adjacent bases is excised from DNA submitted to the UV radiance, and then it is resynthesized correctly (Hassen et al., 2000; Kashimada et al., 2006). Nevertheless, the process of photoreactivation is not general in all bacteria. Different factors affecting photoreactivation are discussed, such as UV dose, wastewater quality, exposure time to photoreactivating light, and particularly the species of the micro-organism.

Conclusions

As compared to overall approach, for all the regression models and based on the two parameters (the correlation coefficient R^2 and ε), the model of Collin-Selleck gave the best results for the description of UV inactivation.

The application of a first order law to the kinetic model of disinfection was therefore possible, if we assumed the existence of two successive steps of different kinetics. Only the second stage was explored during these experiments. The first stage of fast kinetics could only be studied when considering an initial abatement. In the case of the second stage of this model, the presence of suspended particles in water had an important effect on dissipating the radiation energy and therefore on protecting the microorganisms against UV rays. In conclusion, suspended particles affected directly the effectiveness of the UV disinfection.

The examination of cell number obtained after different periods of exposure to visible light showed that only strain S5 had a major revival after UV irradiation but results obtained for S1 and S2 did not show significant difference in the results after 24 h of exposure to visible light exposure.

Results also showed higher photoreactivation rates with increasing fluorescent light intensities. The survival bacteria after UV irradiation would be a consequence of the combined effect of reactivation and temperature shock. Nevertheless, S5, S2, and S1 are each heterogeneous group and the composition of these indicators may change; this new model has the capability of fitting to experiment data from different wastewaters, resulting in new parameters that will permit the prediction of the reactivation process.

In conclusion, the use of special germicidal UV lamps, during a relatively uniform short time release and high flux of energy (notion of flash lamps), and the optimization of UV emission in the irradiation chamber would be of major interest in order to guarantee a good disinfection. This would also present the major advantage of avoiding the phenomena of bacterial revitalization and the release of mutants in the environment.

Conflict of Interests

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

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