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African Journal of Microbiology Research

Full Length Research Paper

Effect of different grains and alternate substrates on oyster mushroom (*Pleurotus ostreatus*) production

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The present investigation was undertaken by using various grains for spawn production, and waste paper, wood chips were used in comparison with wheat husk for mushroom production during 2010-2011 at Department of Botany, Singhania University, Rajasthan. The results of the analysis of variance showed that diameter of colony extension in various grains are different and were significantly affected by substrate type. The maximum and minimum growth rates were seen in the corn and millet substrates, respectively. It is concluded that wheat straw in combination with wood chips are best substrate for oyster mushroom cultivation.

Key words: Oyster mushroom, grains, substrate, wood chips.

INTRODUCTION

Mushroom is a unique horticultural crop. In contrast with the cultivation of higher plants, which started in pre-historical times, the culture of fungi is relatively of recent innovation. Historically, mushrooms were gathered from the wild for consumption and for medicinal use. The first commercial cultivation of edible mushrooms was developed in France in the 18th century since then it has traveled far ahead. Over 200 species of mushrooms have been collected from the wild and utilized for various traditional and medical purposes mostly in the Far East. Till date, about 35 mushroom species have been cultivated commercially. Of these, about 20 are cultivated on industrial scale throughout the world due to their high nutritive and medicinal value which contributes to a healthy diet with rich source of vitamins, minerals and proteins (Garcha et al., 1993). The spawn and spawn making has been primary concern in mushroom cultivation which is achieved by developing mushroom mycelia on supporting medium under controlled environmental conditions. In almost all cases, the supporting matrix is sterilized grain which is preferred due to its bio-chemical properties and practical performance over others (Siddhant et al., 2013). Unfortunately, the ever increasing demand of food grains for human consumption leaves little scope for their use in spawn making. A number of other materials, mostly agricultural wastes, can be used to prepare mushroom spawn. The types of waste available vary from region to region. As

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spawn making, substrate inoculation is also a crucial phase in mushroom growing practices. Depending on the different process parameters the yield of mushroom varied from 74 - 204 g.

Optimum process parameters for maximum yield of *Pleurotus ostreatus* were found to be Bagasse 21.25 g/l, Wheat bran 3.5 g/l, Urea 5.0 g/l and Humic acid 4%. The process parameters also show significant effect on yield, productivity and biological efficiency. Mycelia colonization of compost bags and subsequent growth of oyster mushroom was faster in high Humic acid-based substrates. Hence they produced larger and firmer fruiting bodies (Prakash et al., 2010).

The main nutritional sources for oyster mushroom are cellulose, hemicellulose, lignin and C/N ratio is important factor for optimal substrate composition for oyster mushroom. Oyster mushroom requires much carbon and less nitrogen source than button mushroom *(Agaricus bisporus)* but most of the main substrate materials such as cereal straw (Siddhant et al., 2013) cotton waste and sawdust need supplementation of nitrogen source such as wheat and rice bran to reach optimal C/N ratio for oyster mushroom. Therefore, any type of organic matters containing lignin and cellulose can be used for oyster mushroom substrates and this includes almost all agricultural wastes. Cultivation of mushroom on these solid residues can be viewed as an effective means to utilize bio-resource residue and simultaneously as a sound environmental protection strategy. Furthermore, the use of these residues in bioprocesses may be one of the solutions to bioconversion of inedible biomass residues into nutritious protein rich food in the form of edible mushrooms (Mshandete and Cuff, 2008).

The present study is aimed at using various grains for spawn production and use of waste paper and wood chips in comparison with wheat husk for mushroom production.

MATERIALS AND METHODS

Micro-organism

The pure culture of *P. oestreatus* fungi MTCC 1801 was from IMTECH Chandigarh (edible mushroom species). For the propagation of the main culture, 2.0% malt-extract agar (MEA) was used. MEA plates were inoculated with a mycelium/agar plug (5-mm-diameter) of a young, actively growing margin of the colony. Prior to its use as an inoculum for grain spawn, a mycelium/agar plug was inoculated at the center of the plate and incubated at 25°C in the dark for seven days.

Spawn preparation

Various cereals, viz., wheat grains (*Triticum aestivum*), barley (*Hordeum vulgare*), maize (Zea mays) and millets, (*Setaria italica*), were utilized as grain substrate for planting spawn. These were purchased from the seed market. Some locally available agricultural wastes like wheat straw, sugarcane bagasse, rice husk and mango saw dust and hulled corn cob were also utilized for this purpose. The mother spawn was prepared on traditionally used wheat grains (*Triticum aestivum*). 50 g grain was placed in polythene bags and held in place by rubber bands, it is sterilized at 121°C for 20 min. After cooling, each bag was inoculated with spawns and incubated at 25°C in full darkness for two weeks to enable the mycelia to permeate.

Substrate preparation

Waste paper

Waste paper was collected and cut in 0.5 cm width with different lengths.10 L of water was taken in a bucket and two cups of bleaching solutions were added to this water. Newspapers were added to this bleached water and, allowed to hydrate for about an hour then stirring was done. After an hour papers were taken out and were separated under shade so as to remove the extra water. Then loading of polythene bags was done.

About 3/4 of inch of paper layer was prepared then a coating of spawn on the top of each layer was added. Care is taken on the outer and top layer of news papers. Then bag was tied with the help of thread and holes were made around the bag. The bag was pushed down getting the air out of it and then the bag was kept in shade for a period 2-3 weeks.

Wheat straw and wood chips

Wood chips of 0.5 cm wide were collected. Then, equal quantity of wood chips was mixed with wheat straw. 10 L of water was taken in a bucket and two cups of bleaching solutions were added to the water. Wheat straw and wood chips were added to this bleached water and allowed/to hydrate for about an hour, then stirring was done. After an hour, wheat straw and wood chips were taken out and were separated under shade to remove the extra water. Then loading of polythene bags was done.

Spawn dose

Inoculation of substrate was made with planting spawn of *P. oestreatus* @ 5% w/w on dry weight basis under aseptic conditions.

Spawning techniques

Three methods of spawning were employed for substrate inoculation.

Top spawning: In this method, spawn was planted just above the surface of substrate. It was then covered by a thin layer of substrate.

Thorough spawning: It was achieved by thoroughly mixing of spawn with the substrate while filling the bags.

Layer spawning: The spawn was planted in the substrate in multilayered (3) manner. This mode of spawning was later treated as control and uniformly used in all the experiments.

Method of cultivation

Plastic bag technology was used in this experiment. The beds were

Table 1. The results obtained from different grains.

Different grains	Diameter colony extension after 12 days of inoculation (mm)
Corn	38.60
Wheat	35.80
Barley	32.40
Millet	26.80

prepared from pasteurized substrate. pH of the substrate mixtures were maintained at 6.0. Compost medium was mixed manually. The mixture of varying compositions were packed in polythene bags and sterilized. After sterilization, the substrate is semi dried, inoculated with the spawns in alternate layers and incubated at 25°C in dark for 2 weeks for the ramification of mushroom mycelia. The culture rooms were damped by spraying the top of compost with water once a day. This maintains the relative humidity of 80%. After the development of mycelium on compost bags, they were torn and maintained at 28°C with adequate aeration; watering and high humidity to allow the fruiting bodies to emerge. The harvesting was done in 3 flushes of 1 week intervals. After the 2nd flush, the substrate was turned upside down and regularly watered to harvest the 3rd flush.

Presentation of data and evaluation of biological efficiency of mushroom

The data recorded in respect of yield parameters were time lapsed in spawn run, pin head initiation and maturity of fruit bodies, number of flushes, total yield, number and weight per sporocarp on different kinds of spawn and spawning techniques. The biological efficiency of mushroom was worked out as percentage yield of fresh mushroom in relation to dry weight of the substrate as suggested by Chang and Miles (1989).

$$\text{Biological efficiency} = \frac{\text{Yield of fresh mushroom (gm)}}{\text{Total weight of dry substrate used (gm)}} \times 100$$

Statistical analysis

Completely randomized design (CRD) was employed for all the experiments. All the data were statistically analysed. The critical difference (CD) was worked out at five percent probability level.

RESULTS AND DISCUSSION

The results of the analysis of variance showed that diameter of colony extension in various grains are different and were significantly affected by substrate type. The maximum and minimum growth rate were seen in the corn (Table 1 and Figure 1A) and millet substrates, respectively. The larger surface area and pore of substrates are responsible for the more mycelium growth rate. For the reason that corn seeds size are larger than wheat and millet seeds size, consequently, corn seeds pore is also larger. As a result, it influences mycelia growth rate. Probably, the increased mycelium growth in corn is due to increased ventilation and O_2 concentration in corn substrate as O_2 is one of the most important environmental factors. It performs an important role in metabolism and essential for respiration mushroom. For maximum respiration there is active growth (or mycelia growth). Therefore at the lowest O_2 concentrations, respiration rate relate directly to O_2 concentration of substrate (Siddhant et al., 2013).

The crop of oyster mushroom was harvested in three flushers. Maximum yield was obtained in the first flush than the second and third flushes (Figures 2, 3 and 4). This study has demonstrated that locally available organic substrate is potentially suitable for use in the production of mushrooms. It means that the substrates contain lignin and cellulose which is utilized by the mushroom mycelium as a source of nutrition. The diverse range of substrates indicates that the mushrooms can be grown on almost all available organic wastes. The ability of mushrooms mycelia to degrade organic substrates can also be utilized in management of organic waste in the environment, which is otherwise left to decompose hence causing health hazards. It is one of the method that can help in recycling the organic wastes into profitable products. This is made possible by the ability of mushrooms (basidiomycetes) to secrete a wide range of hydrolyzing and oxidizing enzymes which breaks down natural lignocelluloses waste into simple soluble compounds.

The time taken by the mycelia to start pinning after ramification depends on the substrate used. Materials with high quality lignin and cellulose contents take a longer time to start pinning as compared to the substrates with low contents of the lignin and cellulose. As compared to the substrates with low nutrition values, the substrates with high nutrition value take a short time for full colonization and ramification. This is because the mycelia remains vegetative for a longer period hence the vigorous growth and late pinning. In turn, the highly colonized substrates exhibited low mycelia densities. Poor nutritional substrates exhibited low mycelia densities making them prone to contamination especially by the green mold. The suitability of different substrates for mushroom cultivation was also confirmed by the average biological efficiency which was variable among the substrates (Table 2 and Figure 1B). Wood chips and wheat straw shows highest yield (780 g) and biological efficiency (78%). It is clear that wheat straw in combination with wood chips are best substrate for oyster mushroom cultivation. Comparable results of *Pleurotus*

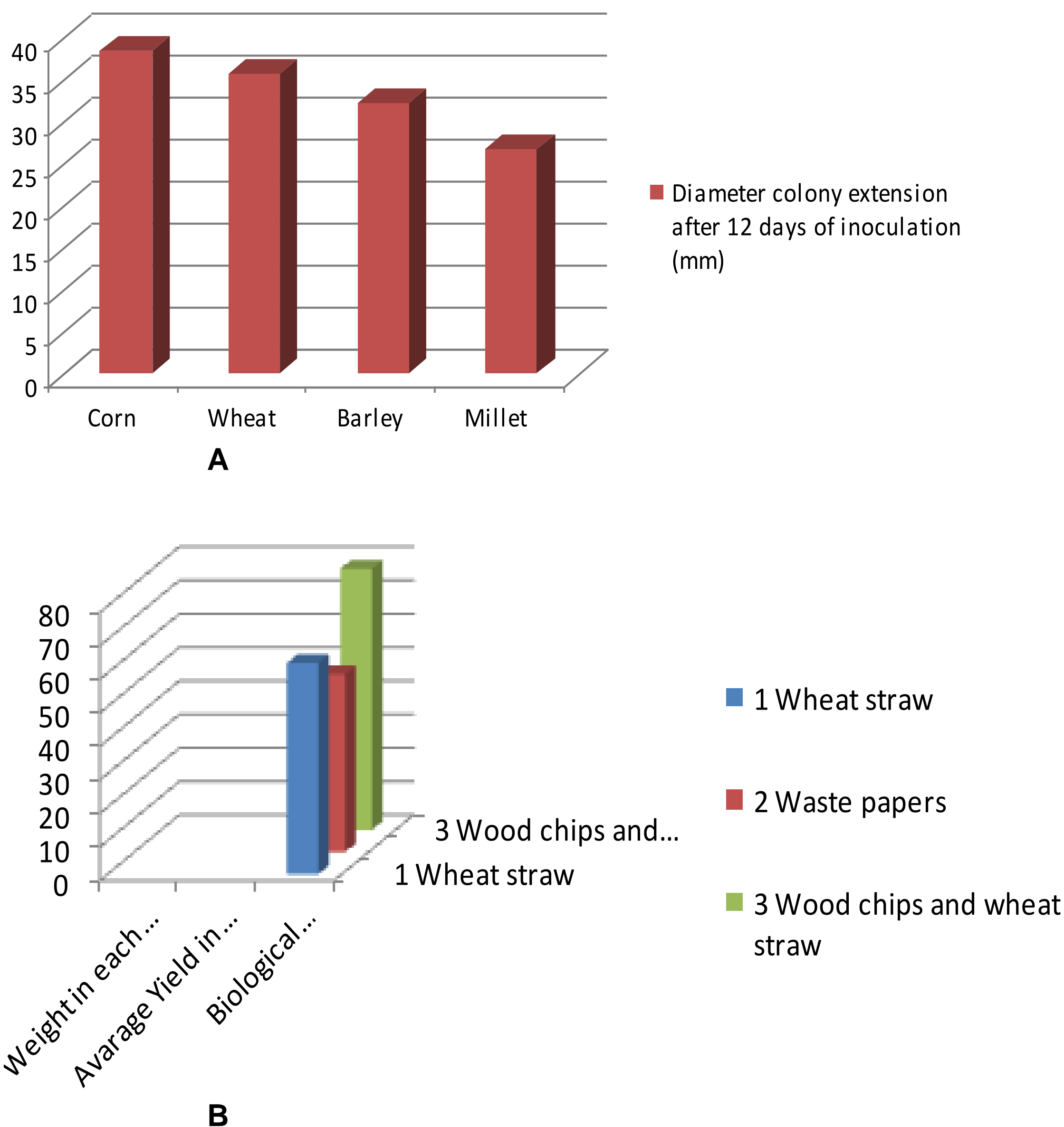

Figure 1. oyster mushroom harvested in three flushers.

Table 2. Showing the average yield and biological actions.

Substrate	Weight in each substrate (g)	Average Yield in three Flushes (g)	Biological efficiency (%)
Wheat straw	1000	630	63.00
Waste papers	1000	530	53.00
Wood chips and wheat straw	1000	780	78.00

species grown on different substrates has been earlier reported (Baysal et al., 2003; Shah et al., 2004; Mshandete, 2011). Mycelia of *Pleurotus* species are well known to colonize various lignocellulosic materials due to their extensive enzyme systems capable of utilizing complex organic compounds, which occurs in organic matter residues (Mane et al., 2007). Thus, a range of 12-14 days has been reported as time period of spawn running for various *Pleurotus* species on lignocelluloses substrates (Mane et al., 2007). Thus, the results from this

Figure 2. Substrate– waste paper.

Figure 3. Substrate– wheat straw.

Figure 4. Substrate– wood chips and wheat straw.

study concur with the previous spawn running periods reported by others on *Pleurotus* species.

Conclusion

Based on the investigations, mushroom growers are advised to develop their spawn on corn and wheat grains. It is evident that many organic substrates have high potential for utilization as substrates in mushroom cultivation. Mushrooms growers are advised to make the right choice of substrates based on the locally available materials. It is clear that wheat straw in combination with wood chips is best substrate for oyster mushroom cultivation.

Conflict of interest

The authors declare that they have no conflict of interest.

REFERENCES

Baysal E, Peker H, Yalinkilic MK, Temiz A (2003). Cultivation of oyster mushroom on waste paper with some added supplementary materials. Bioresour. Technol. 89(1):5-7.

Chang ST, Miles PG (1989). Edible mushrooms and their cultivation, CRC Press, Boca Raton. 256-274.

Garcha HS, Khann PK and Soni GL (1993). Nutritional Importance of Mushroom. In S. T. Chang J.A.Buswell S. Chiu (eds). Mushroom biology and Mushroom Products. The Chinese University Press . 227-235.

Mane VJ, Patil SS, Syed AA, Baig MMV (2007). Bioconversion of low quality lignocellulosic agricultural wastes into edible protein *Pleurotus sajor-caju* (Fr.). Singer J. Zhejiang. Univ. Sci. (8):745-751.

Mshandete AM, Cuff J (2008). Cultivation of three types of indigenous wild edible mushrooms: *Coprinus Cinereus,Pleurotus flabellatus* and *Volvariella volvocea* on composted sisal decortications residue in Tanzania. Afr. J. Biotech. 7(24):4551-4562.

Mshandete AM (2011a). Submerged and solid-state fermentation of the mycelium of *Coprinus cinereus* (Schaeff.) S.Gray s. lato mushroom on sisal bole wastes supplemented with human urine inTanzania. Inter. J. Res. Pure Appl. Microbiol. 1(3):40-55.

Prakash P, Anuradha S, Dhanalakshmi PK, Niveditha N (2010). Application of statistical methods to optimize medium for increased yield of Oyster Mushroom (Pleurotus ostreatus). Int. J. Biol. Technol. 1(1):66-74.

Shah ZA, Ashraf M, Ishtiaq MC (2004). Comparative study on cultivation and yield performance of oyster mushroom (*Pleurotus ostreatus*) on different substrates (Wheat straw, Leaves and Sawdust). Pak. J. Nutr. 3(3):158-160.

Siddhant, Swapnil Y, Singh CS (2013). Spawn and Spawning Strategies for the Cultivation of Pleurotus eous (Berkeley) Saccardo. Int. J. Pharm. Chem. Sc. 2(3):1494-1500.

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Full Length Research Paper

Antimicrobial and antitumor activities of crude secondary metabolites from a marine fungus *Penicillium Oxalicum* 0312F$_1$

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The extract of fermentation product of the *P. oxalicum* strain 0312F$_1$ was isolated through gel (Sephadex LH-20) column chromatography to give fractions A, B, C, D, E and F. Then, antimicrobial activity was evaluated by duel culture method and micro-dilution method. As a result, the water soluble extract of marine fungus *Penicillium oxalicum* 0312F$_1$ had inhibitory activity against *Alternaria solani*. We also tested antitumor activity using methyl thiazolyl tetrazolium (MTT) method. The results showed that the fractions F gave the maximum inhibition rate (higher than 70%) against both human gastric cancer cell line (SGC-7901) (SGC-7901 cell line) and human hepatic cancer cell line (BEL-7404) (BEL-7404 cell line), at the concentration 0.5 mg/mL. While at the concentration of 1 mg/mL, the inhibition rates of fractions D, E, F against SGC-7901 cell line was higher than 70%, the inhibition rates of fractions C and F against BEL-7404 cell line was higher than 70%. There were antimicrobial and antitumor active fractions in secondary metabolites from marine fungus *P. oxalicum* 0312F$_1$.

Key words: Antimicrobial, antitumor, crude secondary metabolites, *Penicillium oxalicum*.

INTRODUCTION

Recently, the interest in studying extreme environments has increased. These habitats may accommodate new species, which may be used as unique sources of secondary metabolites of biotechnological or pharmaceutical potential. The species found in extreme environments may also produce novel chemical compounds (Stierle et al., 2006; Hujslová et al., 2010). Fungal plant pathogens, which infect all major crops, are a threat to global food security; they cause serious losses both in the field and post- harvest, and some may produce mycotoxins (Strange and Scott, 2005). Since the discovery of penicillin, the micromycetes have been

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famous as producers of secondary metabolites with biological activity, including antibacterial, antifungal,antitumor, immunosuppressant, cholesterol-lowering agents and mycotoxins (Shen et al., 2009; Lopes et al., 2012).

Penicillium sp. has been reported as bioactive for antitumor activity (Han et al., 2007) and antimicrobial activity (Li et al., 2001; Yang, 2002; Chen and Zhang, 2006; Tan et al., 2006; Wang et al., 2013). Compounds isolated from the EtOAc extract of *Penicillium* sp. exhibited both cytotoxic and antimicrobial activity (Subramani et al., 2013; Wang et al., 2013). Also, compounds isolated from crude extract of *Penicillium* sp. only showed cytotoxic activity (Rand et al., 2005). It is reported that compounds showed moderate cytotoxic activity against the human hepatocellular liver carcinoma cell line (Gao et al., 2011). There are also new compounds isolated from *Penicillium* sp. with cytotoxic activity, which should be unique resource for developing new pharmaceuticals (Lin et al., 2012; Li et al., 2013).

As part of our previous investigation on the anti-phytoviral, antimicrobial and antitumor activities of marine fungi, fungi attracted our attention because the fungi strains found exhibited relatively high bioactivity (Shen et al., 2009, 2010; Tan et al., 2012).

The aim of this study was to investigate the antimicrobial and antitumor activities of the crude extract of a marine fungus, *Pencilium oxalicum*. The water soluble extract had moderate antimicrobial activity against plant pathogenic fungi. And then, the water soluble extract was concentrated to give a fraction subjected to Sephadex LH-20 to afford six fractions, which had moderate to high potential antitumor activity. The results will guide us to isolate active compounds from active crude fractions by the bio-guided method.

MATERIALS AND METHODS

Tested strains

Marine-derived *P. oxalicum* strain $0312F_1$ (Genbank accession no.EU926977), and the pathogenic fungi (*Colletotrichum orbiculare*, *Fusarium oxysporum*, *Pythium aphanidermatum*, *Colletotrichum graminicola*, *Rhizoctonia cerealis*, *Alternaria solani* and *Fusarium graminearum Schwabe*) were all maintained on potato dextrose agar (PDA) medium. And they were all incubated at 28°C for 48 h and then stored at -20°C in tubes with PDA medium in fridge.

Cancer cell lines

Human gastric cancer cell line (SGC-7901) and hepatic cancer cell line (BEL-7404) were cultured under 5% CO_2 at 37°C in RPMI 1640 medium supplemented with foetal bovine serum and penicillin-streptomycin (100 IU/mL).

Extraction procedure

The marine-derived *P. oxalicum* strain $0312F_1$ was cultured at 28°C for 7 days in Erlenmeyer flasks (1 L), each containing 100 g rice and 200 mL glucose–peptone–yeast medium. The mycelium and medium were twice extracted with MeOH (*ca* 30 L). The extract was concentrated *in vacuo* to yield a residue, which was dissolved in H_2O and filtered. The water-soluble fraction was then desalted by Dianion-HP20 column eluting by H_2O. The MeOH extract was concentrated to give a fraction of 7.8 g, which was subjected to Sephadex LH-20, eluted with MeOH-H_2O (0, 1:20, 1:10, 1:5, 2:5, 1) to afford six fractions (A: 1.2 g, B: 1.7 g, C: 1.8 g, D: 1.9 g, E: 0.8 g, F: 0.4 g).

Antimicrobial activity assay

The inhibitory activity of marine fungus $0312F_1$ against pathogenic fungi was evaluated by duel culture method (Shen et al., 2010). And the inhibitory activity of crude extract of marine fungus $0312F_1$ against pathogenic fungi was evaluated by micro-dilution method (Ríos Dueňas et al., 2011). The concentration of crude extract of marine fungus $0312F_1$ against pathogenic fungi was 2 mg/mL. And the concentrations of crude extract of marine fungus $0312F_1$ in EC_{50} evaluation were 2, 1, 0.5, 0.25 and 0.125 mg/mL, respectively.

Cytotoxic activity assay

Human gastric cancer cell line SGC-7901 and hepatic cancer cells BEL-7404 were plated into 96-well tissue culture dishes at a density of 1×10^3 per well in 180 mL medium and allowed to attach at 37°C for 24 h, followed by the addition of 20 mL solutions of different fractions of crude extract samples (dimethyl sulfoxide (DMSO) was added as the vehicle, ≤1%), incubated at 37°C for another 72 h. Then, 20 mL methyl thiazol tetrazolium (MTT) (2 mg/mL) was added to each well and incubated at 37°C for 4 h. Finally, the supernatants were removed and the formazan crystals were dissolved by adding 200 mL DMSO. The absorbance at 570 nm was determined by a microplate reader. 5'-Fluorouracil and cis-diaminedichloroplatinum (CDDP) were used as positive control (Shen et al., 2009).

Statistical analysis

The IC_{50} values were determined from concentration-effect curves by linear regression analysis. Statistical analysis was performed using SPSS version 13.0, and data were presented as the arithmetic mean ± standard deviation.

RESULTS AND DISCUSSION

Inhibitory activity of marine fungus $0312F_1$ against pathogenic fungi

The inhibitory activity of marine fungus $0312F_1$ against pathogenic fungi was evaluated by duel culture method. Table 1 shows that inhibitory activity of strain $0312F_1$ against pathogenic fungi was weak.

Inhibitory activity of crude extract of marine fungus $0312F_1$ against pathogenic fungi

In Table 2, the crude extract of marine fungus $0312F_1$

Table 1. Inhibitory effect of marine fungus $0312F_1$ against pathogenic fungi.

Pathogenic fungi	Inhibition rate (%)
C. orbiculare	37.21±1.82
F. oxysporum	35.90±1.27
P. aphanidermatum	39.58±2.03
C. graminicola	21.74±0.93
R. cerealis	26.67±1.27
A. solani	12.50±1.22
F. graminearum Schwabe	20.70±0.92

Values are mean of three replicates.

Table 2. Inhibitory activity of crude extract of marine fungus $0312F_1$ against pathogenic fungi.

Pathogenic fungi	Inhibition rate (%)
C. orbiculare	-
F. oxysporum	15.91±1.81[b]
P. aphanidermatum	6.67±2.02[c]
C. graminicola	-
R. cerealis	-
A. solani	58.62±0.92[a]
F. graminearum Schwabe	56.00±0.76[a]

Values are mean of three replicates. The concentration of water soluble extract was 2 mg/mL; -: no inhibitory activity. Means in the same column with different letters are significantly different (n=3, P<0.05).

was determined for inhibitory activity against pathogenic fungi. The metabolites displayed weak to high antimicrobial activity against all pathogenic fungi. The metabolites showed much higher antimicrobial activity against *A. solani* and *F. graminearum Schwabe*, with nearly 60% inhibition. The results indicated that the active fraction of metabolites of strain $0312F_1$ should be in water soluble extract.

A large number of marine-derived fungal extracts have antimicrobial activity mainly related to *Penicillium* species (Shen et al., 2010; Lopes et al., 2012). New antifungal compounds usually come from natural sources, which involves screening of microorganisms and plant extracts (Augustine et al., 2005; Bevan et al., 1995).

Growth of 7 plant pathogens in controlled culture and culture of water soluble extract from strain $0312F_1$ was evaluated by micro-dilution method. *R. cerealis* was the fastest growing of all the controlled plant pathogens (Figure 1E). After inoculation for two days, its radius was 4.5 cm, while *C. graminicola* was the most slowly growing. Growth of 7 plant pathogens increased in a time dependent manner (Figure 1). In Figure 1F, after inoculation with water soluble extract, *A. solani* was growing slowly. The phenomenon indicated that the crude extract of strain $0312F_1$ had definite inhibitory activity against *A. solani*.

The EC_{50} value of water soluble extract of strain $0312F_1$ against *A. solani* was then determined. The inhibition rates were 58.60, 40.00, 32.00, 21.33 and 9.02%, under the concentrations of 2, 1, 0.5, 0.25 and 0.125 mg/mL, respectively. The EC_{50} value was 1.43 mg/mL by calculation (Figure 2). The results indicated that the inhibition rates increased in a concentration dependent manner.

Cytotoxic activity of fractions of crude extract of marine fungus $0312F_1$

The culture extracts of *Penicillium* strains possessing some extent of antifungal ability were evaluated as a possible source of anti-tumor products on human tumor cell lines (Nicoletti et al., 2008). Our MTT assay indicated the inhibitory activity of fractions D, E and F against SGC-7901 cells, which were afforded after water soluble extract was subjected to Sephadex LH-20, and was higher than 70% (with inhibition 70.04, 71.35, and 79.30%, respectively) under the concentration of 1 mg/mL. Furthermore, the inhibition rate of fractions F was high (up to 73.24%) under the concentration of 0.5 mg/mL (Figure 3).

Similarly, the inhibitory activity of fractions C and F against BEL-7404 cells was higher than 70% (with inhibition of 72.38 and 78.13%, respectively). Under the concentration of 0.5 mg/mL, the inhibition rate of fractions F was also high (up to 74.74%) (Figure 4).

Secondary metabolites produced by microorganisms are the main source of the skeletons; some have been used directly in field (Yang et al., 2008). Cytotoxic compounds should be isolated from the active fractions C-F. And fraction F was the most active fraction to obtain cytotoxic compounds against SGC-7901 and BEL-7404 cells.

Conflict of Interests

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

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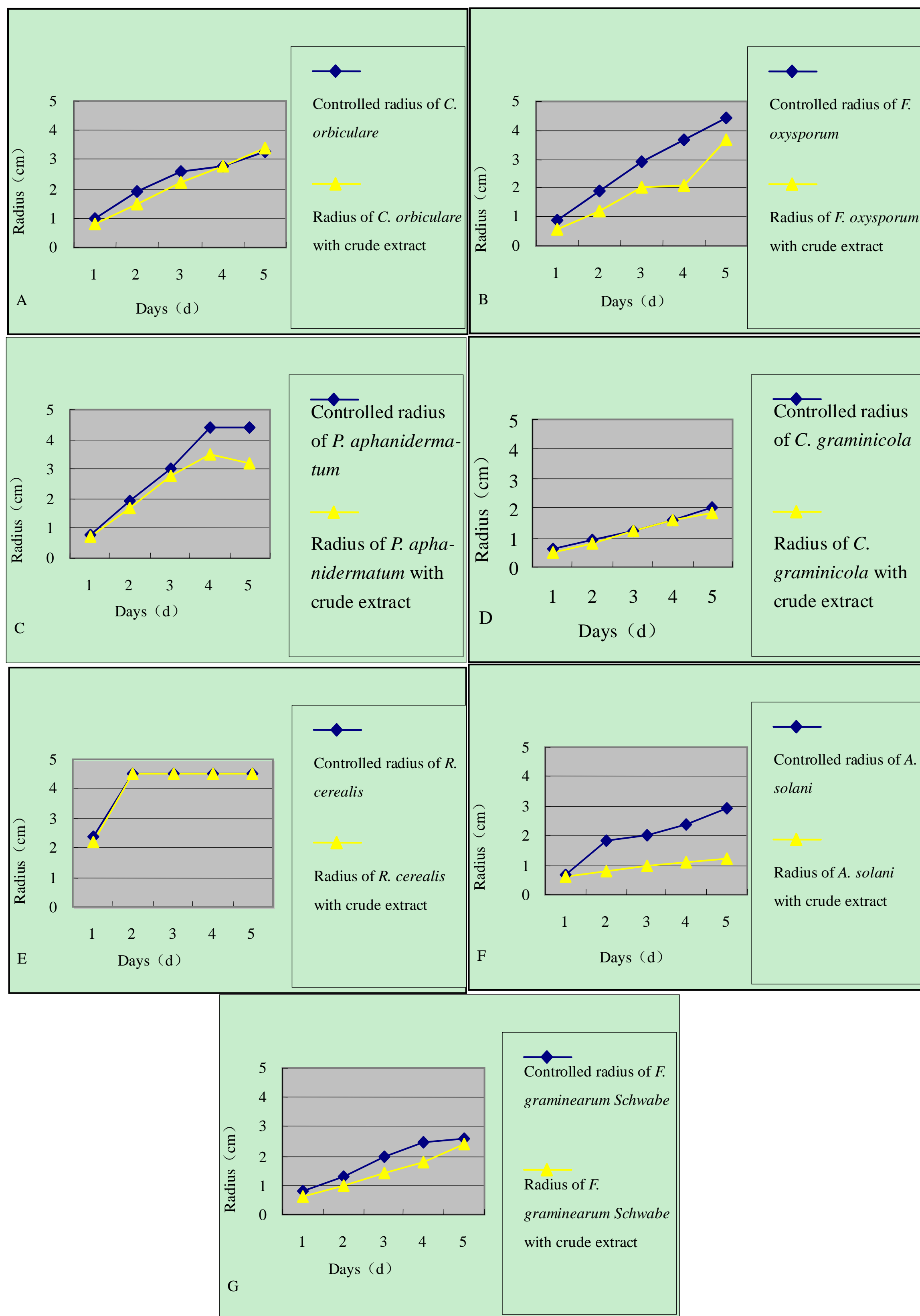

Figure 1. Inhibitory effects of water soluble extract from strain $0312F_1$ on the growth of plant pathogens in controlled culture and culture of water soluble extract from strain $0312F_1$. A-G: Growth of 7 plant pathogens in controlled culture and culture of water soluble extract from strain $0312F_1$.

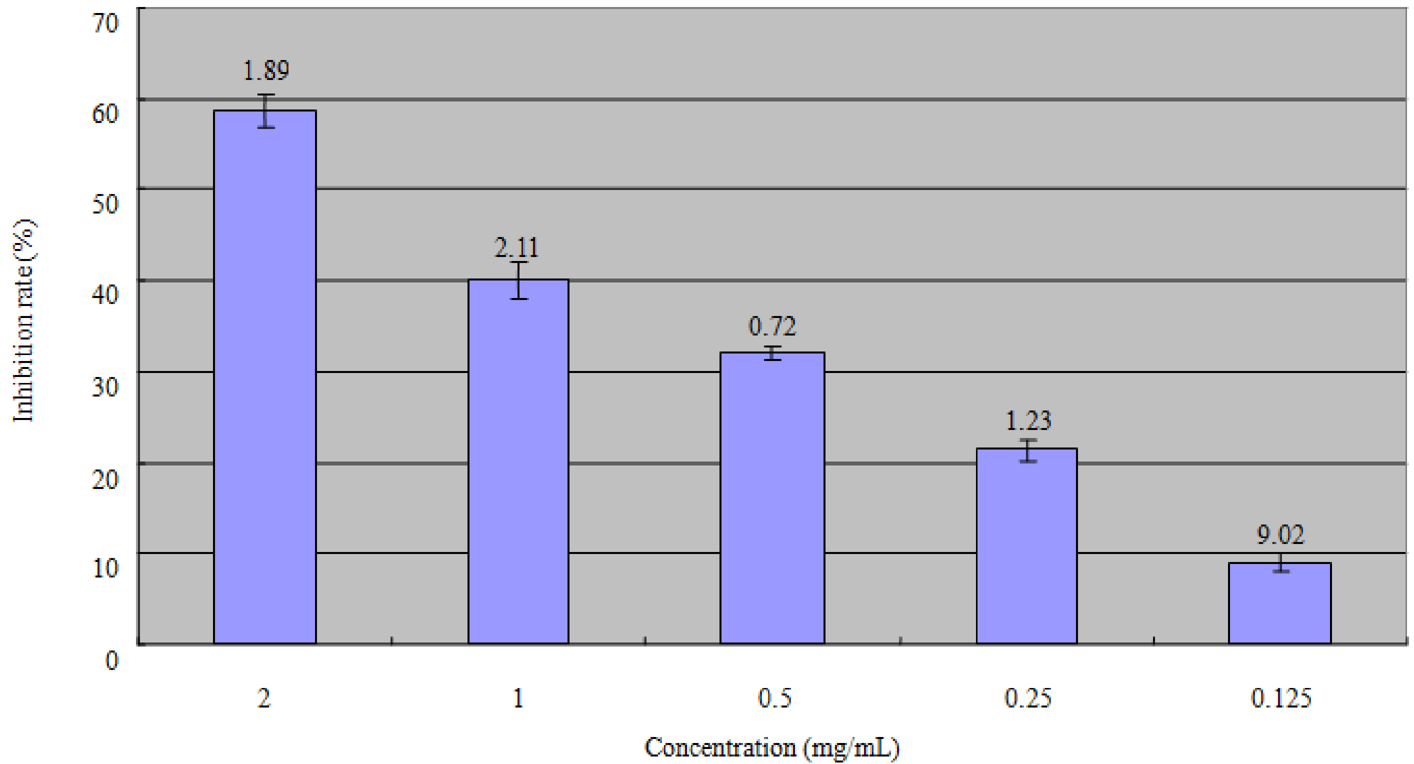

Figure 2. Inhibitory activity of crude extract of marine fungus $0312F_1$ against *A. solani.* Values are mean of three replicates.

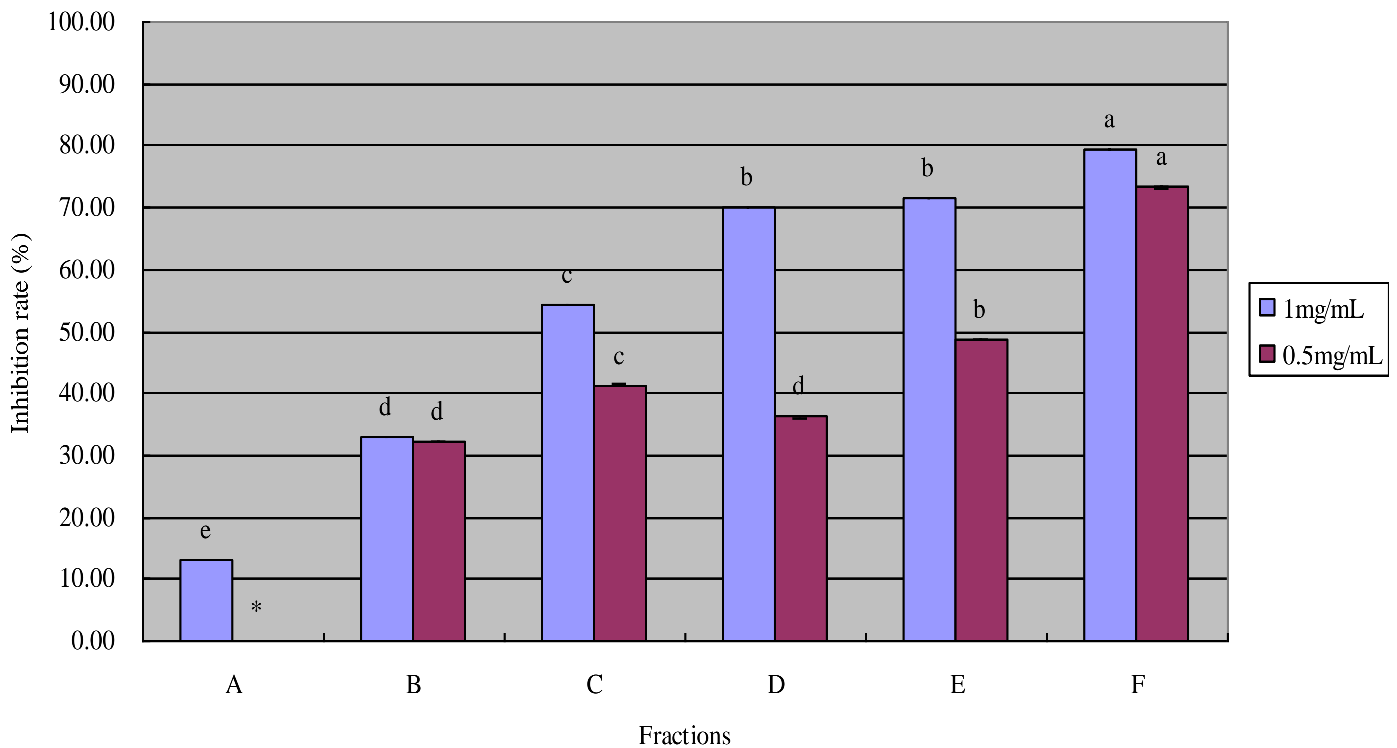

Figure 3. Inhibitory activity of fractions of crude extracts of strain $0312F_1$ against SGC-7901 cells. Values are mean of three replicates. Different letters in the column with the same color (at the same concentration) are significantly different ($P<0.05$); *: no inhibitory activity.

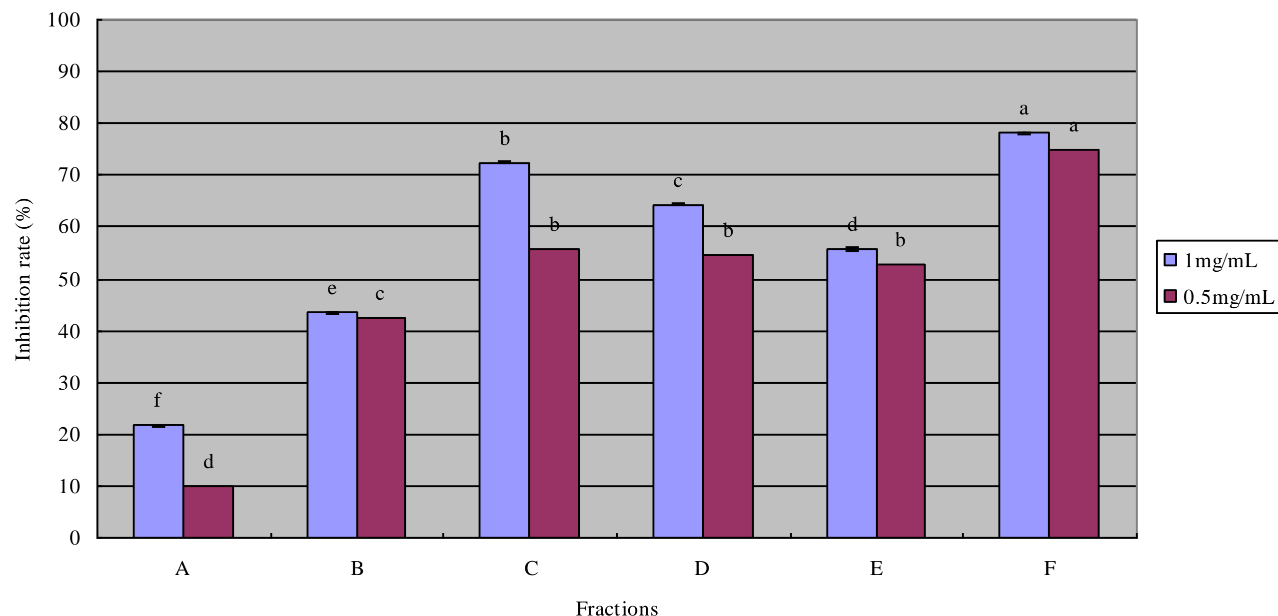

Figure 4. Inhibitory activity of fractions of crude extracts of strain 0312F$_1$ against BEL-7404 cells. Values are mean of three replicates. Different letters in the column with the same color (at the same concentration) are significantly different (P<0.05).

REFERENCES

Augustine SK, Bhavsar SP, Kapadnis BP (2005). A non-polyene antifungal antibiotic from Streptomyces albidoflavus PU 23. J. Biosci. 30(2):201-211.

Bevan P, Ryder H, Shaw I (1995). Identifying small-molecule lead compounds: the screening approach to drug discovery. Trends Biotechnol. 13: 115-121.

Chen BE, Zhang SL (2006). Antimicrobial characteristics of marine fungi Aspergillus sp. MF134. J. Huaqiao Univ. 27: 307-309.

Gao SS, Li XM, Du FY, Li CS, Proksch P, Wang BG (2011). Secondary metabolites from a marine-derived endophytic fungus Penicillium chrysogenum QEN-24S. Mar. Drugs 9: 59-70.

Han XX, Xu XY, Cui CB, Gu QQ (2007). Diketopiperazines produced by marine-derived Aspergillus fumigatus H1-04 and their antitumor activities. Chin. J. Med. Chem. 17: 155-159.

Hujslová M, Kubátová A, Chudíčková M, Kolařík M (2010). Diversity of fungal communities in saline and acidic soils in the Soos National Natural Reserve, Czech Republic. Mycol. Prog. 9: 1-15.

Li CS, Li XM, Gao SS, Lu YH, Wang BG (2013). Cytotoxic anthranilic acid derivatives from deep sea sediment-derived fungus Penicillium paneum SD-44. Mar. Drugs 11: 3068-3076.

Li SB, Wang J, Yang JS, Zhong YC, Zhou SN, Lin YC (2001). Isolation identification and characterization of a fungus strain producing antifungal antibiotic. Mycosystema 20: 362-367.

Lin XP, Zhou XF, Wang FZ, Liu KS, Yang B, Yang XW, Peng Y, Liu J, Ren Z, Liu YH (2012). A new cytotoxic sesquiterpene quinine produced by Penicillium sp. F00120 isolated from a deep sea sediment sample. Mar. Drugs 10:106-115.

Lopes FC, Tichota DM, Sauter IP, Meira SMM, Segalin J, Rott MB, Rios AO, Brandelli A (2012). Active metabolites produced by Penicillium chrysogenum IFL1 growing on agro-industrial residues. Ann. Microbiol. 63(2): 771-778.

Nicoletti R, Buommino E, De Filippis A, Lopez-Gresa MP, Manzo E, Carella A, Petrazzuolo M, Tufano MA (2008). Bioprospecting for antagonistic Penicillium strains as a resource of new antitumor compounds. World J. Microbiol. Biotechnol. 24: 189-195.

Rand TG, Giles S, Flemming J, Miller JD, Puniani E (2005). Inflammatory and cytotoxic responses in mouse lungs exposed to purified toxins from building isolated Penicillium brevicompactum Dierckx and P. chrysogenum Thom. Toxicol. Sci. 87(1): 213-222.

Ríos Dueňas E, Rodríguez-Avial I, Picazo JJ (2011). In vitro activity of ceftobiprole and seven other antimicrobial agents against invasive Streptococcus pneumoniae isolates in Spain. Eur. J. Microbiol. Infect. Dis. 30: 1621-1625.

Shen S, Li W, Ouyang MA, Wu ZJ, Lin QY, Xie LH (2009). Identification of two marine fungi and evaluation of their antivirus and antitumor activities. Acta. Microbiol. Sin. 49: 1240-1246.

Shen S, Li W, Ouyang MA, Xie LH (2010). Identification of two marine fungi and anti-microbial activities of their metabolites. Chin. J. Biol. Control 26: 62-68.

Stierle AA, Stierle DB, Kelly K (2006). Berkelic Acid, a novel spiroketal with selective anticancer activity from an acid mine waste fungal extremophile. J. Org. Chem. 71:5357-5360.

Strange RN, Scott PR (2005). Plant disease: a threat to global food security. Annu. Rev. Phytopathol. 43: 83-116.

Subramani R, Kumar R, Prasad P, Aalbersberg W (2013). Cytotoxic and antibacterial substances against multi-drug resistant pathogens from marine sponge symbiont: citrinin, a secondary metabolite of Penicillium sp. Asian Pac. J. Trop. Biomed. 3(4):291-296.

Tan QW, Ouyang MA, Shen S, Li W (2012). Bioactive metabolites from a marine-derived strain of the fungus Neosartorya fischeri. Nat. Prod. Res. 26: 1402-1407.

Tan ZQ, Zhang RY, Yang JS, Peng XF (2006). Antagonistic action comparison of marine *Penicillium* sp. T03 to four plant pathogens. Chin. J. Biol. Control 22: 112-114.

Wang LW, Lu CH, Xu QY, Song SY, Hu ZY, Zheng ZH (2013). Four new citrinin derivatives from a marine-derived Penicillium sp. fungal strain. Molecules 18:5723-5735.

Wang MH, Li XM, Li CS, Ji NY, Wang BG (2013). Secondary metabolites from Penicillium pinophilum SD-272, a marine sediment-derived fungus. Mar. Drugs 11:2230-2238.

Yang JS (2002). Antimicrobial activity of secondary metabolites of marine Penicillum spp. Chin. J. Trop. Crops. 23: 73-76.

Yang LP, Xie JT, Jiang DH, Fu YP, Li G.Q, Lin FC (2008). Antifungal substances produced by Penicillium oxalicum strain PY-1—potential antibiotics against plant pathogenic fungi. World J. Microbiol. Biotechnol. 24: 909-915.

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Full Length Research Paper

Phylogenetic analysis of β subunit of the sulfite reductase gene from *Colletotrichum lindemuthianum* and fungi with different lifestyles

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Previous studies have demonstrated that the machinery for sulfate assimilation influences the capacity of pathogenic microorganisms to invade a minor or major range of hosts, and this machinery appears to be dispensable when the lifestyle of the organism allows it. *Colletotrichum lindemuthianum* is the etiological agent of anthracnose, which is an important disease of beans (*Phaseolus vulgaris*) and an interesting model of study due to its hemibiotrophic lifestyle. We report the isolation, sequence and analysis of a 442 bp DNA fragment from the *Clsir* gene, which encodes the β subunit of sulfite reductase from *C. lindemuthianum*. The *Clsir* nucleotide sequence showed an identity of 97% with *Trichoderma atroviride*, 81% with *Gibberella zeae*, 77% with *Penicillium chrysogenum*, *Arthroderma otae* and 74% with *Leptosphaeria maculans*. In addition, we performed a phylogenetic analysis of the deduced amino acid sequence of *Clsir* and 48 reported *SiR* sequences from fungi with different lifestyles. The distance tree showed diverse clusters of fungi related with different lifestyles and host types: pathogens of animals, saprophytic/opportunistic, phytopathogens, endophytes and mycoparasites/saprophytes. The deduced amino acid sequence of ClSiR from *C. lindemuthianum* was grouped with the sequences of SiRs from *T. atroviride*, *Trichoderma virens* and *Trichoderma reesei*. Based on the fact that *T. atroviride* is a mycoparasite, this result suggests horizontal gene transfer.

Key words: *Colletotrichum lindemuthianum*, sulfite reductase, pathogen, lifestyle, host type.

INTRODUCTION

The ascomycete *Colletotrichum lindemuthianum* is an economically important phytopathogen, and this fungus and its host, *Phaseolus vulgaris,* provide a convenient model for studying the physiological and molecular bases of plant-pathogen interactions (Perfect et al., 1999). *C. lindemuthianum* is an intracellular hemibiotrophic pathogen with physiological races that invade the plant in an interaction consistent with the gene-for-gene model (Flor, 1971). Monogenic dominant resistance in common bean cultivars leads to the appearance of localized

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necrotic spots that are typical of the hypersensitive response (HR) (O'Connell and Bailey, 1988). After penetration of a host epidermal cell in a susceptible cultivar, the pathogenic races of *C. lindemuthianum* develop an infection vesicle and extend into adjacent cells by producing large primary hyphae. These hyphae invaginate without penetrating the host cell membrane, allowing the fungus to persist in a biotrophic interaction. Once a large area of the plant tissue has been colonized, necrotrophic hyphae develop (O'Connell and Bailey, 1988); this event closely correlates with the production of a number of host cell wall-degrading enzymes that are characteristic of phytopathogenic fungi (Wijesundera et al., 1989; Knogge, 1998; Dodds et al., 2009). For *C. lindemuthianum,* as for other phytopathogenic ascomycetes, basidiomycetes and oomycetes, their lifestyle requires hydrolytic enzymes that degrade the cell wall of plants and genes that induce cellular death, a metabolic process necessary for free-living organisms (Dodds, 2010). Obligate biotrophic fungi and oomycetes have lost many of the hydrolytic enzymes that digest the host wall cell, which suggests a selection for stealth, allowing microorganisms to avoid triggering host defense responses during extended biotrophic interactions (Dodds, 2010). In addition, it has been reported that the oomycete *Hyaloperonospora arabidopsidis,* an obligate biotrophic pathogen of *Arabidopsis thaliana,* has lost sulfate and nitrate assimilation enzymes; as this oomycete grows exclusively in living plant leaves, *H. arabidopsidis* relies on access to reduced nitrogen and sulfur from host cells (Dodds, 2010).

The metabolic processes of nitrate and sulfate assimilation require the enzymes nitrite reductase (NiR) (EC 1.7.7.1.) and sulfite reductase (SiR) (EC 1.8.7.1.). These enzymes are found in archaea, bacteria and eukaryotes, and they catalyze the six-electron reductions of sulfite to sulfide and of nitrite to ammonium during the assimilation and biological dissimilation of compounds containing sulfur and nitrogen. Both enzymes are metalloproteins that belong to a small group of proteins that contain a siroheme group, which is a tetrahydroporphyrin of the bacterioclorin class. SiRs are a family of homoproteins with two to four identical subunits; each subunit consists of a siroheme group bound to a [Fe4-S4] complex by endogenous cysteine connections (Stroupe and Getzoff, 2001). The sequences of these enzymes present a conserved domain for the union of the siroheme group and the [Fe-S] complex, which facilitates electron transport to the substrate. These enzymes have a high level of polymorphism at the structural level and display differences in absorption spectrum, electrophoretic mobility and redox properties (Wagner et al., 1998; Dhillon et al., 2005).

Due to the evolutionary importance of this process, phylogenetic studies of sulfur metabolism genes have been performed. The results of these studies showed a low correlation with results obtained using other methods or phylogenetic tools, but interest in such studies is rising due to the structural variability of these enzymes, the relationship between the genes required for sulfur metabolism and those required for nitrogen metabolism, and the ability of SiRs and NiRs to act on each other's substrates. Phylogenetic studies of sulfur metabolism include alignments of genes and/or structural comparisons of ATP sulfurilase (ATPS), adenosil sulfate reductase (ASR), and dSiR from eukaryotes and prokaryotes (Patron et al., 2008). In eukaryotes, analyses of ATPS, sulfide quinone oxidoreductase (SQR), adenosine 5'-phosphosulfate reductase (APR) and aSiR genes resulted in the grouping of super-families/cohorts; however, the results did not reflect supported lines. The reported data suggest different origins for the known lineages, but the data available in the GenBank database are limited. An idea that continues to take force is the process of lateral transference of genes in eucaryotes-procaryotes for enzymes of different assimilatory and disassimilatory complexes (Patron et al., 2008).

Since phylogenetic studies of sulfur metabolism genes can contribute to an improved understanding of the evolution of this system in fungi, the objective of this study was to identify and isolate a conserved fragment of the β subunit of the *Clsir* sulfite reductase from *C. lindemuthianum* and perform a phylogenetic comparison of deduced amino acid sequence of this fragment with the corresponding sequences of SiRs from fungi with different lifestyles.

MATERIALS AND METHODS

Strain and culture conditions

C. lindemuthianum race 1472 was kindly provided by Dr. June Simpson (CINVESTAV-IPN, Unidad Irapuato, México) which was reported and characterized by interaction with differential varieties of bean (*Phaseolus vulgaris*) and molecular strategies (RAPD and AFLP) as the pathotype 1472 by González et al. (1998) and subsequently analyzed by Rodríguez et al. (2006). *C. lindemuthianum* was maintained on potato dextrose agar (PDA) (Difco, México) at 20°C. For induction, 125-ml Erlenmeyer flasks containing 50 ml of modified Mathur's medium, which was made according to the protocol of Acosta-Rodríguez et al. (2005), supplemented with 2.5% cellulose (Sigma-Aldrich, St. Louis, MO, USA) (w/v), were inoculated with 1.6 mg dry weight (approximately 5 cm^2) of a 9 day-old colony growing grown on PDA and incubated at 20°C with continuous shaking (150 rpm). After 10 days, the culture was centrifuged at low speed, and the mycelia were harvested.

DNA extraction, PCR amplification and cloning of a fragment of the *Clsir* gene

To amplify a fragment of the *SiR* gene from *C. lindemuthianum,* total DNA from *C. lindemuthianum* was purified according to standard protocols (Sambrook and Russell, 2001) and PCR amplified using an upstream primer (5´-GATGGACGACATTGCCAAC-3´) and a downstream primer (5´-ACCACCGGCGAGCAGGTTGAAAC -3´) that were designed using

the sequence from *Neurospora crassa* (GenBank Access XM_956909.2). The PCR reaction mixture was heated at 94°C for 2 min in an thermocycler (Eppendorf Master Cycler Gradient Brinkmann, Westbury, NY), followed by 30 cycles of denaturation for 30 s at 94°C, annealing for 35 s at 50°C, extension for 2 min at 72°C and a final extension for 7 min at 72°C. PCR products of 300 to 650 bp were obtained, ligated into the pGEM-T vector system I (Promega, Madison, WI) and introduced into an *Eco*RI XL1-Blue strain (Sambrook and Russell 2001). Four clones were selected after digestion with the *Eco*R1 restriction enzyme (New England Biolabs, Beverly, MA), and the DNA *Cl/p6* clone was selected for sequencing.

DNA sequencing and analysis

The sequences of both strands of the DNA were determined using the dideoxy-chain termination method by automatized sequencing (ABI Prism Dye Cycle Sequencing Ready Reaction Kit) in an ABI PRISM 310 DNA sequencer (Applied Biosystems, Foster City, CA). The obtained nucleotide sequences were analyzed using DNAsis software (Hitachi) and BLAST GenBank data.

Phylogenetic analysis

Phylogenetic analysis was performed using the deduced amino acid sequences (141 bp) of *Clsir* fragment and those of 48 previously reported *SiR* genes (Table 1). The protein sequences were aligned using Clustal W softwar e (Larkin et al., 2007) with a BLOSUM 30 matrix and the default parameters.

Phylogenetic analysis was performed under Maximum Likelihood (ML), Bayesian method and Neighbor-Joining (NJ) criteria using the Mega v5 (Tamura et al., 2011) and MrBayes v 3.1.2 (Huelsenbeck et al., 2001) software programs. For the NJ method, a JTT matrix was used (Dayhoff et al., 1978), and 1000 bootstrap replicates were performed. For ML analyses, a WAG+F matrix was used, according the ProTest 2.4 analyses (Abascal et al., 2005), with gamma correction and 1000 bootstrap replicates were performed. We used the amino WAG+F evolution model (Whelan et al., 2001) for Bayesian analysis. In total, 10,001 trees were obtained based on the settings ngen = 1,000,000 and simple freq = 100 for Bayesian criteria. We checked the convergence of overall chains (4) when the log likelihood values reached the stationary distribution. The first 2,500 trees were burn-in and discarded, and a 50% majority rule consensus tree of the remaining trees was generated. We used the *Bacillus coagulans Sir* [GenBank: AEH53460] as an outgroup for the analyses. The phylogenetic tree was edited using Dendroscope software (Huson et al., 2007).

RESULTS

Sequence analysis

The DNA fragment of *Clsir* (GenBank Access: KF487128) has 442 bp. A comparison of the *Clsir* nucleotide sequence showed 97% identity with the corresponding *SiR* sequence from *Trichoderma atroviride*, 81% with *Gibberella zeae*, 77% with *Penicillium chrysogenum* and *Arthroderma otae,* and 74% with *Leptosphaeria maculans*. The deduced amino acid sequence of ClSiR (147 aa) showed 75% identity with the conserved domain of the β subunit that contains the [4Fe-4S] group from *T. atroviride*, 65% identity with the sequence from *T. reesei* and 64% identity with the sequence from *G. zeae*.

Phylogenetic analyses

A Clustal alignment of 49 deduced amino acid sequences for SiR from fungi with different lifestyles indicated that these proteins, including the *B. coagulans* sequence that was used as external group for the phylogenetic analysis, share highly conserved domains (Figure 1). The general topology of the phylogenetic analysis of the amino acid sequences is represented in the tree generated by the Maximum Likelihood method, in which the ML and NJ bootstrap support and the Bayesian posterior probabilities as indicated on the branches (Figure 2).

According to the results, the SiR sequences from the analyzed fungi were separated into two basal clades. One of these clades includes most of the SiRs, which are distributed among a number of sub-clades that correspond to different lifestyles: saprophytic/oppor-tunistic fungi, such as *Aspergillus* species, that are often associated with plants and human diseases (99% posterior probability for the Bayesian analysis and 71 and 53% bootstrap support for the ML and NJ methods, respectively); common fungal pathogens of humans and animals that possess the ability to develop as saprophytes for part of their life cycle (84% posterior probability and 82 and 80% bootstrap support); endophytic fungi of the genera *Epichloe* and *Metarhizium* that commonly form symbiotic relationships with plants and are insect pathogens (81 and 96% bootstrap support); plant pathogenic fungi that are positioned near the endophytic group in the phylogenetic analysis (94% posterior probability and 89 and 89% bootstrap support); plant pathogenic fungi of the genus *Colletotrichum* (98% posterior probability and 79 and 89% bootstrap support); and a cosmopolitan group of pathogenic fungi, such as *Fusarium* and *Verticillium* species, that have a wide host range and often behave as saprophytes (posterior probability 89, 38 and 61% bootstrap support).

In addition, a second basal clade was observed in which the ClSiR from *C. lindemuthianum* was grouped with the amino acid sequences from the mycoparasites/saprophytes *T. atroviride*, *T. virens* and *T. reesei* (72% posterior probability and bootstrap support of 89 and 92% for the ML and NJ methods, respectively) (Figure 2).

DISCUSSION

The phylogenetic analysis of the deduced amino acid sequences of 49 fungal SiRs (including ClSiR) corresponding to the conserved domain of the β subunit of sulfite reductase indicated that SiRs tend to group together according to the lifestyle and the host type of the analyzed fungi.

The increased availability of sequenced fungal and

Table 1. Sequences of *SiRs* genes used on phylogenetic analysis.

Microorganism	GenBank Access
Aspergillus flavus	XP_002375562
Aspergillus oryzae	XP_001727314
Aspergillus terreus	XP_001211603
Aspergillus niger	XP_001397106
Aspergillus clavatus	XP_001275842
Aspergillus fumigatus	XP_755894
Neosartorya fischeri	XP_001261021
Aspergillus nidulans	XP_680869
Coccidioides posadasii	EFW18322
Coccidioides immitis	XP_001246498
Uncinocarpus reesii	XP_002540834
Arthroderma otae	XP_002844117
Arthroderma gypseum	XP_003172274
Trichophyton rubrum	XP_003237040
Trichophyton tonsurans	EGD97005
Paracoccidioides brasiliensis	EEH18359, EEH47297
Ajellomyces dermatitidis	EGE82222, XP_002623052
Ajellomyces capsulatus	EEH06826, EER38492, EGC47637
Penicillium marneffei	XP_002145392
Talaromyces stipitatus	XP_002487153
Glomerella graminicola	EFQ30629, ACOD010
Colletotrichum higginsianum	CCF35724
Magnaporthe oryzae	AHZT0100203
Epichloe typhina	AFSE01000003
Epichloe brachyelytri	AFRB0100
Neotyphodium gansuense	AFRE010
Metarhizium acridum	EFY85783
Metarhizium anisopliae	AFZ01510
Leptosphaeria maculans	CBX93782
Phaeosphaeria nodorum	XP_001799010
Pyrenophora tritici-repentis	XP_001939342
Verticillium dahliae	EGY20529
Verticillium albo atrum	302406933
Cordyceps militaris	AEVU010000
Gibberella zeae	XP_382658
Nectria haematococca	ACJF01000
Gibberella moniliformis	AAIM02
Fusarium oxysporum	AFQF0100343, AGNF0100022
Trichoderma reesei	EGR52094
Trichoderma virens	EHK15840
Trichoderma atroviride	EHK46277
Bacillus coagulans	AEH53460

oomycete genomes has allowed for the observation of important differences between related microorganisms that have different lifestyles. Among the most notable differences can mention genome size, differences in the number of genes encoding pathoge-nesis factors or lack of them, as well as secreted proteins associated with pathogenesis, genes that encode the enzymes of nitrogen and sulfur assimilation systems and in general genes involved in primary and secondary metabolism (Baxter et al., 2010; Dodds, 2010; Kubicek et al., 2011). These differences were first documented between obligate biotrophic and fungal pathogens (Baxter et

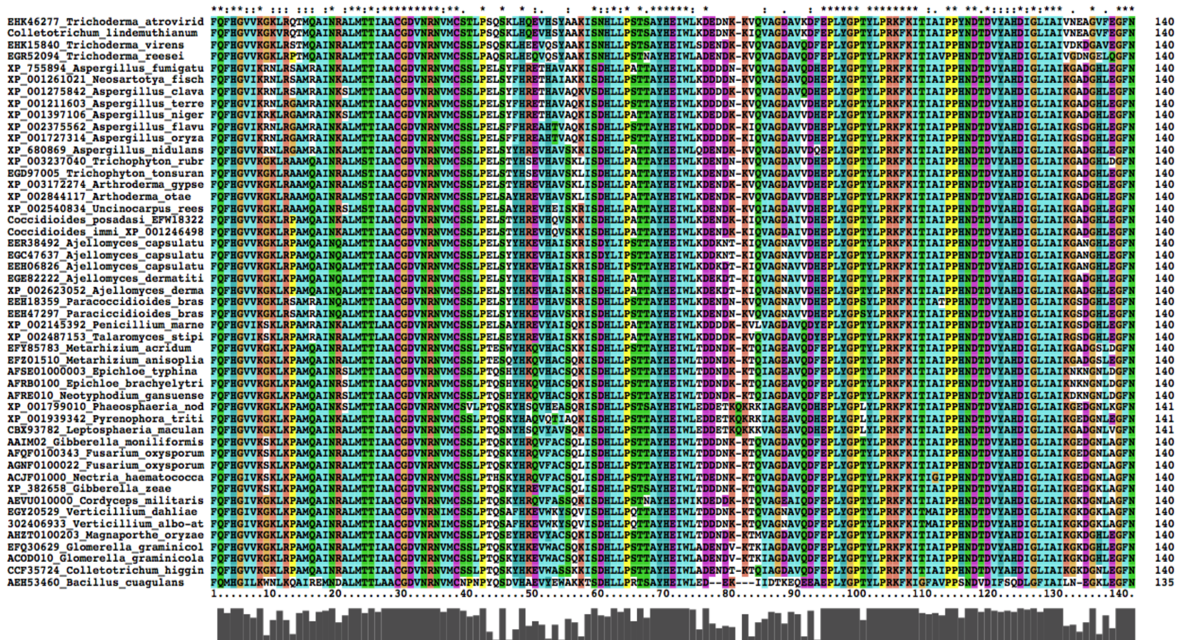

Figure 1. Cluster alignment of amino acid sequences for SiRs from fungi. Identical amino acids are marked with (*), the dashes represent gaps introduced to preserve alignment. The graph at the bottom represents amino acids conserved.

al., 2010); however, this phenomenon has also been observed between organisms with different lifestyles (e.g., saprophytic/opportunistic, pathogenic and mycoparasitic fungi) and pathogenesis strategies (biotrophic, hemibiotrophic and necrotrophic fungi) (Kubicek et al., 2011; Brunner et al., 2013; Ohm et al., 2012). Based on these findings and the results obtained in this study, we hypothesized that the molecular evolution of the fungal sulfur assimilation pathway has been influenced by fungal lifestyle and by coevolution with different types of hosts.

Since *Trichoderma* species are fungi with mycoparasitic/saprophytic lifestyles, it is surprising that the ClSiR of *C. lindemuthianum* clustered in this group and not with plant pathogenic fungi, particularly fungi from the genus *Colletotrichum*. In addition, *Clsir* showed high identity with the corresponding nucleotide and amino acid sequences from *T. atroviride*. This phenomenon has been observed previously for the *CbhII* gene fragment from *C. lindemuthianum* that encodes cellobiohydrolase II; this fragment showed 99% identity with the corresponding nucleotide sequences from *T. reesei* and *T. viride* and 94 and 84% identity with the corresponding amino acid sequences from *T. reesei* and *T. longibrachiatum,* respectively (Acosta-Rodríguez et al., 2005).

Horizontal gene transfer is now considered an important factor in fungal evolution, and this process may be involved in niche specification, disease emergence and metabolic capabilities (Fitzpatrick, 2011). *Trichoderma* species such as *T. atroviride,* also known as *T. harzianum,* are primarily ancestral mycoparasites of phytopathogens, which could facilitate transfer events that provide new capabilities to the receptor fungus (Kullnig et al., 2000; Kubicek et al., 2011). The transfer of genes, groups of genes and intact chromosomes among fungi of the genus *Aspergillus* spp., *Pyrenophora tritici-repentis* and other fungal species has been reported (Fitzpatrick, 2011; Friesen et al., 2006; Fedorova et al., 2008). Thus, it is likely that the phenomenon observed in this work was caused by horizontal gene transfer between species of the genus *Trichoderma* (e.g., *T. atroviride*) and *C. lindemuthianum.* This hypothesis requires future study.

C. lindemuthianum is a hemibiotrophic or facultative biotrophic fungus that establishes an initial asymptomatic biotrophic phase during the infection process, in which it invades the tissues of its host (*Phaseolus vulgaris*) undetected, and a subsequent necrotrophic phase, in which it degrades the cell walls of its host, killing its host and allowing the fungus to complete its life cycle as a saprophyte (Perfect et al., 1999). Although *C. lindemuthianum* is closely related to *G. graminicola* and *C. higginsianum* and uses similar invasion strategies, the host ranges of these organisms differ considerably (Perfect et al., 1999).

It is possible that the evolution of the genes encoding fungal SiR has been influenced not only by the lifestyle of the microorganisms but also by the host range of the microorganisms, particularly in the case of fungi such as *C. lindemuthianum,* which have undergone coevolution with their hosts. Thus, the study of fungi that have different lifestyles, particularly fungi like *C.*

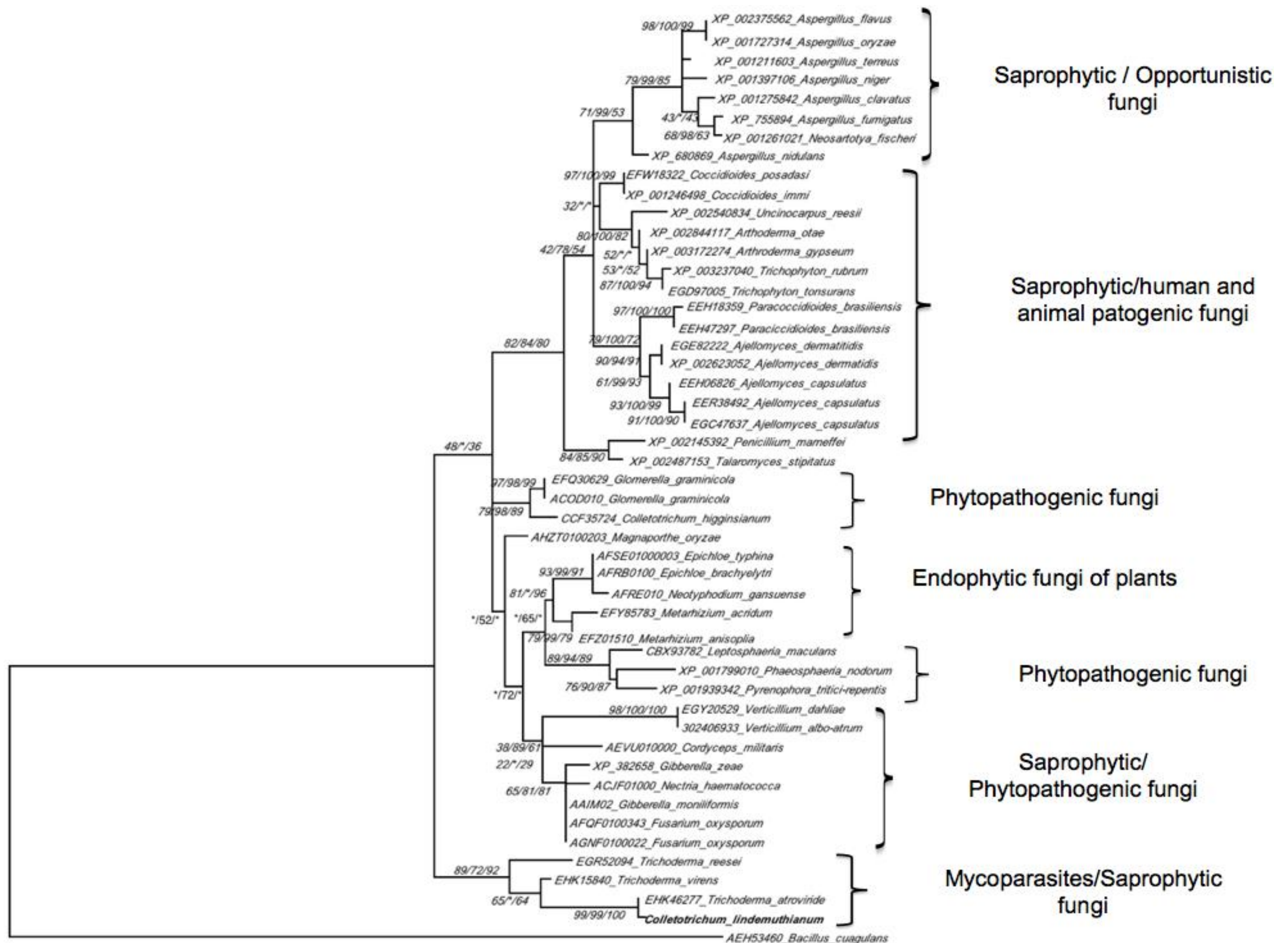

Figure 2. Phylogenetic tree of amino acid sequences for SiRs from fungi. The phylogeny shows the topology of the ML method. Numbers above the diagonal indicate bootstrap support values from ML analysis and below the diagonal the posterior probability of Bayes analysis and bootstrap support for NJ, respectively. *B. cuagulans* SiR was used as an out-group. The asterisks represent branches that were not supported for one of the analysis.

lindemuthianum, is of great importance for understanding the processes that influence the evolution of fungi.

Conflict of Interests

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

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REFERENCES

Abascal F, Zardoya R, Posada D (2005). ProtTest: selection of best-fit models of protein evolution. Bioinformatics. 21:2104-2105.

Acosta RI, Piñon EC, Zavala PMG, López RE, Cano CH (2005). Degradation of cellulose by the bean-pathogenic fungus *Colletotrichum lindemuthianum*. Production of extracellular cellulolytic enzymes by cellulose induction. A. Van. Leeuw. 87(4):301-310.

Baxter L, Tripathy S, Ishaque N, Boot N, Cabral A (2010). Signatures of adaptation to obligate biotrophy in the Hyaloperonospora arabidopsidis genome. Science. 330(6010):1549-1551.

Brunner PC, Torriani SFF, Croll D, Stukenbrock EH, McDonald BA (2013). Coevolution and life cycle specialization of plant cell wall degrading enzymes in a hemibiotrophic pathogen. Mol. Biol. Evol. 30:1337-1347.

Dayhoff MO, Schwartz RM (1978). A model of evolutionary change. In: Proteins Atlas of protein sequences and structure. Nat. Biomed. Res. Found. Washington, DC. pp. 363-368.

Dhillon A, Goswami S, Riley M, Teske A, Sogin M (2005). Domain evolution and functional diversification of sulfite reductases. Astrobiology. 5(1):18-29.

Dodds PN (2010). Genome evolution in plant pathogens. Science 330(6010):1486-1487.

Dodds PN, Rafiqi M, Gan PHP, Hardham AR, Jones DA, Ellis JG (2009). Effectors of biotrophic fungi and oomycetes: pathogenicity factors and triggers of host resistance. New Phytol. 183(4):993-1000.

Fedorova ND, Khaldi N, Joardar VS, Maiti R, Amedeo P, Anderson MJ, Crabtree J, Silva JC, Badger JH, Albarraq A, Angiuoli S, Bussey H, Bower P, Cotty PJ, Dyer PS, Egan A, Galens K, Fraser-Liggett CM, Hass BJ, Inman JM, Kent R, Lemieux S, Malavazi I, Orvis J, Roemer T, Ronning CM, Sundaram JP, Sutton G, Turner G, Venter JC, White OR, Whitty BR, Youngman P, Wolfe KH, Goldman GH, Wortman JR, Jiang B, Denning DW, Nierman WC (2008). Genomic islands in the pathogenic filamentous fungus *Aspergillus fumigatus*. PLoS genet. 4(4): e1000046.

Fitzpatrick DA (2011). Horizontal gene transfer in fungi. FEMS Microbiol. lett. 329(1):1-8.

Flor HH (1971). Current status of the gene-for-gene concept. Annu. Rev. Phytopathol. 9:275-296.

Friesen TL, Stukenbrock EH, Liu Z, Meinhardt S, Ling H, Faris JD, Rasmussen JB, McDonald BA, Oliver RP (2006). Emergence of a new disease as a result of interspecific virulence gene transfer. Nat. Genet. 38(8):953-956.

González M, Rodríguez R, Zavala ME, Jacobo JL, Hernández F, Acosta J, Martínez O, Simpson J (1998). Characterization of Mexican isolates of Colletotrichum lindemuthianum by using differential cultivars and molecular markers. Phytopathology 88(4): 292-299.

Huelsenbeck JP, Ronquist F (2001). MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17(8):754-755.

Huson DH, Richter DC, Rausch C, Dezulian T, Franz M, Rupp R (2007). Dendroscope: An interactive viewer for large phylogenetic trees. BMC Bioinformatics 8:460.

Knogge W (1998). Fungal pathogenicity. Curr. Opin. Plant. Biol. 1(4):324-328.

Kubicek CP, Herrera EA, Seidl SV, Martinez DA, Druzhinina IS, Thon M, Zeilinger S, Casas FS, Horwitz BA, Mukherjee PK, Mukherjee M, Kredics L, Alcaraz LD, Aerts A, Antal Z, Atanasova L, Cervantes-Badillo MG, Challacombe J, Chertkov O, McCluskey K, Coulpier F, Deshpande N, von Döhren H, Ebbole DJ, Esquivel NEU, Fekete E, Flipphi M, Glaser F, Gómez REY, Gruber S, Han C, Henrissat B, Hermosa R, Hernández OM, Karaffa L, Kosti I, Le Crom S, Lindquist E, Lucas S, Lübeck M, Lübeck PS, Margeot A, Metz B, Misra M, Nevalainen H, Omann M, Packer N, Perrone G, Uresti REE, Salamov A, Schmoll M, Seiboth B, Shapiro H, Sukno S, Tamayo RJA, Tisch D, Wiest A, Wilkinson HH, Zhang M, Coutinho PM, Kenerley CM, Monte E, Baker SE, Grigoriev IV (2011). Comparative genome sequence analysis underscores mycoparasitic as the ancestral life style of *Trichoderma*. Genome Biol. 12(4): R40

Kullnig C, Mach RL, Lorito M, Kubicek CP (2000). Enzyme diffusion from Trichoderma atroviride (= T. harzianum P1) to Rhizoctonia solani is a prerequisite for triggering of Trichoderma ech42 gene expression before mycoparasitic contact. Appl. Environ. Microbiol. 66(5): 2232-2234.

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG (2007). Clustal W and Clustal X version 2.0. Bioinformatics. 23(21):2947-2948.

O'Connell RJ, Bailey J (1988). Differences in the extent of fungal development, host cell necrosis and symptom expression during race-cultivar interactions between Phaseolus vulgaris and *Colletotrichum lindemuthianum*. Plant Pathol. 37(3):351-362.

Ohm RA, Feau N, Henrissat B, Schoch CL, Horwitz BA, Barry KW, Condon BJ, Copeland AC, Dhilon B, Glaser F, Hesse CN, Kosti I, LaButti K, Lindquist EA, Lucas S, Salamov AA, Bradshaw RE, Ciuffetti L, Hamelin RC, Kema GHJ, Lawrence C, Scott JA, Spatafora JW, Turgeon BG, deWit PJGM, Zhong S, Goodwin B, Grigoriev IV (2012). Diverse lifestyles and strategies of plant pathogenesis encoded in the genomes of eighteen Dothideomycetes fungi. PLoS Pathog. 8(12): e1003037.

Patron NJ, Durnford DG, Kopriva S (2008). Sulfate assimilation in eukaryotes: fusions, relocations and lateral transfers. BMC Evol. Biol. 8:39.

Perfect SE, Hughes HB, O'Connell RJ, Green JR (1999). Colletotrichum: A model genus for studies on pathology and fungal-plant interactions. Fungal Genet. Biol. 27(2-3): 186-198.

Rodríguez GR, Acosta GJA, González CMM, Simpson J (2006). Pathotypes of Colletotrichum lindemuthianum and their implications for the development of resistant vean cultivars. Agric. Tec. Mex. 22(1): 101-114.

Sambrook J, Russell DW (2001). Molecular Cloning: A Laboratory Manual, E. Golemis Ed. 3rd ed. Vol. 1, 2, 3. Cold Spring Harbor Laboratory Press.

Stroupe ME, Getzoff ED (2001). Sulfito reductase hemoprotein. In: Handboock of metalloproteins, pp. 471-485.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011). MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28(10):2731-2739.

Wagner M, Roger AJ, Flax JL, Brusseau GA, Stahl DA (1998). Phylogeny of dissimilatory sulfite reductases supports an early origin of sulfate respiration. J. Bacteriol. 180(11): 2975-2982.

Whelan S, Goldman N (2001). A general empirical model of protein evolution derived from multiple protein families using a maximum-likelihood approach. Mol. Biol. Evol. 18(5): 691-699.

Wijesundera RLC, Bailey JA, Byrde RJW, Fielding AH (1989). Cell wall degrading enzymes of Colletotrichum lindemuthianum: their role in the development of bean anthracnose. Physiol. Mol. Plant Pathol. 34(5):403-413.

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Full Length Research Paper

Bacterial profiles and antibiotic resistance patterns in Xiangya Hospital, China

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The aim of this study was to characterize the bacterial profiles and antibiotic-resistance patterns in Xiangya Hospital in 2012, and provide guidance for rational use of antimicrobial agents. Clinical strains were identified by the Vitek 2 automatic microbe analysis system and API test strips, and minimal inhibitory concentrations (MICs) for each antibiotic agent was determined. Data were analyzed in the WHONET 5.4 software. 12,407 non-repetitive strains were identified in 2012, including 3,579 Gram-positive bacterial strains (28.85%), 7,579 Gram-negative bacterial strains (61.09%) and 1,249 fungi (10.06%). 53.63% *Staphylococcus aureus* are methicillin-resistant and 62.39% coagulase-negative *Staphylococci* are methicillin-resistant, but susceptible to vancomycin, teicoplanin or linezolid. Four *Enterococcus faecium* and 3 *Enterococcus faecalis* strains were resistant to vancomycin. 72.12% *Escherichia coli* and 56.23% *Klebsiella pneumoniae* were extended spectrum β-lactamases (ESBLs) positive, and carbapenem showed high activity against both bacteria (resistant rates <10%). Therefore, the number of bacterial pathogens isolated in this hospital and their antibiotic resistance situation were not optimistic. It is urgent and necessary to promote a wide, systematic, continuous and high-quality bacterial-resistance surveillance.

Key words: Antibiotic resistance pattern, bacterial profile, pathogen.

INTRODUCTION

According to a recent report, 80,000 deaths per year in China directly or indirectly resulted from antibiotics misuse, causing increases in bacterial resistance and a n enormous damage to health (Yan et al., 2013). The irrational use of antibacterial agents includes incorrect selection of antibiotics, insufficient management of preventive medication and patients taking medicines without a doctor's prescription, which was one of the foremost causes of antimicrobial resistance. Reports from various regions of China showed differences in bacterial profiles and antibiotic resistance patterns. Therefore, a long-term and continuous bacterial-resistance surveillance

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Abbreviations: MICs, Minimal inhibitory concentrations; **ESBLs,** extended spectrum β-lactamases; **CLSI,** Clinical and Laboratory Standards Institute; **ISO,** International Standardization Organization; **CNS,** coagulase negative *staphylococci*; **MRSA,** methicillin-resistant *Staphylococcus aureus*; **MRCNS,** methicillin-resistant coagulase negative *Staphylococci*; **VRE,** vancomycin-resistant *Enterococcus*.

program should be established and carried out, to understand the variation in local bacteria antibiotic resistance patterns and also guide in rational selection of antibiotics, and assist in developing relevant management measures of hospital infection control. This study characterized the profile of bacterial pathogens that were isolated in Xiangya Hospital in 2012, and analyzed the antibiotic resistance patterns.

MATERIALS AND METHODS

Bacterial strains

The specimens were collected from out-patients and in-patients who were undergoing a bacteriological examination in 2012 with bacterial infections. Pathogenic bacteria were cultured and isolated with appropriate media and environment. Concurrent quality control tests were performed by using the following standard strains: *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, *Enterobacter cloacae* ATCC 700323, *Staphylococcus aureus* ATCC 25923 and *Staphylococcus aureus* ATCC29213 (all strains were provided by the National Center for Clinical Laboratories).

Identification

Clinical strains were identified using the Vitek 2 automatic microbe analysis system and API test strips (the identification system and API test strips were purchased from bioMerieux in France), then the minimal inhibitory concentrations (MICs) of antibiotic agents for each strain were tested by the Vitek 2 automatic microbe analysis system with its ancillary drug susceptibility cards (using broth microdilution method). The antibiotic susceptibility of a small number of the isolates was determined manually using the Kirby-Bauer method (drug slips were purchased from Oxoid Company in England). The antibacterial agents were tested following Clinical and Laboratory Standards Institute (CLSI) recommendations for antimicrobial sensibility tests, MRSA test, and extended spectrum β-lactamases (ESBLs) test.

Quality control

Xiangya Hospital is a well-known tertiary general hospital in China, the Department of Clinical Laboratory has acquired certification of International Standardization Organization (ISO) and all clinical microbiologists participating in this program have a laboratory qualification certificate and at least 3 years of work experience.

Data analysis

The results of the antimicrobial susceptibility tests were interpreted per CLSI standards. Data were analyzed using the WHONET 5.4 software. The same strain from the same type of specimen from one patient was counted once to avoid double counting of strain.

RESULTS

Bacterial profiles

A total of 12,407 non-repetitive strains were identified in 2012, including 3,579 (28.85%) Gram-positive bacterial strains, 7,579 (61.09%) Gram-negative bacterial strains and 1,249 (10.06%) fungi. The Gram-positive strains mostly consist of *S. aureus* (1,020 strains, 8.22%), coagulase negative *Staphylococci* (787 strains, 6.34%), *E. faecium* (420 strains, 3.39%), *E. faecalis* (398 strains, 3.21%) and *S. intermedius* (87 strains, 0.70%). The main Gram-negative bacterial strains are *Acinetobacter baumannii* (1,524 strains, 12.28%), *Pseudomonas aeruginosa* (1,517 strains, 12.23%), *E. coli* (1,397 strains, 11.26%), *Klebsiella pneumoniae* (1,280 strains, 10.32%) and *Enterobacter cloacae* (352 strains, 2.84%) (Table 1).

Among the total 12,407 bacterial strains, 11,102 strains were isolated from in-patient's specimens, and the rest were from out-patients. The strains were isolated from respiratory tract specimens (sputum, throat, bronchial, broncho-alveolar lavage, etc.) (51.24%), genitourinary tract specimens (urine, prostatic fluid, vaginal secretions, etc.) (13.60%), wound secretions and pus (13.07%), paracentesis fluid (9.16%), blood and bone marrow (7.65%) and others (5.28%).

Antibiotic resistance patterns

Staphylococcus

For *S. aureus* or coagulase negative *Staphylococci* (CNS), methicillin-resistant *S. aureus* (MRSA) and methicillin-resistant coagulase negative *staphylococci* (MRCNS) were 53.63 (547/1020) and 62.39% (491/787), but all strains were fully susceptible to vancomycin, teicoplanin or linezolid (Table 2). *S. aureus* was susceptible to doxycycline (81.76%) and chloramphenicol (79.61%), but strongly resistant (resistant rate over 60%) to penicillin, ceftazidime, cefazolin, erythromycin and azithromycin (Table 2).

Enterococcus

E. faecium and *E. faecalis* were fully susceptible to teicoplanin and linezolid, but four isolates of *E. faecium* and three isolates of *E. faecalis* strains were resistant to vancomycin (Table 3). *E. faecium* were more resistant to almost all tested agents than *E. faecalis*, except tetracycline (Table 3). *E. faecalis* were highly susceptible to ampicillin (92.21%) and penicillin (84.92%) (Table 3).

Enterobacteriaceae

A majority of *E. coli* strains (72.12%) and *K. pneumoniae* strains (56.23%) were ESBLs positive, but carbapenem antibiotics are very effective, with resistant rates below 10% (Table 4). *E. coli* and *K. pneumoniae* were susceptible to the β-lactamase inhibitor compounds

Table 1. Percentages of main strains (%).

Bacterial pathogen	No.	Percentage (%)
Acinetobacter baumannii	1,524	12.28
Pseudomonas aeruginosa	1,517	12.23
Escherichia coli	1,397	11.26
Klebsiella pneumoniae	1,280	10.32
Staphylococcus aureus	1,020	8.22
Candida albicans	841	6.78
Coagulase negative *staphylococci*	787	6.34
Enterococcus faecium	420	3.39
Enterococcus faecalis	398	3.21
Enterobacter cloacae	352	2.84
Stenotrophomonas maltophilia	290	2.34
Smooth candida	225	1.81
Enterobacter aerogenes	202	1.63
Burkholderia cepacia	135	1.09
Serratia marcescens	117	0.95
Haemophilus influenzae	108	0.87
Proteus mirabilis	102	0.82
Str.intermedius	87	0.70
Streptococcus pneumoniae	81	0.65
Klebsiella oxytoca	74	0.60
Candida tropicalis	62	0.50
Streptococcus Feacalis	50	0.41
Streptococcus agalactiae	48	0.39
Others	1,291	10.40

Table 2. Antibiotic resistance patterns of *Staphylococcus*.

Antibacterial agents	*Staphylococcus aureus*			coagulase negative *staphylococci*		
	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)
Penicillin	3.14	0.00	96.86	10.29	0.00	89.71
Oxacillin	46.37	0.00	53.63	37.61	0.00	62.39
Cefazolin	31.67	1.66	66.67	35.96	4.07	59.97
Ceftazidime	20.00	11.67	68.33	32.02	14.99	52.99
Meropenem	37.55	4.31	58.14	54.13	8.39	37.48
Vancomycin	100.00	0.00	0.00	100.00	0.00	0.00
Teicoplanin	100.00	0.00	0.00	100.00	0.00	0.00
Azithromycin	32.84	3.63	63.53	13.34	0.00	86.66
Erythromycin	31.96	0.29	67.75	12.58	0.25	87.17
Tetracycline	41.37	2.06	56.57	56.16	1.15	42.69
Doxycycline	81.76	16.77	1.47	87.93	9.27	2.80
Ciprofloxacin	47.65	1.96	50.39	44.85	9.66	45.49
Levofloxacin	46.18	4.21	49.61	52.10	12.96	34.94
Clindamycin	47.55	0.69	51.76	58.07	3.05	38.88
Chloromycetin	79.61	12.35	8.04	77.38	1.27	21.35
Rifampicin	55.69	2.45	41.86	79.29	0.38	20.33
Linezolid	100.00	0.00	0.00	100.00	0.00	0.00

S, Susceptibility; I, insensitivity; R, resistance.

Table 3. Antibiotic resistance patterns of *Enterococcus faecium* and *Enterococcus faecalis*.

Antibacterial agent	*Enterococcus faecium*			*Enterococcus faecalis*		
	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)
Penicillin	14.29	0.00	85.71	84.92	0.00	15.08
Ampicillin	7.86	0.00	92.14	92.21	0.00	7.79
Vancomycin	95.00	4.05	0.95	96.98	2.27	0.75
Teicoplanin	100.00	0.00	0.00	100.00	0.00	0.00
erythromycin	3.33	5.96	90.71	10.80	24.88	64.32
tetracycline	41.67	1.43	56.90	21.11	1.50	77.39
Ciprofloxacin	14.05	3.81	82.14	67.59	10.55	21.86
Levofloxacin	17.14	2.86	80.00	76.13	4.27	19.60
Linezolid	100.00	0.00	0.00	100.00	0.00	0.00

S, Susceptibility; I, insensitivity; R, resistance.

Table 4. Antibiotic resistance patterns of *Escherichia coli* and *Klebsiella pneumoniae*.

Antibacterial agent	*Escherichia coli*			*Klebsiella pneumoniae*		
	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)
Ampicillin	7.16	0.21	92.63	0.00	0.00	100.00
Ampicillin/Sulbactam	24.27	24.91	50.82	35.86	14.14	50.00
Piperacillin/Tazobactam	88.69	6.44	4.87	79.22	8.05	12.73
Cefazolin	20.54	0.00	79.46	26.25	11.80	61.95
Cefepime	51.18	6.37	42.45	50.63	22.73	26.64
Cefotaxime	30.99	6.95	62.06	27.50	13.12	59.38
Ceftriaxone	25.34	11.24	63.42	46.88	0.39	52.73
Cefotetan	93.99	1.72	4.29	91.72	2.11	6.17
Ceftazidime	56.48	15.82	27.70	47.03	19.30	33.67
Cefoperazone/Sulbactam	92.56	6.29	1.15	86.72	7.19	6.09
Cefuroxime	17.32	8.02	74.66	25.55	7.03	67.42
Aztreonam	48.46	0.29	51.25	41.88	6.48	51.64
Ertapenem	95.92	0.50	3.58	82.19	12.26	5.55
Imipenem	98.07	0.21	1.72	95.63	2.18	2.19
Meropenem	92.63	5.01	2.36	92.50	1.48	6.02
Gentamicin	42.95	0.64	56.41	54.77	1.01	44.22
Tobramycin	45.88	31.07	23.05	57.27	19.29	23.44
Amikacin	89.69	5.51	4.80	86.64	0.78	12.58
Levofloxacin	37.87	4.15	57.98	74.14	7.66	18.20
Ciprofloxacin	35.86	1.65	62.49	67.50	5.62	26.88
Trimethoprim/Sulfamethoxazole	34.57	0.00	65.43	45.94	2.58	51.48

S, Susceptibility; I, insensitivity; R, resistance.

cefoperazone/sulbactam, but highly resistant to ampicillin (> 90%), and also resistant to cefazolin, cefuroxime, cefotaxime and ceftriaxone (Table 4).

Non-fermenters

A. baumannii were resistant to ceftriaxone (78.99%), trimethoprim/sulfamethoxazole (76.63%), cefotaxime (72.75%) and ciprofloxacin (71.77%), but susceptible to amikacin (74.20%), minocycline (72.69%) and meropenem (53.84%) (Table 5). *P. aeruginosa* were susceptible (> 50%) to all tested antibacterial agents, and meropenem, amikacin and ciprofloxacin were the top three potential agents (Table 5).

DISCUSSION

Among the total 12,407 bacterial strains isolated in

Table 5. Antibiotic resistance patterns of *Acinetobacter baumannii* and *Pseudomonas aeruginosa*.

Antibacterial agents	*Acinetobacter baumannii*			*Pseudomonas aeruginosa*		
	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)
Ampicillin/Sulbactam	41.37	10.17	48.46	-	-	-
Piperacillin/Tazobactam	19.11	14.05	66.84	62.69	11.73	25.58
Ceftazidime	21.60	11.36	67.04	67.17	12.66	20.17
Cefepime	23.24	13.79	62.97	67.63	13.45	18.92
Aztreonam	-	-	-	54.12	15.69	30.19
Cefotaxime	21.08	6.17	72.75	-	-	-
Ceftriaxone	8.27	12.74	78.99	-	-	-
Imipenem	29.81	1.58	68.61	65.46	6.86	27.69
Meropenem	53.84	25.67	20.49	84.57	7.32	8.11
Gentamicin	18.58	15.30	66.12	56.69	3.56	39.75
Tobramycin	31.58	3.29	65.13	67.11	6.13	26.76
Amikacin	74.20	1.51	24.29	75.81	3.75	20.44
Minocycline	72.69	27.31	0.00	-	-	-
Ciprofloxacin	23.90	4.33	71.77	68.16	5.14	26.70
Levofloxacin	38.61	29.54	31.85	62.69	13.18	24.13
Ofloxacin	-	-	-	56.23	15.69	28.08
Trimethoprim/Sulfamethoxazole	18.45	4.92	76.63	-	-	-

S, Susceptibility; I, insensitivity; R, resistance; -, no break point in CLSI.

Xiangya Hospital in 2012, there were more Gram-negative bacterial strains (61.09%) than Gram-positive ones (28.85%), and the top 5 pathogens were *A. baumannii*, *P. aeruginosa*, *E. coli*, *K. pneumoniae* and *S. aureus*, which composed of 54.31% of the total strains isolated and identified. The most common strains were *A. baumannii* (Gram-negative) and *S. aureus* (Gram-positive). *E. coli* (11.26%) had the highest relevance ratio in *Enterobacteriaceae*. The most frequent *Non-fermenters* was *Acinetobacter baumannii* (12.28%), followed by *P. aeruginosa* (12.23%). Of all the specimens, only 10.52% were collected from out-patients, suggesting that clinicians should be more aware of the importance of routine microbiological detections and apply appropriate tests for out-patients.

The relevance ratios of MRSA and MRSCN in 2012 were close to the results of last year, and the susceptibility rates of *S. aureus* and CNS to vancomycin, teicoplanin and linezolid also remained similar (Qun et al., 2011). MRSA had relatively high resistance rates to β-lactam antibiotics and may be resistant to many other antibiotics, leading to strong pathogenicity and high death rates. As compared to a national surveillance result, there is no obvious difference in the relevance ratio of MRSA (50.50%), and few strains were resistant to teicoplanin and linezolid (Yong-hong et al., 2012). According to a report from United States, an estimated 80,461 invasive MRSA infections occurred nationally in 2011, 48,353 were HACO infections, 14,156 were hospital-onset infections and 16,560 were community-associated infections (Dantes et al., 2013). Therefore, the burden of invasive MRSA infections was heavy.

Among clinical isolations of *Enterococci*, *Enterococcus faecium* and *Enterococcus faecalis* were the most prevalent ones. *E. faecium* displayed higher antibiotic resistance than *E. faecalis* (Qing et al., 2012; Sharifi et al., 2013). Our data shows a similar antibiotic resistance pattern, in which *E. faecalis* but not *Enterococcus faecium* were highly susceptible to ampicillin and penicillin. *E. faecium* were highly resistant to ciprofloxacin (82.14%) and levofloxacin (80.00%), which could be due to overuse of these antibiotics. We identified seven isolates of vancomycin-resistant *Enterococcus* (VRE), including four isolates of *E. faecium* (0.95%) and three isolates of *E. faecalis* (0.75%). The prevalence of VRE seemed more severe in India as shown by a report that 128 *Enterococcus* strains (2.30%) were isolated from a total of 5,555 clinical samples in one year (Sreeja et al., 2012). Among all the isolates, there were 97 isolates of *E. faecalis* (76%) and 31 isolates of *E. faecium* (24%).

For *Enterobacteriaceae*, our bacterial resistance data showed that they were susceptible to carbapenems (above 80%). Carbapenems were considered to be the most effective antimicrobial agents against *Enterobacteriaceae* infection, but there are more reports on *Enterobacteriaceae* resistance to carbapenems in China and other countries (Shi-guo, 2012; Castanheira, 2011) which should be noticed generally. *E. coli* and *K. pneumoniae* showed relatively high resistant rates to cefazolin, cefuroxime, cefotaxime and ceftriaxone, mainly due to ESBLs (Pitout and Laupland, 2008). From the results, 72.12% *E. coli* and 56.23% *K. pneumoniae* were

ESBL-positive, which were much higher than the ESBL-positive rate from other regions' reports (9.7 and 12.7%; 13.51 and 16.55%) (Hawser et al., 2014; Chander and Shrestha, 2013). *E. coli* were intermediately resistant to ciprofloxacin (62.49%) and levofloxacin (57.98%), so clinicians should pay more attention to antibiotics selection when dealing with urinary system infection.

A. baumannii, *P. aeruginosa* and *S. maltophilia* were the most commonly identified Gram-negative non-fermenters in this hospital. These bacteria could survive all kinds of moisture environment in a hospital, naturally resistant to a variety of antibiotics and tend to develop into multi-drug resistant bacteria, which was quite a challenge for hospital infection control and clinical treatment. The analysis showed that the susceptibility rates of *A. baumannii* to most antibiotics were below 50%, except meropenem, amikacin and minocycline. *A. baumannii* were resistant to imipenem with a rate of 68.61%. A recent meta-analysis of carbapenem-resistant *A. baumannii* indicated the resistance mechanisms mainly contained carbapenemase production, outer membrane proteins and the Ade ABC efflux pump (De-song et al., 2013).

In summary, the number of bacterial pathogens isolated in Xiangya Hospital was very significant and the antibiotic resistance situation was not optimistic. It is urgent and necessary to promote a wide, systematic, continuous and high-quality bacterial-resistance surveillance. On the basis of this surveillance, clinicians should be more cautious when selecting and using antibiotics and the management of hospital infection control should be optimized. It is highly recommended, in order to avoid the bacteria resistance increasing and prevent the new antibiotic-resistant strains, to strictly control and rationally use antibiotics, enhance the overall effects on hospital infection control measures, and pays more attention to hospital disinfection and isolation.

Conflict of Interests

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

REFERENCES

Castanheira M, Mendes RE, Woosley LN, Jones RN (2011). Trends in carbapenemase-producing *Escherichia coli* and *Klebsiella spp.* from Europe and the Americas: report from the SENTRY antimicrobial surveillance programme (2007-09). J. Antimicrob. Chemother. 66(6):1409-1411.

Chander A, Shrestha CD (2013). Prevalence of extended spectrum beta lactamase producing *Escherichia coli* and *Klebsiella pneumoniae* urinary isolates in a tertiary care hospital in Kathmandu, Nepal. BMC Res Notes. 6:487.

Dantes R, Mu Y, Belflower R, Aragon D, Dumyati G, Harrison LH, et al (2013). National burden of invasive methicillin-resistant *Staphylococcus aureus* Infections, United States, 2011. JAMA Intern. Med. 173(21):1970-1978.

De-song Ming, Yong Deng, Yu-ying Xu (2013). Meta-analysis of resistance mechanisms in carbapenem-resistant *Acinetobacter baumannii*. J. Pathog. Biol. 8(4):358-360.

Hawser SP, Badal RE, Bouchillon SK, Hoban DJ, Hackel MA, Biedenbach DJ, Goff DA (2014). Susceptibility of gram-negative aerobic bacilli from intra-abdominal pathogens to antimicrobial agents collected in the United States during 2011. J. Infect. 68(1):71-76.

Pitout JD, Laupland KB (2008). Extended-spectrum beta-lactamase-producing *Enterobacteriaceae*: an emerging public-health concern. Lancet Infect. Dis. 8(3):159-166.

Qing Y, Yun-song Y, Yu-xing N, Jing-yong S, Ying-chun X, Xiao-jiang Z, et al (2012). CHINET 2010 surveillance of antibiotic resistance in *Enterococcus* in China. Chin J. Infect. Chemother. 12(2):92-97.

Qun Yan, Jun Li, Hong-ling Li, Jing-min Wu, Ming-xiang Zou, Wen-en Liu (2011). Analysis of the distribution and antibiotic resistance of pathogenic bacteria from Xiangya Hospital in 2011. J. Pathog. Biol. 7(7):539-543.

Sharifi Y, Hasani A, Ghotaslou R, Naghili B, Aghazadeh M, Milani M, Bazmany A (2013). Virulence and Antimicrobial Resistance in *Enterococci* Isolated from Urinary Tract Infections. Adv. Pharm. Bull. 3(1):197-201.

Shi-guo He (2012). The resistance mechanisms of resistant *enterobacteriaceae* bacterial of carbapenem and its control. China Modern Doctor. 50(20):70-73.

Sreeja S, Babu PRS, Prathab AG (2012). The prevalence and the characterization of the *enterococcus* species from various clinical samples in a tertiary care hospital. J. Clin. Diagn. Res. 6(9):1486-1488.

Yan Hu, Ji-geng Bai, Xian-ming Hu, Si-jie Wang, Jian-kang Wu, Li-li Kong, et al (2013). Exploring on the Status, Reasons and Countermeasures of Antibiotics Abuse. Chin. J. Soc. Med. 30(2):128-130.

Yong-hong Xiao, Ping Shen, Ze-qing Wei, Yun-bo Chen, Hai-shen Kong, Qing Yang, et al (2012). Mohnarin report of 2011: motitoring of bacterial resistance in China. Chin J. Nosocomio. 22(22):4946-4952.

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Full Length Research Paper

Distribution and characterization of microbial communities in *Chrysoperla zastrowi sillemi*, an important predator of sap sucking insect pests

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Common green lacewing, *Chrysoperla zastrowi sillemi* is one of the important biological control agents and is used effectively to manage various insect pests. Chrysopid predators are found to harbor many endosymbiotic yeasts and bacteria. Keeping this in view, a study on the distribution of yeast and bacteria in the adult diverticulum and larval gut was conducted using transmission electron microscopy (TEM) and molecular techniques. TEM showed the presence of a load of bacterial cells towards the periphery of inner side of the epithelial lining and the dividing bacterial cells in the larval gut. Numerous oval and kidney shaped yeast fauna were found to be distributed within the lumen and diverticular folds of the diverticulum of adult. Our study reveals the presence and distribution of yeast and bacterial cells from the adult diverticulum and gut of larva. Microbial isolates were identified by sequencing 16S rRNA gene for bacteria and ITS region of yeast including partial rRNA genes ITS-1 and partial 5.8S rRNA gene for yeast, respectively which revealed the presence of yeast isolates namely *Kodamaea ohmeri*, *Torulaspora delbrueckii, Wickerhamomyces anomalus* and bacterial isolates namely: *Enterobacter hormaechei*, *E. cloacae* and *Enterobacter* sp. as most common from adult and larvae, respectively.

Key words: Diverticulum, diverticular folds, epithelial lining, lumen, yeast, bacteria.

INTRODUCTION

Lacewings are one of the important biological control agents that are used effectively to manage various insect pests especially sucking pests in different agro-ecosystems (Cannard et al., 1984; Carvalho et al., 2002; Symondron et al., 2002; Venkatesan et al., 2009; Henry et al., 2010). It has long been considered as a promising candidate for pest management worldwide due to its wide prey range and geographical distribution, resistant to insecticides, voracious larval feeding capability as well as ensured commercial availability (New 1975; Tauber et al., 2000; Mc Ewen et al., 2001; Medina et al., 2003; Pathan et al., 2008; Sayyed et al., 2010). Adult lacewings feed solely on nectar, pollen and plant secretions containing sugar (Hagen, 1950; Hassan, 1974; Freistas, 2002) although a few are predatory (Coppel and Mertins, 1977). There is growing interest to understand microbial diversity like symbiotic yeast and bacteria because yeasts are known to provide amino acids, vitamins, degradation of xenobiotic compounds, play a role in host finding and fermentation of food (Dowd, 1989, 1991; Vega and Dowd, 2005; Peter et al., 2012). Symbiotic bacteria are known to fix the atmospheric nitrogen as well

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synthesize other essential nutrients (Dillon and Dillon, 2004; Nardi et al., 2002; Lilburn et al., 2001; Breznak, 2000) and enhancing the internal defence mechanism against toxic compounds. In this regard the chrysopid predators are also found to harbor many endosymbiotic yeasts like symbionts in adults which may provide essential amino acids that are normally absent in their diet (Hagen and Tassan, 1966, 1972). Hagen et al. (1970) identified the yeasts *Torulopsis* sp. in *Chrysopa carnea*, Nguyen et al. (2007) isolated five novel *Candida* sp. from Neuropteran, some of the yeasts like *Metschnikowia noctiluminum, Candida picachoensis* and *C. pimensis* were obtained from gut of adult lacewings (Woolfolk et al., 2003; Suh et al., 2004; Nguyen et al., 2006). Chrysopid predators harbor bacteria in their mid gut region which degrades the digestive residues (Mc-Dunnough, 1909; Spiegler, 1962; Jepp, 1984). Therefore in the present study we described the internal anatomy of the diverticulum of adult as well as midgut of both larvae and adult using TEM and also characterized the yeast and bacterial microflora in *C. z. sillemi* through molecular studies.

MATERIALS AND METHODS

Collection of Chrysopid predator

Adults of lacewings collected from various cotton fields of states (district) namely; Tamil Nadu (Coimbatore), Darward (Karnataka), AndhraPradesh (Guntur), Bangalore (Karnataka), Delhi, Haryana (Sirsa), Punjab (Ludhiana), Rajasthan (Sriganganagar & Udaipur), lab population, Gujarat (Anand), Orissa (Bhuvaneshwar) and Maharashtra (Nagpur). The collections were made in the early morning hours (7-9 am).

Rearing of Chrysopid predator

The adults were transferred in to the oviposition chambers (14 × 9 cm) covered with muslin cloth. Cotton swabs dipped in water and the other with 50% honey, protein mixture [Protein X® (PFIZER Ltd, Mumbai, India): commercial yeast (Gloripan, DEV, INC, China): honey: sucrose in the ratio of 1:1:1:1] and castor pollen grains was provided for adult and provided with perforated brown paper for facilitating egg laying. Eggs were collected every two days interval and kept for hatching with UV treated (to prevent them from hatching) *Corcyra cephalonica* (Stainton) eggs and the containers (14 × 9 cm) were covered with perforated brown paper. Freshly emerged larvae were secured individually in glass vials (4 × 2.5 cm) plugged with cotton. *Corcyra* eggs were provided in the vials for larvae alternate days. Entire rearing was carried out at 26±1°C, 65±5% RH, photoperiod of 14L: 10D in a plant growth chamber.

Transmission electron microscopy (TEM)

Single population collected from Coimbatore (Tamil Nadu) named as CZS-1 was taken for TEM analysis. Larvae (n= 15) and adults (n= 18) of *C. z. sillemi* were processed according to standard protocols (Woolfolk et al., 2004). The third instar larvae were dissected to obtain the intact midgut after removing the fat bodies.
Similarly the adults were dissected to obtain diverticulum. The image of such dissected samples was digitized using Auto Montage software (Leica Stereozome microscope M205A) (Figure 1A and 1B). The gut tissues were fixed in 3% glutaraldehyde and subjected to TEM studies. Briefly, tissues fixed in 3% glutaraldehyde for 24 h were washed in 0.1M phosphate buffer (pH=7.2-7.4), post fixed in 1% osmium tetroxide, washed and dehydrated 1 h each with 70, 80, 90 and 100 % ethanol. After dehydration, the tissues were cleared in propylene oxide, followed by infiltration over night in the mixture of propylene oxide and epoxy resin (resin mixture contains Araldite CY212 resin, DDSA, Dibutyl Phthalate and DMP30), subsequently transferred to pure araldite and finally embedded using flat embedding moulds and kept at 60°C for 48 h for polymerization. Plastic blocks containing the specimen were cut under Leica EMUC6 Ultra microtome using glass knives. Initially 1 µm thick sections were cut and stained with 1% Toluidine blue for light microscopic examination (Carl Zeiss, Primostar), later 70 nm thick ultrathin sections collected on copper grids were stained using Uranyl acetate and lead citrate. The stained grid containing specimens were scanned under Tecnai G^2 Spirit Bio-twin TEM at 80 KVA and representative areas were captured using *Mega View-III* digital CCD camera.

Isolation of microflora associated with Chrysopid predator

Thirteen field-collected populations of *C. z. sillemi* adults and larvae were separated individually into sterilized glass tube (4.5 × 2.5 cm) and kept in 95% ethanol for 2-3 min to disinfect the surface; followed by a wash with saline solution (0.5%). Further, the adults were dissected in a sterile condition and obtained intact tissues of larval gut and adult diverticulum which were used for isolation of microorganism. The tissues were placed in a micro centrifuge tube containing 100 µl of sterile saline solution (0.9% NaCl) and were then mashed. The suspension thus obtained was spread on to YPDA (Yeast Peptone Dextrose Agar) media (consisting of 10 g of yeast extract, 20 g of peptone, and 20 g dextrose and 15 g of agar in 1 L of distilled water) and was incubated for 48 h at 25°C. The colonies obtained were streaked in to media plates to obtain pure culture. Single yeast and bacterial colonies were purified at least twice. Pure culture were then inoculated into YPDA broth and incubated to obtain reaches optimum growth.

Molecular characterization of resident microflora of Chrysopid predator

Yeast and bacterial genomic DNA were isolated from the pure culture by slightly modified method (Kim et al., 1997). The extracted DNA was used as template for amplification of ITS & 16S r RNA.

Characterization of yeast isolates

For yeast isolates, the primers ITS-1F (5’-TCCGTAGGTGAACCTGCGG-3’) and ITS-1R (5’-GCTGCGTTCTTCATCGATGC-3’) (White et al., 1990) were subsequently used for the amplification of partial rRNA genes and ITS-1 and partial 5.8S rRNA gene. The conditions for amplification were initial denaturation at 94°C for 3 min followed by 30 cycles of 1min at 94°C, 1 min at 60°C and ending with an extension 1 min at 72°C (C1000™ Thermal cycler). The mixtures consisted of total volume of 50 µl containing 10x-reaction buffer, 10 mM dNTP, and 2 µl of each primer and 1U *Taq* polymerase. Each PCR was performed with a total of 10 µl of genomic DNA.

Characterization of bacterial isolates

For bacteria isolates the primers 16S-F (5'-AGAGTTTGATCCTGGCTCAG-3') and 16S-R (5'-

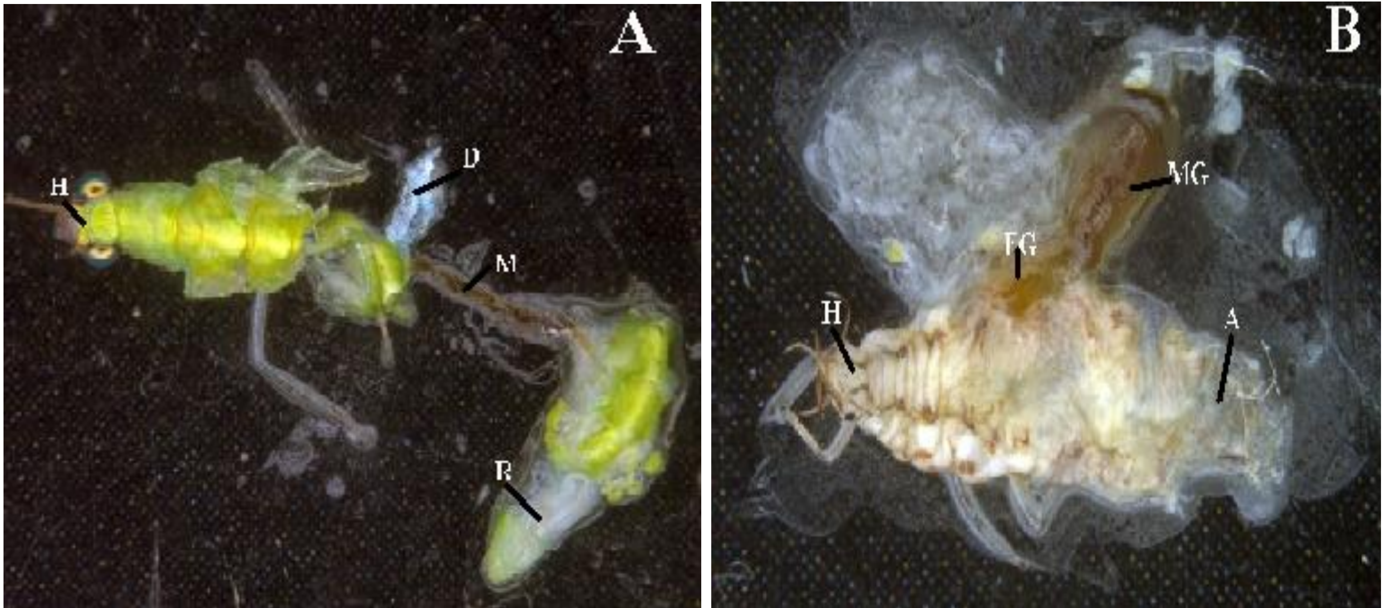

Figure 1. (A) Gut of *C. z. sillemi* adult. H, Head; D, diverticulum; MG, midgut; R, rectum. (B) Gut of *C. z. sillemi* larva. H, Head; FG, foregut; MG, midgut; H, head; A, abdomen.

CGGTGTGTACAAGACCC-3') (Universal primers) were subsequently used for the amplification of 16S r RNA gene. Standard PCR conditions were followed for amplification (O Neill et al., 1991).

The resulting PCR product was electrophoresed in a 1.5% TBE - agarose gel, and a 100 bp ladder was used to size products. The resulting PCR product was purified by PCR purification kit (Min elute spin column PCR purification kit) and were sequenced by Sanger's dideoxynucleotide sequencing. All sequence data were analyzed using basic local alignment search tool (BLAST) (www.ncbi.nlm.nih.gov). The partial rRNA gene sequence including sequences for the 18S rRNA, ITS 1 and 5.8S rRNA genes and 16S rRNA gene were deposited in GenBank.

RESULTS

Collection of chrysopid predator

Field collected chrysopid predators were identified as *Chrysoperla zastrowi sillemi* based on morphology, behavior and acoustic analysis (Henry et al., 2010) and were named as CZS-1, CZS-2, CZS-3, CZS-4, CZS-5, CZS-6, CZS-7, CZS-8, CZS-9, CZS-15, CZS-19 and CZS-20. Lab population was named as CZS-10.

Rearing of chrysopid predators

Different stages like larvae, pupae and adults were collected from field to maintain the uniform cultures and to obtain all stages for the studies *C. z. sillemi* was successfully maintained under laboratory conditions and diet.

Internal anatomy of larval gut and adult gut and diverticulum

TEM showed a thick epithelial lining surrounding the lumen and the lumen harbor load of bacterial cells towards the periphery of inner side of the epithelial lining and the dividing bacterial cells (Figure 2; 1-4), confirming that bacterial cells are widely distributed within the lumen of the gut of larvae.

The adult diverticulum was folded internally and numerous kidney, oval and other shapes of yeast fauna were found distributed within the lumen of the diverticulum and between the diverticular folds of the diverticulum of adult (Figure 3: 1-6). The numerous shape of yeast is due to the ascospores production, different species of yeast produce different types of ascospores revealing that the diverticulum harbors different species of yeast.

Non-dividing bacterial fauna were observed in the lumen of adult gut lined by epithelia (Figure 4: 1-4). TEM analysis of adult gut showed the distribution of bacterial cells but not the yeast cells.

Molecular characterization of yeast and bacteria

Characterization of yeast

The amplified product checked against the 100 bp ladder on 1.5% agarose gel stained with ethidium bromide (1%) showed an amplification length of ≈175 bp for yeast isolates isolated from diverticulum of adults from strains

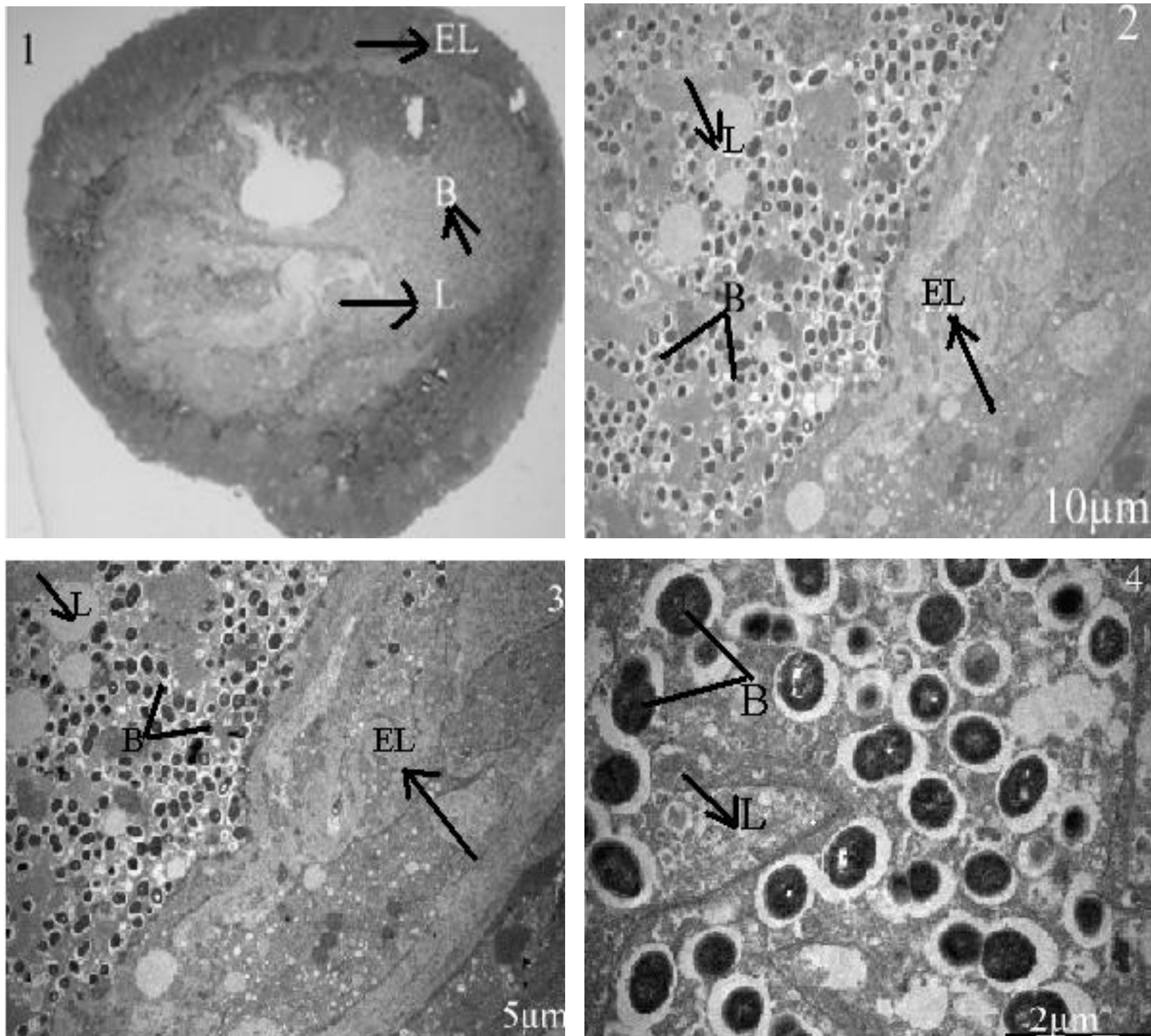

Figure 2. (1) Light microscopic view of semi ultra thin section of larval midgut stained with toluidine blue showing L-lumen, EL-Epithelial lining; B-Bacterial cells; (2-4) Transmission electron micrographs of larval gut of *C. z. sillemi*; Dividing bacterial (B) oval cells observed in the lumen (L) lined by epithelial lining (EL). Scale bar.10, 5 and 2 µm.

CZS-1, CZS-5, CZS-9, CZS-15 and CZS-16. Similarly ≈ 300 bp for stains CZS-4, CZS-7 and CZS-10.

Eight yeast isolates were identified from adult diverticulum, the sequence when analysed by BLAST revealed the yeast genera as well as species and the percentage matching was found to be 100, 99, 100, 97, 99, 99, 97 and 97%, respectively. The yeast isolates were identified as *Wickerhamomyces anomalus* (strain CZS-1 & 5), *Pichia anomala* (CZS-2, 8 &15), *Candida blankii* (CZS-3), *Can. apicola* (CZS-2), *Torulaspora delbrueckii* (CZS-4), *Zygosaccharomyces rouxii* (CZS-7), *Kodamea ohmeri* (CZS-9 &16), *Can. pimensis* (CZS-10). *W. anomalus* was most commonly found yeast isolate in populations (CZS-1, CZS-2, CZS-5 and CZS-8). The characterization of yeast confirms that the diverticulum of adults harbour various species of yeast.

Characterization of bacteria

Bacterial isolates showed amplification length of ≈1200bp when checked on the 1.5% agarose gel stained by ethidium bromide (1%). Seventeen bacterial isolates were isolated from larval gut of strains CZS-1, CZS-2, CZS-3, CZS-6, CZS-8, CZS-9, CZS-10, CZS-15, CZS-19 and CZS-20.

The bacterial isolates were found to be *Enterobacter cloacae* (CZS-1 & 8), *Enterobacter* sp. (CZS-1,2,3 & 8), *Pantoea dispersa* (CZS-2), *Bacillus* sp.(CZS-3 & 9), *Agrobacterium tumefaciens* (CZS-6), *Enterobacter hormaechei* (CZS-8, 9 & 10), *Enterobacter asburiae* (CZS-9), *Bacillus cereus* (CZS-9), *Enterococcus faecium* (CZS-10), *Empedobacter* sp., (CZS-10), *Lactococcus garvieae* (CZS-15), *Enterococcus gallinarum* (CZS-19), *B. subtilis*

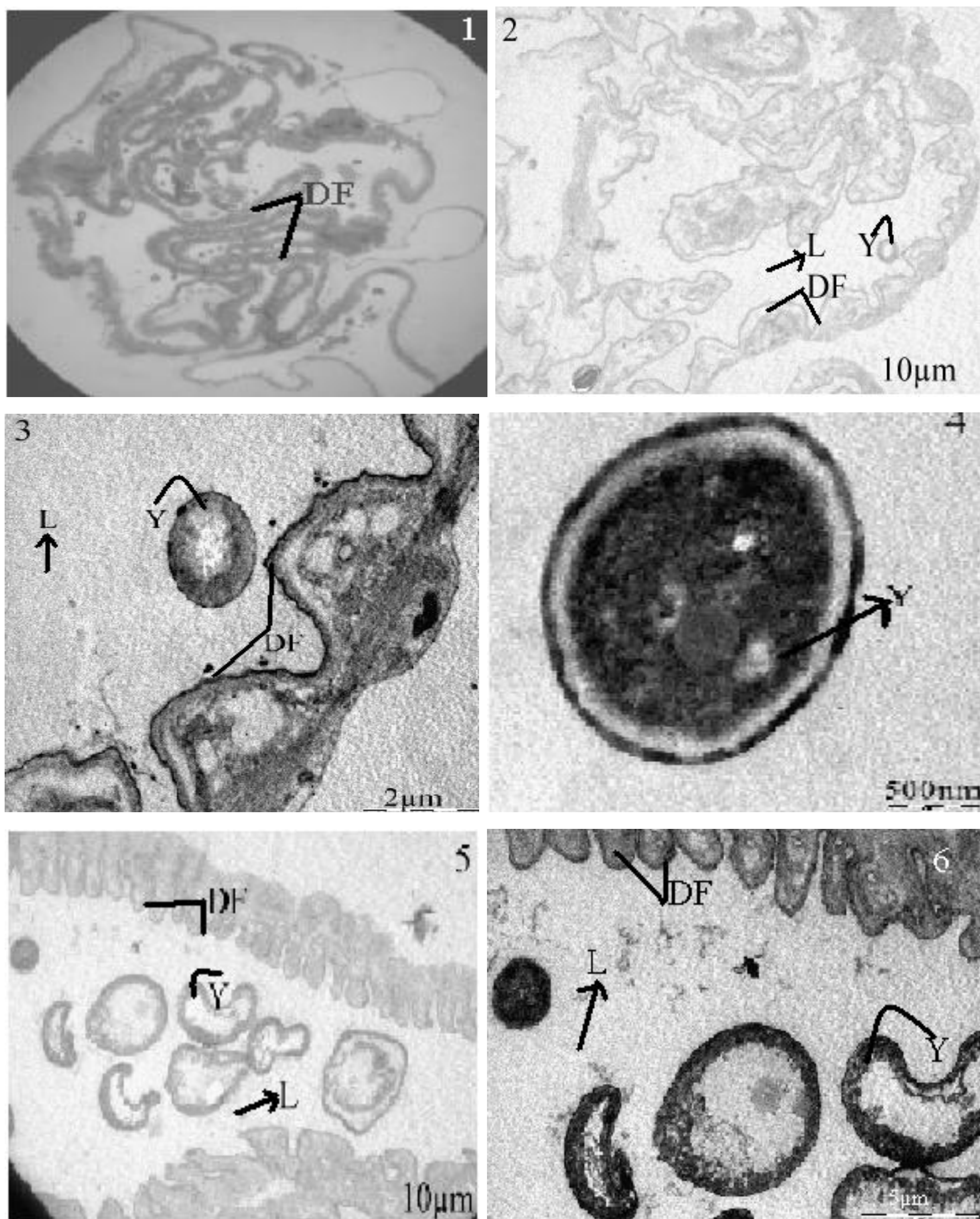

Figure 3. (1) 1 µm thick semi thin section showing diverticulum. (2-6) Transmission Electron micrographs of adult diverticulum of *C. z. sillemi* shows oval and kidney shape yeast (Y) cells observed in the lumen (L) lined by diverticular folds (DF). Scale Bar.10 µm, 5 µm, 2 µm and 500 nm.

(CZS-20), *Enterococcus faecalis* (CZS-20), *B. pumilus* (CZS-20), *Enterococcus* (CZS-10), *Leclercia adecarbxylata* (CZS-15) and their percentage matching were 90-100%. *Enterobacter* sp. was most commonly found bacterial isolates in populations (CZS-1, CZS -2, CZS -3 & CZS- 8). The sequences of these bacteria and yeast isolates were submitted to Gen Bank and accession numbers were obtained (Table 1).

DISCUSSION

TEM studies showed the presence of yeast and bacteria in larval gut and adult diverticulum and similar observations were made by (Chen et al., 2006; Woolfolk et al., 2003). We have observed yeast cells of different shapes like spherical, kidney and irregular; Woolfolk et al. (2003) however, observed only spherical shape.

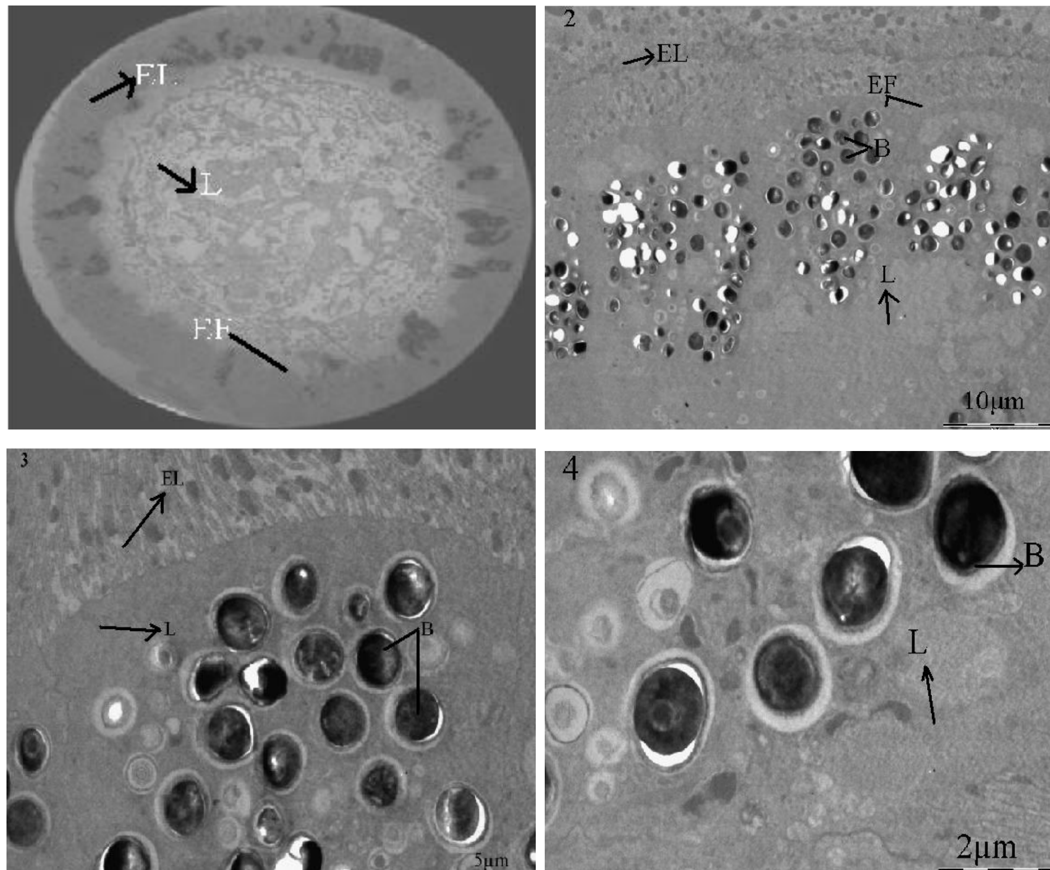

Figure 4. (1) Light microscopic view of 1μm thick semi thin section stained with toluidine blue showing adult gut having epithelial line (EL), epithelial folds (EF) and lumen (L). (2-4) Transmission Electron micrographs of adult gut of *C. z. sillemi* shows oval shaped bacteria (B) present in the lumen (L) lined by epithelial lining (EL) and Epithelial folds (EF). Scale Bar.10 μm, 5 μm, 2 μm and 500 nm.

Bacterial symbionts are present intracellular in bacteriocytes which are often present in certain organs like caeca of the digestive tract, malphigian tubules, appendages of the reproductive tract (Buchner, 1953; Peterson et al., 1994; Kikuchi et al., 2009) in some insects where as in *Chrysoperla carnae* the bacteria were observed free in the lumen (Chapman, 1985). Our study reveals that *C. z. sillemi* harbored bacterial cells in the lumen but not in specialized structures like bacteriocytes.

There was no instance of yeasts in the eggs and larvae. For further confirmation whether adult's posses yeasts in their gut upon eclosion from pupae no instances of yeast were found. Only the field collected adult chrysopids showed the presence of yeast isolates. This indicates that the yeast are transients and have been acquired through the diet and can be indicated as facultative endosymbionts and transmission through is not obligatory. This is in accordance with (Hagen et al., 1970) who suggested that *C. carnea* obtain yeasts from environment and they did not observe any yeasts from larvae. However, this result was contradicted by (Woolfolk et al., 2004) who isolated yeasts from the field collected larvae of *C. rufilabris* and could not isolate from the newly eclosed adults.

Yeast symbionts in the diverticulum provide the amino acid, valine to adult females which may increase the fecundity of the same (Hagen et al., 1970). Further, Woolfolk et al. (2004) observed that the diverticulum was associated with large tracheal trunk which could involve gas exchange in order to support the activity of yeast and other obligate anaerobes or facultative anaerobes (Barnett et al., 1990; Canard et al., 1990; Woolfolk et al., 2004). Similarly we had isolated most of the yeasts from the diverticulum and not from gut.

Several yeasts were isolated from the adult diverticulum, however, *Kodamaea ohmeri*, *Torulaspora delbrueckii* and *Wickerhamomyces anomalus* were found to be prevalent in many populations. Chrysopids are found to have association with variety of micro flora namely *Torulopsis* sp. (Hagen et al., 1970, 1972); *Candida multigemmis* (Buhagiar) Meyer and Yarrow; *T. multigemmis* (Johnson, 1982); *Metschnikowia chrysoperla*, *C. picachoensis* and *C. pimensis* (Suh, 2004; Gibson et al., 2005); *M. pulcherrima* (Woolfolk et

Table 1. Diversity of yeast and bacterial isolates from *C. z. sillemi.*

Population	Yeast isolate from adult diverticulum and accession number	Bacterial isolate from larval gut and accession number
CZS-1 (Coimbatore)	*Wickerhamomyces anomalus* (JQ061141)	*Enterobacter cloacae* (KC 333898), *Enterobacter* sp., (KC 333890), *Enterobacter* sp., (KC 333891)
CZS-2 (Dharwad)	Not observed	*Pantoea dispersa* (JX873957), *Enterobacter* sp., (JX873958)
CZS-3 (Guntur)	*Candida blankii* (JQ340778)	*Enterobacter* sp., (JX 873959), *Bacillus* sp., (JX 873960)
CZS-4 (Bangalore)	*Torulaspora delbrueckii* (KC507190)	Not observed
CZS-5 (Delhi)	*Wickerhamomyces anomalus* (JQ340781)	Not observed
CZS-6 (Sirsa)	Not observed	*Agrobacterium tumefaciens* (KC 333915)
CZS-7 (Ludhiana)	*Zygosaccharomyces rouxii* (JQ410172)	Not observed
CZS-8 (Sriganganagar)	Not observed	*Enterobacter* sp., (KC407909), *E. hormaechei* (KC 333906), *E. cloacae* (KC 333907), *Enterobacter* sp., (KC 333908)
CZS-9 (Udaipur)	*Kodamaea ohmeri* (KC473466)	*Bacillus* sp., (KC 333897), *E. asburiae* (KC 333899), *E. hormaechei* (KC 333901), *B. cereus* (KC 333903)
CZS-10 (Lab population)	*Candida pimensis* (KC473468)	*E. faecium* (KC 333895), *E. hormaechei* (KC 333894), *Empedobacter* sp., (KC 333902)
CZS-15 (Anand)	*Pichia anomala* (KC473464)	*Lactococcus garvieae* (KC 333889)
CZS-19 (Bhuvaneshwar)	Not observed	*Enterococcus gallinarum* (KC 333905)
CZS-20 (Nagpur)	Not observed	*B. subtilis* (KC 333910), *Enterococcus faecalis* (KC 333911), *B. pumilus* (KC 333997)

al., 2004). *T. delbrukii* was isolated from the gut of female *Chauliodes rastricornis* (Neuroptera) in LA, USA. Yeast isolates belonging to the *Metschnikowia* were isolated from the digestive tracts of lacewings (Nguyen et al., 2006). *Kodamaea ohmere* was isolated from gut of female *Corydalus cornutus* (Neuroptera) (Nguyen et al., 2007) found *M. pulcherrima* was predominant yeast found in the alimentary canal of *Chr. rufilabris* which are acquired from the environment. However, we did not observe *M. pulcherrima* in Indian populations of *C. z. sillemi* may be due to the difference in cropping pattern and diversity grown in different countries.

Our study reveals that bacterial isolates *viz.*, *Enterobacter hormaechei*, *E. cloacae* and *Enterobacter* sp. were observed as most common from larvae which are culturable. Some of the facultative symbionts like Enterobacteriaceae are found in aphids, whiteflies, tsetse flies and mealy bugs (Baumann, 2005; Chiel et al., 2007;

Darby et al., 2001; Moran et al., 2005; Novakov, 2007; Russell et al., 2003; Sauer et al., 2000; Weiss et al., 2006). The facultative mutualists play a role in insect host like enhancing the host reproduction, providing protection against natural enemies of host (Oliver et al., 2008; Piel, 2002; Scarborough et al., 2005; Holt et al., 1994). Though TEM analysis revealed the presence of bacteria in adult gut which were unculturable on the media indicates that both the larvae and adult of *C. z. sillemi* harbor different types of bacterial fauna showing that the bacteria are facultative.

However, Woolfolk et al. (2004) isolated *E. areogenes* and *E. cloacae* from the adult diverticulum. Chen et al. (2006) observed the midgut structure and contents of *Chrysoperla carnea* larvae and is hypothesized that the several species of *Chrysoperla* including *Chr. carnea* (Stephens), *Chrysopa oculata* Say, *Ceratochrysa cubana* (Hagen), and the alimentary canal is closed between the midgut and the hindgut. Hence, microflora present in the larval gut may decompose the digestive residues in the midgut.

Conclusion

The study reveals the distribution of microbial diversity of *C. z. sillemi*, as the larvae showed the presence of bacteria and the adults showed the presence of yeast. The route of transmission may be horizontal. This is the first study established the presence of yeast and bacterial cells in adult diverticulum and larval midgut of *C. z. sillemi* through TEM and molecular studies. Further, it is suggested to study on the role of yeast on the fitness attributes of the adult predator especially on reproducing capacity and bacteria on insecticide degradation in larvae as the larvae of the predator was found to have high resistance to various groups of insecticides.

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REFERENCES

Barnett JA, Payne RW, Yarrow D (1990). Yeasts characteristics and identification, 2nd edition: (Cambridge University Press, New York).

Baumann P (2005). Biology of bacteriocytes-associated endosymbionts of plant sap-sucking insects. Annu. Rev. Microbiol. 59:155-189.

Buchner P (1953). Endosymbiose der Tiere mit pflanzlichen Mikroorganismen (Birkhäuser Basel, Stuttgart).

Breznak JA (2000). Ecology of prokaryotic microbes in the guts of wood- and litter-feeding termites. In: Abe T, Bignell DE, Higashi M (ed) Termites: Evolution, sociality, symbiosis, ecology, Boston: Kluwer Academic. pp. 209-231.

Canard M, Semeria Y, New TRR (1984). Biology of Chrysopidae: Dr. W. Junk, Publisher: The Haque, The Netherlands.

Canard M, Kokubu K, Duelli P (1990). Tracheal trunks supplying air to the foregut and feeding habits in adults of European green lacewing species (Insecta: Neuroptera: Chrysopidae), Pretoria, Republic of South Africa: Department of Agricultural Development, pp.277-286.

Carvalho CF, Souza B, Ulhôa JLR (2002). Selectividade de insecticide as a *Chrysoperla externa* (Hagen) (Neuroptera: Chrysopidae), Neotrop Entomol. 31:615-621.

Chapman RF (1985). Structure of the digestive system, In GA Kerkut & LI Gilbert [eds.], Comprehensive insect physiology, biochemistry and pharmacology: Pergamon: Oxford, United Kingdom, 4:p.165 p.121.

Chiel E, Gottlieb Y, Zchori-Fein E, Mozes-Daub N, Katzir N (2007). Biotype-dependent secondary symbiont communities in sympatric populations of *Bemisia tabaci*. Bull. Entomol. Res. 97:407-413.

Chen C, Chu C, Hu J Mu, Henneberry TJ (2006). Observations on midgut structure and content on *Chrysoperla carnea* (Neuroptera: Chrysopidae). Ann. Entomol. Soc. Am. 99:917-919.

Coppel HC, Mertins JW (1977). Biological Insect Pest Suppression. Spinger-Verlag, Berlin, Germany,

Darby AC, Birkle LM, Turner SL, Douglas AE (2001). An aphid-borne bacterium allied to the secondary symbionts of whitefly. FEMS Microbiol. Ecol. 36:43-50.

Dillon RJ, Dillon VM (2004). The gut bacteria of insects: nonpathogenic interactions. Annu. Rev. Entomol. 49:71-92.

Dowd PF (1989). *In situ* production of hydrolytic detoxifying enzymes by symbiotic yeasts in the cigarette beetle (Coleoptera: Anobiidae). J. Econ. Entomol. 82:396-400.

Dowd PF (1991). Symbiont-mediated detoxification in insect herbivores. In: Barbosa P, Krischik VA, Jones CG (ed.). Microbial mediation of plant-herbivore interactions, New York: John Wiley & Sons. pp. 411-440.

Hagen KS (1950). Fecundity of *Chrysopa californica* as affected by synthetic foods. J. Econ Entomol. 43:101-104.

Hagen KS, Tassan RL (1966). The influence of protein hydrolysates of yeasts and chemically defined upon the fecundity of *Chrysopa carnea* (Neuroptera). Vestn Cesk Spol Zool. 30:219-227.

Hagen KS, Tassan RL, Sawall EF (1970). Some ecophysiological relationships between certain Chrysopa, honeydews and yeasts. Boll Lab Entomol Agric Portici. 28:113-134.

Hagen KS, Tassan RL (1972). Exploring nutritional roles of extracellular symbiotes on the reproduction of honeydews feeding adult chrysopids and tephritids, In Insect and Mite Nutrition. Significance and Implications in Ecology and Pest Management. pp. 323-351.

Hassan SA (1974). Mass-culturing and utilization of *Chrysopa* spp. (Neuroptera: Chrysopidae) in the control of insect pests. Zeitschrift fur Pflanzenkrankheiten und Pflanzenschutz. 81:620-637.

Henry CS, Brooks SJ, Johnson JB, Venkatesan T , Duelli P (2010) .The most important lacewing species in Indian agricultural crops, *Chrysoperla sillemi* (Esben-Petersen), is a subspecies of *Chrysoperla zastrowi* (Esben-Petersen) (Neuroptera: Chrysopidae). J. Nat. Hist, 44:2543-2555.

Holt JG, Krieg NR, Sneath PHA, Staley JT, Williams ST (2010). Bergey's manual of Determinative Bacteriology, ninth (ed) Williams and Wilkins, Baltimore MD.

Johnson JB (1982). *Bionomics of some symbiote using Chrysopidae (Insecta: Neuroptera)* from the Western United States, Ph.D dissertation, University of California, Berkeley.

Jepp J (1984). Morphology and anatomy: morphology and anatomy of the preimaginal stages of Chrysopidae: a short survey. *In* Canard M, Semeria Y, New TR [eds.] *Biology of Chrysopidae*. Dr. W. Junk Publishers, The Hague, the Netherlands. pp. 9-18.

Kim CS, Lee CH, Shin JS, Chung YS, Hyung NI (1997). A simple and rapid method for isolation of high quality genomic DNA from fruit trees and conifers using PVP. Nucleic Acids Res. 25:1085-1086.

Kikuchi Y, Hosokawa T, Nikoh N, Meng XY, Kamagata Y, Fukatsu T (2009). Host-symbiont co-speciation and reductive genome evolution in gut symbiotic bacteria of acanthosomatid stinkbugs, BMC Biol. 7(1):2.

Lilburn TC, Kim KS, Ostrom NE, Byzek KR, Leadbetter JR, Breznak JA (2001). Nitrogen fixation by symbiotic and free-living spirochetes. *Science.* 292:2495-2498.

Mc Ewen PK, New TR, Whittington AE (2001). Lacewings in the crop improvement. Cambridge University Press, New York.

Medina P, Budia F, Del Estal P, Viñuela E (2003). Effect of three modern insecticides, pyriproxyfen, spinosad and tebufenozide, on survival and reproduction of Chrysoperla carnea adults. Ann. Appl. Biol. 142:55.

Moran NA, Russell JA, Koga R, Fukatsu T (2005). Evolutionary relationships of three new species of Enterobacteriaceae living as symbionts of aphids and other insects. Appl. Environ. Microbiol. 71:3302-3310.

Nardi JB, Mackie RI, Dawson JO (2002).Could microbial symbionts of arthropod guts contribute significantly to nitrogen fixation in terrestrial ecosystems? J. Insect Physiol. 48:751-763.

New TRR (1975).The biology of Chrysopidae and Hemerobiidae (Neuroptera) with reference to their usage as biocontrol agents. R. Entomol. Soc. Lond. Proc. A. 127:115-140.

Nguyen NH, Suh SO, Blackwell M (2007). Five novel *Candida* species in insect- associated yeast clades isolated from Neuroptera and other insects, *Mycologia,* 99:842-858.

Nguyen NH, Suh SO, Erbil CK, Blackwell M (2006). *Metschnikowia noctiluminum* sp. nov, *Metschnikowia corniflorae* sp. nov., and *Candida chrysomelidarum* sp. nov., isolated from green lacewings and beetles. Mycol. Res. 110:346-356.

Novakov AE, Hypsa V (2007). A new Sodalis lineage from bloodsucking fly *Craterina melbae* (Diptera, Hippoboscoidea) originated independently of the tsetse flies symbionts *Sodalis glossinidius.* FEMS Microbiol. Lett. 269:131-135.

Oliver KM, Campos J, Moran NA, Hunter MS (2008). Population dynamics of defensive symbionts in aphids. Proc. R. Soc. 275:293-299.

O'Neill S, Giordano R, Colbert AM, Karr TL, Robertson HM (1992). 16S rRNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic incompatibility in insects. Proc. Natl. Acad. Sci. USA 89(7):2699-2702.

Pathan AK, Sayyed AH, Aslam M, Razaq M, Jilani G, Saleem MA (2008). Evidence of field-evolved resistance to organophosphates and pyrethroids in *Chrysoperla carnea* (Neuroptera: Chrysopidae). J. Econ. Entomol. 101:1676-84.

Peter W, Magali P, Elzbieta R, Paul GB, Stefanos A, Miryan C, Tobias UTL, Lee JR, Arne H, Marie B, Cletus PK, Jure P, Alan K (2012). “This is not an apple” -yeast mutualism in codling moth. J. Chem. Ecol. 38:949-957.

Peterson JK, Schalk JM (1994). Internal bacteria in the Chrysomelidae. In Novel aspects of the biology of Chrysomelidae. Edited by: PH Jolivet ML, Cox and E Petitpierre, Kluwer Academic Publisher, Dordrecht, the Netherlands. pp. 393-405.

Piel J (2002). A polyketide synthase-peptide synthetase gene cluster from uncultured bacterial symbionts of Paederus beetles: Proc. Natl. Acad. Sci. USA. 99:14002-14007.

Russell JA, Latorre A, Sabater-Munoz B, Moya A, Moran NA (2003). Side-stepping secondary symbionts: widespread horizontal transfer across and beyond the Aphidoidea. Mol. Ecol. 12:1061-1075.

Sauer C, Stackebrandt E, Gadau J, Holldobler B, Gross R (2000). Systematic relationships and co-speciation of bacterial endosymbionts and their carpenter ant host species: proposal of the new taxon *Candidatus blochmannia* gen. nov. Int. J. Syst. Evol. Microbiol. 50:1877-1886.

Sayyed AH, Pathan AK, Faheem U (2010). Cross-resistance, genetics and stability of resistance to deltamethrin in a population of *Chrysoperla carnea* from Multan: Pakistan. Pestic. Biochem. Physiol. 98:325-332.

Scarborough CL, Ferrari J, Godfray HCJ (2005). Aphid protected from pathogen by endosymbionts. Science 310:1781-1781.

Spiegler PE (1962). The origin and nature of the adhesive substance in larvae of the genus *Chrysopa* (Neuroptera, Chrysopidae). Ann. Entomol. Soc. Am. 55:69-77.

Suh SO, Blackwell M (2004). *Metschnikowia chrysoperlae* sp. nov, *Candida picachoensis* sp. nov. and *Candida pimensis* sp. nov, isolated from the green lacewings *Chrysoperla Comanche* and *Chrysoperla carnea* (Neuroptera: Chrysopidae). Int. J. Syst. Evol. Microbiol. 54:1883-1890.

Symondron WUC, Sunderland KD, Greenstone MH (2002). Can generalist predator be effective biocontrol agents? Annu. Rev. Entomol. 47:561-594.

Tauber MJ, Tauber CA, Daane KM, Hagen KS (2000). Commercialization of predators: recent lessons from green lacewings (Neuroptera: Chrysopidae). Ann. Entomol. Soc. Am. 46:26-38.

Vega FE, Dowd PF (2005). The role of yeasts as insect endosymbionts. insect-fungal associations: ecology and evolution, New York: (Oxford University Press). pp. 211-243.

Venkatesan T, Jalali SK, Murthy KS, Rabindra RJ, Lalitha Y (2009). Occurrence of insecticide resistance in field populations of *Chrysoperla zastrowi arabica* (Neuroptera: Chrysopidae) in India. Indian J. Agric. Sci. 79:910-912.

Weiss BL, Mouchotte R, Rio RV, Wu YN, Wu Z (2006). Interspecific transfer of bacterial endosymbionts between tsetse fly species: infection establishment and effect on host fitness: Appl. Environ. Microbiol. 72:7013-7021.

White TJ, Bruns T, Lee S, Taylor JW (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR Protocols: a guide to methods and applications. Academic Press, San Diego. pp. 315-322.

Woolfolk SW, Cohen AC, Inglis GD (2004). Morphology of the alimentary canal of *Chrysoperla rufilabris* (Neuroptera: Chrysopidae) adults in relation to microbial symbionts. Ann. Entomol. Soc. Am. 97:795-808.

Woolfolk SW, Inglis GD (2003). Microorganisms associated with field collected *Chrysoperla rufilabris* (Neuroptera: Chrysopidae) adults with emphasis on yeast symbionts. Biocontrol 19:155-168.

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Full Length Research Paper

Biological control of citrus green mould, *Penicillium digitatum*, by antifungal activities of Streptomyces isolates from agricultural soils

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Streptomyces strains can control postharvest citrus fruit diseases caused by *Penicillium digitatum*. One hundred and ten (110) *Streptomyces* strains were isolated from agricultural soils and screened against *P. digitatum*. The antagonistic activities of the isolates were determined through dual culture technique. In the 110 isolates, only strain 328 showed strong antifungal inhibitory effects. *Streptomyces* strain 328 showed a maximum biomass value (1.85 g/L) after 72 h incubation in yeast extract, malt extract, glucose (YMG) medium; in starch casein medium, the highest value was 0.68 g/L after 96 h incubation. The metabolites produced in starch casein medium showed the maximum inhibition zone against *P. digitatum* (28 mm diameter). *Streptomyces* strain 328 metabolites have molecular mass higher than 2000 and they have fungistatic mode of action against *P. digitatum*. In *in vivo* assays, the selected isolate has significantly reduced severity of green mold on crops which have been stored under 24°C for 28 days as compared to the controls. The inhibition effect of about 95% was shown by strain 328.

Key words: *Penicillium digitatum*, *Streptomyces* spp., Actinomycetes, biocontrol.

INTRODUCTION

Postharvest losses of fresh fruits and vegetables may reach very high values depending on species, harvest methods, length of storage and marketing conditions, Postharvest diseases often account for a major part of losses (Brown and Chambers, 1996; Bull et al., 1997; Pailly et al., 2004) and their control requires use of a large amount of fungicides (Wilson et al., 1993; Holmes and Eckert, 1999). Biological control of postharvest diseases by antagonistic microorganisms seems to be a promising alternative to fungicides (Qing and Shiping, 2000; Fan and Tian, 2001; Long et al., 2005; Zhang et al., 2005). Understanding the methods of action of antagonisms is essential to allow the use of antagonists under partial conditions and to enhance their biological control while protecting the human health and the environment. Several modes of action have been documented for the antagonistic activity of biological control agents: they act by multiplying on the fruit surface or within wounds on the fruit, then by competing for space and nutrient at an infection court on the

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commodity, by antibiosis, by restricting the action of hydrolytic enzymes produced by the pathogen, by producing enzyme to degrade pathogen cell walls, and/or by direct parasitism of the pathogen (Jijakli and Lepoivre, 1998; Bar-Shimon et al., 2004; Long et al., 2005). Green mould, caused by *Penicillium digitatum* Sacc., is among the most economically important postharvest diseases of citrus worldwide. It has been estimated that losses of citrus fruit due to green mould infection is 30-50% in China (Boxun et al., 2002), the world's largest producer of citrus fruit. One major research focus in this area has been the selection and development of effective and environment-friendly agents for biological control of this disease. The purpose of these experiments was to evaluate the commercial potential of Streptomycetes for the control of postharvest decay of citrus fruit. At present, we have targeted our efforts toward the control of green mould.

Streptomyces spp. are one of the most attractive sources of biologically active substances such as vitamins, alkaloids, plant growth factors, enzymes and enzyme inhibitors (Omura, 1986; Shahidi, 2003). Soil Streptomycetes are one of the major contributors to the biological buffering of soils and have roles in decomposition of organic matter conductive to crop production (Gottlieb, 1973; Kieser et al., 2000). For this research, 110 isolates of *Streptomycetes* were isolated from agricultural soils of Kerman of Iran and screened against *P. digitatum in vitro* and *in vivo*. The objective of this study is to isolate *Streptomyces* strains having antagonistic properties with the aim that they can serve as gene donors in developing resistant transgenic plants and used for soil amendments as biofertilizer or biofungicide in biological control of the tested pathogen. From all tested isolates of *Streptomycetes*, 11 *Streptomyces* strains showed high *in vitro* anti green-mold activity.

MATERIALS AND METHODS

Culture media

P. digitatum was obtained from Iran Plant Protection Research Institute (IPPI). The pathogen was cultured on potato dextrose agar (PDA). Spores were harvested by flooding the surface of 10 days-old cultures with distilled water; the inoculum concentration used was 10^6 spores/ml. This concentration is commonly used in citrus postharvest experiments (Eckert and Brown, 1986). Casein glycerol agar (CGA) was prepared from basic ingredients as described by Küster and Williams (1964) and used as *Streptomycetes* culture.

Soil sampling and isolation of Streptomycetes

Samples of soil were collected from grassland, orchards and vegetable fields in different parts of Kerman, Iran (Kerman is one of the major region that produces varied agricultural crop, particularly citrus). Several samples were selected using an open-end soil borer (20 cm in depth, 2.5 cm in diameter) as described by Lee and Hwang (2002). Soil samples were taken aseptically from a depth of 10-20 cm below the soil surface. Samples were air-dried at room temperature for 7 to 10 days and then passed through a 0.8 mm mesh sieve and were preserved in polyethylene bags at room temperature. Ten gram samples were mixed with sterile distilled water (100 ml) and the mixtures were shaken vigorously for one hour and then allowed to settle. One milliliter of soil suspensions (diluted 10^{-1}) were transferred to 9 ml of sterile distilled water and subsequently diluted to 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}. Inoculum consisted of adding aliquots of 10^{-3} to 10^{-6} soil dilutions to autoclaved CGA (1, 25 ml^{-1}CGA) at 50°C and then poured into plates. Three independent replicates were considered for each dilution. Plates were incubated at 30°C for up to 20 days. The *Streptomyces* colonies were isolated and incubated at 28°C for one week and stored in a refrigerator as pure cultures (growing a single clone on medium is indicative of pure culture) and at the end 110 *Streptomyces* isolates were screened.

In vitro studies

To estimate the antifungal activity of the *Streptomyces* isolates against pathogen, bioassays were done using agar disk method as described by Shahidi Bonjar (2003) and Aghighi et al. (2004) and the ratings used were modified by Lee and Hwang (2002) and El-Tarabily (2000). Antifungal activity was defined as growth inhibition against pathogenic fungi and was calculated by subtracting the radius distance (mm) of fungal growth obtained in the control and antagonist plates. Reference values to evaluate inhibition were: slight (5 to 9 mm), moderate (10 - 19 mm) and strong (> 20 mm).

Monitoring activity

Growth kinetics and metabolites production for Streptomyces strain 328

Kinetics studies were carried out with starch casein and YMG media (g/l : yeast extract, 4; malt extract,10; glucose, 4; K2HPO4 , 2; casaminoacids, 0.1%; pH 7) to optimize *Streptomyces* strain 328 growth and metabolites production. 50 ml of YMG and starch casein media were inoculated with 5 ml of a cell suspension (1.5 10^8cel/ml) of *Streptomyces* strain 328, incubated for 7 days at 28°C, samples were taken every 24 h. Biomass was separated by filtration and was then dried. The supernatant was used to carry out antagonism assays in solid medium and *in vivo* (Maldonado et al., 2010).

Determination of minimum inhibitory concentrations (MIC)

To measure the MIC values, two-fold serial dilutions of 50, 25, 12.5, 6.25, 3.125, 1.562, 0.781, 0.390 and 0.187 mg ml^{-1} of the crude extract were prepared in DM solvent and assayed by well diffusion-method as described by Shahidi (2004). The MIC was defined as the lowest concentration able to inhibit any visible fungal growth. All data represent average of three replicated experiments.

Polarity detection rate of active crude extract in organic solvents

To evaluate the relative polarity of the active principle(s) present in the crude extract, 2 ml of each of H_2O, methanol and chloroform were added to 20 mg pulverized-crude samples separately and vortex for 20 min. Each sample was then centrifuged at 3000 rpm for 15 min using a bench low speed centrifuge. Supernatants and pellets were separated at 50°C and assayed at concentration of 20 mg ml^{-1} by agar diffusion-method (Bonjar, 2004).

Determination of thermal inactivation point (TIP)

Small aliquots (10 mg ml^{-1}) of soluble crude extract were exposed to 30, 40, 50, 60, 70, 80 and 90°C for 10 min and cooled on ice afterwards to monitor the effect of temperature on bioactivity. For temperatures over 90°C, heat oil was used. Bioactivity of treated samples was evaluated using well diffusion method. Control included incubation of an untreated sample at 28°C (Nawani and Kapadnis, 2004).

Detection of fungicidal activity and metabolites mode of action

Small blocks of inhibition zones (1 mm^3) of *Streptomyces* strain 328 against *P. digitatum* was transferred to fresh PDA plates and incubated for seven days at 24-28°C. During incubation, growth or lack of growth of the fungus was investigated both visually and microscopically. Rejuvenation of growth was indicative of fungistatic and lack of growth represented fungicidal properties of the antagonist.

Chloroform assay for detection of antibiotic

Spore suspensions (approximately 10^8 spores ml^{-1}) of individual isolates were dotted (10^{-6} l per spot) onto 15 ml starch casein agar plates, five dots per plate. Plates were incubated at 28°C for 3 days (Davelos et al., 2004). Dotted isolates were killed by inverting the uncovered Petri plates over 4 ml of chloroform in a Watch glass for 1 h. Watch glasses were removed, and plates were aerated in a fume hood for 30 min to permit evaporation of chloroform. Plates were subsequently overlaid with 15 ml of 1% water agar and inoculated with 10^{-6} l of test isolate, *P. digitatum*, (approximately 10^8 spores ml^{-1}) spread uniformly over the surface of the agar. Plates were incubated at 24°C for four to five days. The size of any zone of growth inhibition of the overlaid isolate surrounding any dotted isolate were measured in millimeters from the edge of the dotted colony to the edge of the cleared zone.

Metabolite molecular mass estimation

A benzoylated dialysis tube (SIGMA) capable of separating molecular mass compounds between 1200 and 2000 M.W. was used. Five millilitres of metabolites were dialyzed at 4°C, 6 h in phosphate buffer 0.2 M, pH 7. Recovered metabolites from the dialysis tube were sterilized by filtration and assayed for *in vitro* antagonism against *P. digitatum*.

Scanning electronic microscopic

Mycelial morphology of *Streptomyces* strain 328, was showed by using scanning electronic microscopic (CAM SCAN – MV 2300). Preparation for the scanning electron microscope consisted of using the culture held by a piece of double stick scotch tape. The stubs were coated in a sputter coater for 2 min. Afterwards, the specimens were viewed and digital electron micrograph were prepared at magnification of 6000 to 20000x with an accelerating voltage of 20 kv accordingly.

***In vivo* antagonism assays**

In vivo assays were carried out with *Streptomyces* strain 328 to study its inhibitory activity against *P. digitatum*. Fresh orange cultivars Valencia of uniform size and maturity, without wounds, were used in this study. They were picked and washed for 10 min in running tap water, surface sterilized in 1% sodium hypochlorite for 2 min and aseptically rinsed carefully with sterile distilled water,. Four batches of 10 oranges each were used: (1) oranges without treatment (control disease); (2) oranges treated with solution of sodium bicarbonate (200 ppm) (control without disease); (3) oranges treated with 50 ml of sodium bicarbonate (200 ppm), plus 50 ml of metabolites obtained after centrifugation (200 rpm) of *Streptomyces* culture; (4) oranges treated with 50 ml of sodium bicarbonate (200 ppm) with 50 ml of Spore of *Streptomyces* culture; (5) Oranges treated as in 3 and 4, but with the addition of 20% wax-water emulsion. The fruits were sprayed, allowed to dry, placed in boxes with moistened paper towels to maintain 85% humidity and covered with plastic. They were kept for three weeks at 4°C and 1 week at room temperature to reproduce the storage conditions until reaching their final destination. The results were expressed as percentage of diseased oranges. The results reported are the average of triplicate determinations (Maldonado et al., 2010).

Experimental design and data analysis

The experiments were arranged in a completely randomized design. Each treatment was replicated three times with ten fruits for each replication. The experiments were repeated twice. Extent of disease was planned at each of the four inoculation sites by assessing presence or absence of soft rot symptoms after four day. Statistical analysis was performed with SPSS software package version 15.00 for windows. Analysis of variance and Tukey test of Post Hoc were used for analysis of recorded percentages of mean values. Significant factors in the univariate analysis at first step were entered to multivariate comparisons.

RESULTS

Antifungal bioassays

From tested *Streptomyces* isolates, eleven isolates were active in dual culture methods, showing two antagonists and the suppressive reaction of *P. digitatum*. Figure 1 shows bioassay results of *Streptomyces* strain 328 against *P.digitatum* measured in agar disk-method. In both methods *Streptomyces* strain 328 show antifungal inhibitory effects on the pathogen.

Streptomyces strain 328 in *in vitro* antagonism against several fungal strains

The *Streptomyces* strain 328 culture and its metabolites against *P. digitatum* and *P. italicum* (because *P. italicum* like *P. digitatum* produce common mold on citrus) were studied and both could inhibit fungal growth (Figure 2). Table 1 shows that the inhibitory effect was higher in the test carried out with *Streptomyces* strain 328 culture.

Monitoring antagonistic activity and growth curve

Activity reached maximum after five days in rotary cultures. In shake cultures, this interval was used to harvest cultures to prepare crude extract for use in further investigations. Activity versus post seeding time in rotary

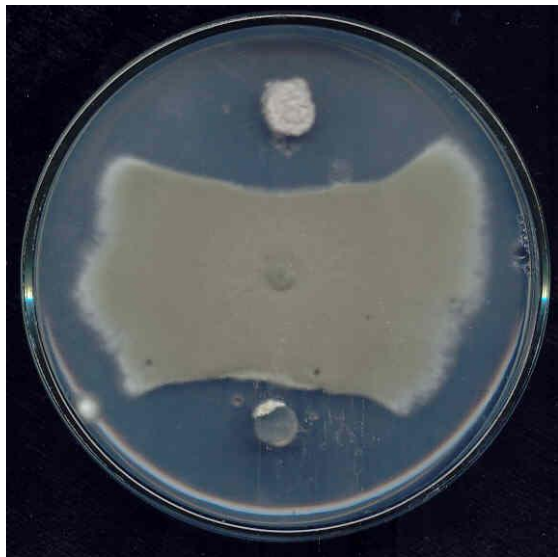

Figure 1. Suppressive reaction of two *Streptomyces* isolates on *Penicillium digitatum*. up: *Streptomyces* isolate 328 and down: *Streptomyces* isolate 223. Center: colony of *Penicillium digitatum* agar disk which its growth towards the antagonists is inhibited clearly.

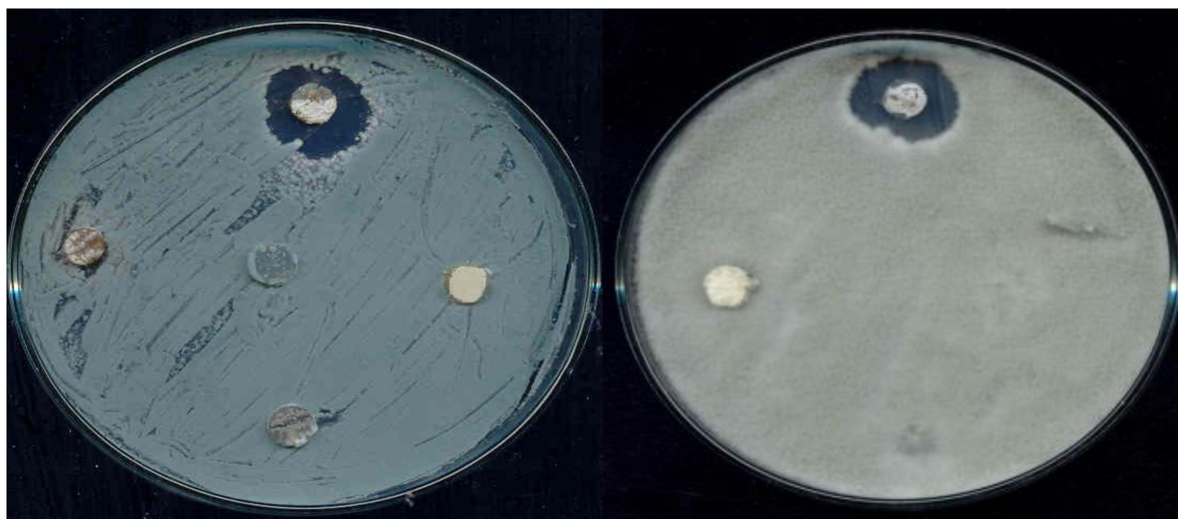

Figure 2. Bioassay results of *Streptomyces* isolates against *Penicillium digitatum* and *P. italicum*. Left; Clockwise from top: *Streptomyces* strain 328 against *P. italicum*, Center: blank agar disk (control). Right; Clockwise from top: *Streptomyces* strain 328 against *P. digitatum*, Down: blank agar disk (control).

Table 1. Antifungal activity of *Streptomyces* isolate 328 metabolites and culture against *P. digitatum* and *P. italicum*.

***Penicillium* spp.**	**Inhibition (%)**	
	***Streptomyces* isolate 328 metabolites**	***Streptomyces* isolate 328 culture**
P. digitatum	20.22	70.10
P. italicum	23.70	76.55

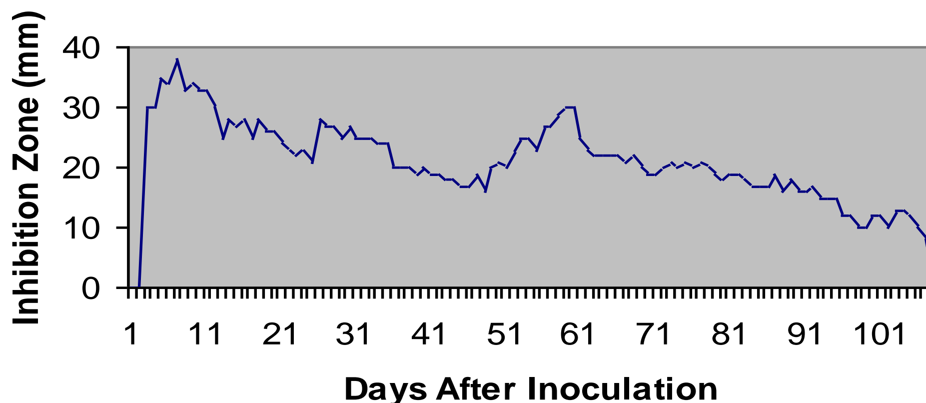

Figure 3. *In vitro* bioassay results of *Streptomyces* strain 328 against *Penicillium digitatum* in rotary cultures indicative of production time versus inhibition zones.

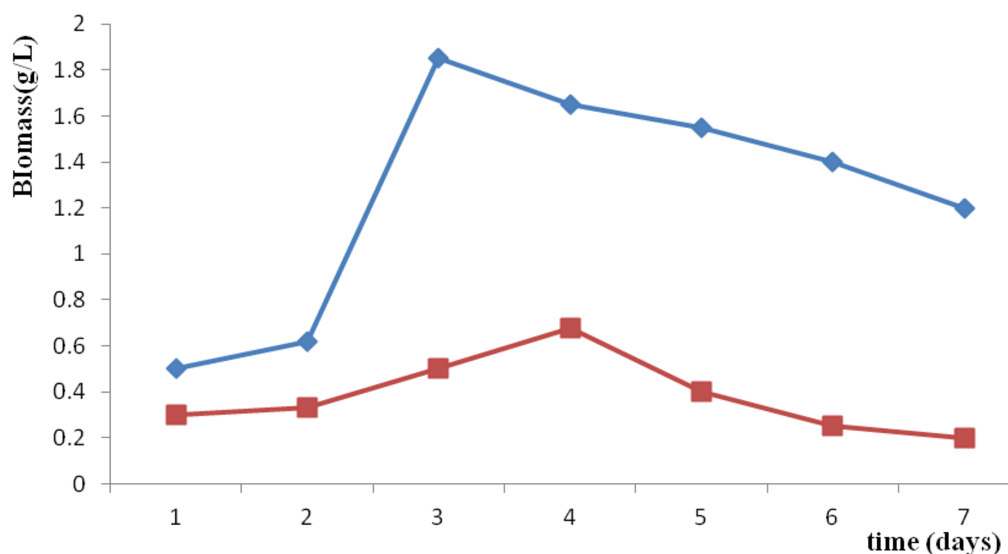

Figure 4. Growth kinetics of *Streptomyces* isolate 328 in starch casein (■) and YMG (♦) media pH 7 at 28°C during seven days.

cultures is presented in Figure 3.

Streptomyces strain 328 growth and metabolites production in YMG and starch casein media

Growth kinetics for *Streptomyces* strain 328 in YMG medium showed a maximum biomass value of 1.85 g/l after 72 h incubation, while in starch casein medium the highest value was 0.68 g/l after 96 h incubation (Figure 4). The metabolites obtained from one to seven days fermentations in starch casein and YMG media were assayed against *P. digitatum* and *P. italicum* in liquid media antagonism tests. The metabolites produced in YMG after 48 to 72 h incubation showed the maximum inhibition against *P. italicum* and *P. digitatum* (54.80 and 63.27%, respectively) while those produced in starch casein presented the highest inhibition levels after 5 days fermentation (12.75 and 13.25%, respectively) as seen in Table 2. All metabolites from more than 6 days fermentation kept inhibitory activity against both phytopathogens, but their inhibition percentages were lower than the maximum values obtained in previous days.

Determination of MIC

In well diffusion-method, MIC of the crude was determined as 0.39 mg ml^{-1} against *P. digitatum* (Figure 5).

Table 2. Inhibition (%) produced by of *Streptomyces* isolate 328 metabolites from YMG and starch casein media against *P. digitatum* and *P. italicum*.

Day		Inhibition (%)						
		1	2	3	4	5	6	7
P. digitatum	Metabolites from YMG	47.22	56.12	63.27	61.30	39.03	22.41	13.70
	Metabolites from starch casein	2.5	2.75	4.75	7.25	13.25	11.65	6.21
P. italicum	Metabolites from YMG	33.5	47.25	52.03	54.80	23.5	22.01	18.69
	Metabolites from starch casein	0.36	1.83	4.02	10.50	12.75	12.33	9.67

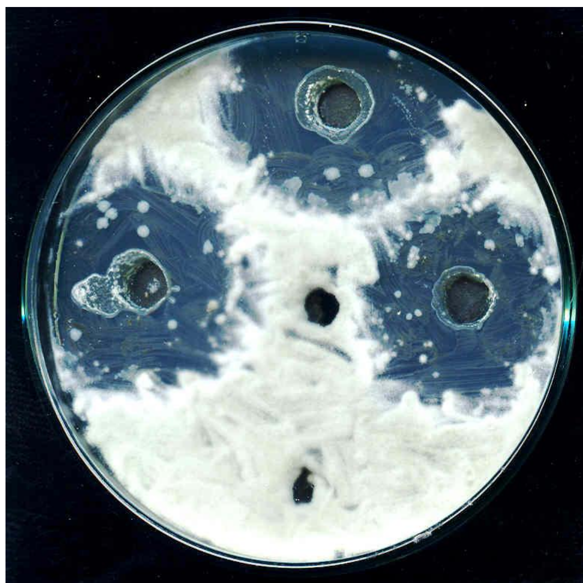

Figure 5. MIC bioassay results of *Streptomyces* isolates against *Penicillium digitatum*. Clockwise from top: *Streptomyces* strain 328 after 11 days, the *Streptomyces* after 19 days, the *Streptomyces* after 20 days, blank agar hole (control).

Polarity detection rate of active crude extract in organic solvents

Solubility results are indicated in Table 3. The results show more than one active principle involved since activity is traceable in polar solvents of H_2O, methanol.

Determination of TIP

Bioactivity of active isolate diminished to zero at 160°C.

Detection of fungicidal activity and metabolites mode of action

P. digitatum was able to grow in PDA medium after their spores were exposed to *Streptomyces* strain 328 metabolites at 28°C for five days. Based on the result we assumed that *Streptomyces* strain 328 metabolites presents fungistatic mode of action.

Table 3. Bioassay results of solubility tests of the antifungal principle(s) of *Streptomyces* isolate 328 against *Penicillium digitatum* in fractions of different solvents indicated by well diffusion-method at 10 mg ml^{-1} of dry crude.

Solvent	Fraction	Activity
H_2O	Supernatant	+
	Pellet	+
Methanol	Supernatant	+
	Pellet	+
Chloroform	Supernatant	-
	Pellet	+

Chloroform assay

Among 11 effective *Streptomyces* strain, 3 strains of 328, 263 and 394 retained their antifungal activities after exposure to chloroform.

Molecular mass evaluation for *Streptomyces* strain 328 metabolites

P. digitatum growth inhibition produced by *Streptomyces* strain 328 metabolites showed identical levels as experiential without dialysis and after this treatment. We assumed that benzoylated dialysis tube retained *Streptomyces* strain 328 metabolites and they have a molecular mass higher than 2000.

Scanning electron microscope studies

Scanning electron micrograph of mycelia of *Streptomyces* strain 328 is indicated in Figure 6 (this picture is necessary for determination of spore level and chain morphology to identification of streptomyces species).

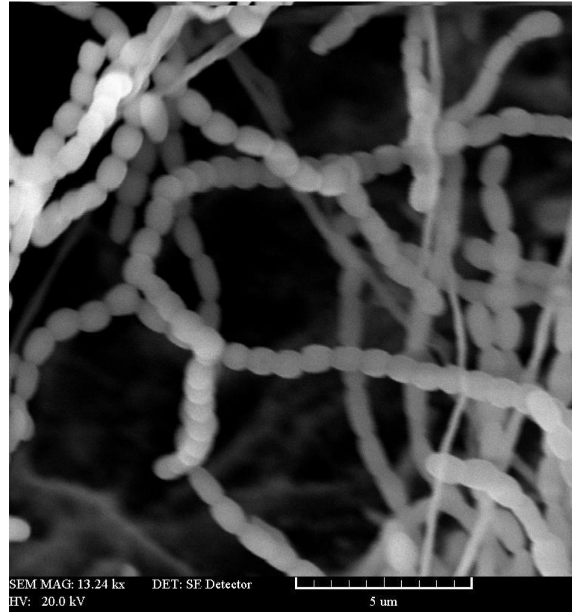

Figure 6. Scanning electron micrographs of mycelia of *Streptomyces* isolate No. 328

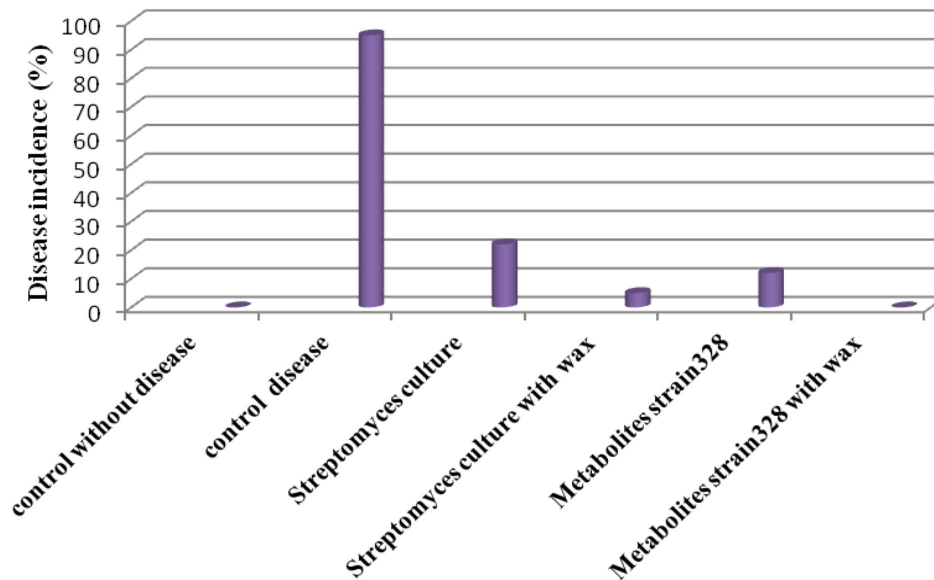

Figure 7. *In vivo* assays of *Streptomyces* isolate 328 metabolites and *Streptomyces* isolate 328 culture treated and not treated with wax against *P. digitatum*

In vivo antagonism assays

Oranges without treatment showed disease incidence percentages of 80 to 100%, while oranges with chemical fungicide treatment showed no signs of disease. When biological treatment was applied, the results were as follows: (1) *Streptomyces* culture without wax reduced disease to 22%; with wax, the disease was reduced to 5%. (2) Metabolites without wax produced disease incidence levels of 12%; with wax disease, was totally controlled (Figure 7).

Statistical data analysis

Statistical analysis of data with multiple comparisons of

Tukey test indicated 95% significance level. There was significance difference indicative of high level of antagonistic effect of *Streptomyces* strain no. 328 against *P. digitatum.*

DISCUSSION

In vitro assays of *Streptomyces* isolate no. 328 metabolites presents higher inhibitory effect than *Streptomyces* culture. This effect could be attributed to competition for space, nutrients, etc. The YMG medium was better than starch casein medium for *Streptomyces* growth and its metabolites production. These results partially coincide with those obtained by Sabaratnam and Traquair (2002) and Maldonado et al. (2010); the authors also observed that maximum biomass production occurred after three days fermentation in the same conditions. The highest *Streptomyces* isolate no. 328 biomass was 1.85 g/l in YMG medium after 96 h incubation, but grew poorly in starch casein medium, which, Sabaratnam and Traquair (2002) suggested as better than other media. In the case of dual culture technique, only moderate to slight inhibitions were observed. Aghighi et al. (2004) found that *Streptomyces* metabolites assayed in solid medium tests presented a slight inhibition against *Fusarium solani*, but it was moderate to strong against *Phytophtora megasperma*. With the previous antagonist incubation assay, the results were better than the obtained with dual culture. Trejo-Estrada et al. (1998) demonstrated that *Streptomyces violaceusniger* YCED9 showed 50% inhibition with this same technique against different phytopathogens (*Rhizoctonia solani, Gaeumannoyces graminis, Pythium ultimun* and *S. homeocapa*. When treatments were carried out *in vivo*, citrus green mold caused by *P. digitatum* was significantly reduced and wax addition contributed to fruit protection, to avoid orange dehydratation. In this case, better results were realized with *Streptomyces* isolate No. 328 metabolites, in *in vitro* assays. Similar results of disease inhibition produced by *Streptomyces* sp. R03 metabolite against lemon pathogens has been reported earlier by Maldonado et al. (2010). Plaza et al. (2004) pointed out that it is not possible to extrapolate all *in vitro* results to natural systems since *in vivo* there are other factors, those in the environment as the nature of its factors, interfere with other microorganisms, etc. *Streptomyces* 328 metabolites was not inhibited by heat and choloroform; this effect could be due to their chemical nature and could not inhibit the lytic enzymes activity in fermentation broth. Mahadevan and Crawford (1997) and Trejo-Estrada et al. (1998) founded that, although antibiosis is one of the main mechanisms found in *Streptomyces*, the production of lytic enzymes (chitinases, β 1-3 glucanases) capable of acting on the fungus cell wall by altering growth and possibility is also common among them. *Streptomyces* isolate No. 328 metabolites have molecular mass higher than 2000 and their mode of action is fungistatic, but several authors report *Streptomyces* ability to produce fungicidal substances like Streptomycin, Kasugamycin, Polyoxin and Validamycin that have different action modes and the active ingredients of many biological plaguicides (Duran, 2004). Our findings represent the presence of potential antifungal metabolite(s) *Streptomyces* strain 328 against *P. digitatum*. Antifungal activity of the isolate found in this study highlights its importance as a candidate for further investigation in biological control of the world-wide destructive citrus postharvest disease. Further works on the project would be focused on field and long-term storage evaluation of the antagonist against the pathogen in the cosmopolitan malady of citrus postharvest disease. The genes encoding many antifungal characteristics are currently being used by agribusiness to create genetically modified plants that have increased fungal resistance in the field or packing house. We believe that the results of these findings can form the avenue for production of resistant transgenic-plants with recombinant DNA having antifungal genes cloned from biologically active *Streptomyces* isolates which would lead to environmentally safer measures in plant-pest management.

Conflict of Interests

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

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REFERENCES

Aghighi S, Shahidi Bonjar GH, Rawashdeh R, Batayneh S,Saadoun I (2004). First report of antifungal spectra of activity of Iranian Actinomycetes strains against Alternaria solani, Alternaria alternate, Fusarium solani, Phytophthora megasperma, Verticillium dahliae and Saccharomyces cerevisiae. Asian J. Plant Sci. 3:463-471.

Bar-Shimon M, Yehuda H et al (2004). Characterization of extracellular lytic enzymes produced by the yeast biocontrol agent Candida oleophila. Curr. Genet. 45(3):140-148.

Bonjar G (2004). New approaches in screening for antibacterials in plants. Asian J. Plant Sci. 3(1):55-60.

Boxun D, Lanju W et al (2002). Storage-Transportation and Marketing of Horticultural Products Experiment, Chinese Agricultural Publishing Company, Beijing.

Brown GE, Chambers M (1996). Evaluation of biological products for the control of postharvest diseases of Florida citrus. Proc. Fla. State Hort. Soc. 109:278-282

Bull CT, Stack JP, Smilanick JL (1997). Pseudomonas syringae Strains ESC-10 and ESC-11 Survive in Wounds on Citrus and Control Green and Blue Molds of Citrus. Biol. Control 8(1):81-88.

Davelos AL, Kinkel LL, Deborah AS (2004). Spatial variation in frequency and intensity of antibiotic interactions among

Streptomycetes from prairie soil. Appl. Environ. Microbiol. 70(2):1051-1058.

Durán J (2004). Gua técnica de ingredientes activos de bioplaguicidas. Centro Agronómico Tropical de Investigación y Enseñanza (CATIE/GTZ), Proyecto Fomento de Productos Fitosanitarios No Sintéticos. Turrialba, Costa Rica.

Eckert J, Brown G (1986). Evaluation of postharvest fungicide treatments for citrus fruits. In: Methods for Evaluating Pesticides for Control of Plant Pathogens. K. D. Hickey, ed. American Phytopatological Society, St. Paul, MN. pp. 92-97.

El-Tarabily K, Soliman M, Nassar AH, Al-Hassani HA, Sivasithamparam K, McKenna F, Hardy GEStJ (2000). Biological control of Sclerotinia minor using a chitinolytic bacterium and actinomycetes. Plant Pathol. 49(5):573-583.

Fan Q, Tian S (2001). Postharvest biological control of grey mold and blue mold on apple by Cryptococcus albidus (Saito) Skinner. Postharvest Biol. Technol. 21(3):341-350.

Gottlieb D (1973). General consideration and implications of the Actinomycetales. Society for Applied Bacteriology symposium series.

Holmes GJ, Eckert JW (1999). Sensitivity of *Penicillium digitatum* and *P. italicum* to postharvest citrus fungicides in California. Phytopathology 89(9):716-721.

Jijakli MH, Lepoivre P (1998). Characterization of an exo-β-1, 3-glucanase produced by Pichia anomala strain K, antagonist of Botrytis cinerea on apples. Phytopathology 88(4):335-343.

Kieser T, Bibb MJ et al (2000). Practical streptomyces genetics, John Innes Foundation Norwich.

Küster E, Williams S (1964). Selection of media for isolation of streptomycetes. Nature 202:928-929.

Lee JY, Hwang BK (2002). Diversity of antifungal actinomycetes in various vegetative soils of Korea. Can. J. Microbiol. 48(5):407-417.

Long C-A,.Wu Z, Deng B-X (2005). Biological control of *Penicillium italicum* of citrus and *Botrytis cinerea* of grape by strain 34–9 of Kloeckera apiculata. Eur. Food Res. Technol. 221(1-2):197-201.

Mahadevan B, Crawford DL (1997). Properties of the chitinase of the antifungal biocontrol agent *Streptomyces lydicus* WYEC108. Enzyme Microbial Technol. 20(7):489-493.

Maldonado MC, Orosco CE, Gordillo MA, Navarro AR (2010). *In vivo* and *in vitro* antagonism of Streptomyces sp. RO3 against *Penicillium digitatum* and *Geotrichum candidum*. Afr. J. Microbiol. Res. 4(22):2451-2456.

Nawani N, Kapadnis B (2004). Production dynamics and characterization of chitinolytic system of Streptomyces sp. NK1057, a well equipped chitin degrader. World J. Microbiol. Biotechnol. 20(5):487-494.

Omura S (1986). Philosophy of new drug discovery. Microbiol. Rev. 50(3):259.

Pailly O, Tison G, Amouroux A (2004). Harvest time and storage conditions of ‘Star Ruby’grapefruit (Citrus paradisi Macf.) for short distance summer consumption. Postharvest Biol. Technol. 34(1):65-73.

Plaza P, Usall J, Teixidó N, Viñas I (2004). Effect of water activity and temperature on competing abilities of common postharvest citrus fungi. Int. J. Food Microbial. 90(1):75-82.

Qing F, Shiping T (2000). Postharvest biological control of Rhizopus rot of nectarine fruits by *Pichia membranefaciens*. Plant Dis. 84(11):1212-1216.

Sabaratnam S, Traquair JA (2002). Formulation of a Streptomyces Biocontrol Agent for the Suppression of Rhizoctonia Damping-off in Tomato Transplants. Biol. Control 23(3):245-253.

Shahidi Bonjar G (2003). *In vitro* monitoring of antibacterial properties of soil Streptomyces. Research project report. Department of Plant Protection, College of Agriculture, Bahonar University of Kerman, Iran.

Trejo-Estrada S, Paszczynski A, Crawford DL (1998). Antibiotics and enzymes produced by the biocontrol agent Streptomyces violaceusniger YCED-9. J. Ind. Microbiol. Biotechnol. 21(1-2):81-90.

Wilson CL, Wisniewski ME, Drobyb S, Chalutz E (1993). A selection strategy for microbial antagonists to control postharvest diseases of fruits and vegetables. Sci. Hortic. 53(3):183-189.

Zhang H, Zheng X, Fub C, X Yufang (2005). Postharvest biological control of gray mold rot of pear with *Cryptococcus laurentii*. Postharvest Biol. Technol. 35(1):79-86.

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Full Length Research Paper

The inhibitory effect of different chemical food preservatives on the growth of selected food borne pathogenic bacteria

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The inhibitory effect of different chemical food preservatives (potassium metabisulphite, sodium benzoate, citric acid, ascorbic acid and potassium sorbate) on the growth of selected food borne pathogenic bacteria (*Staphylococcus aureus, Klebsiella aerogenes, Proteus mirabilis, Pseudomonas aeruginosa and Escherichia coli*) were investigated using agar diffusion technique. The concentrations used on test bacteria ranged from 0.125 - 1.5 mg/ml. The minimum inhibitory concentrations (MIC) of the five preservatives were 0.5, 1.5, 1.5, 1.5 and 1.5 mg/ml for potassium metabisulphite, sodium benzoate, citric acid, ascorbic acid and potassium sorbate, respectively. The zone of inhibition of the test bacteria ranged from 4.0 - 21 mm. *S. aureus* was susceptible to all the five chemical food preservatives. All the MIC of chemical food preservatives used against selected pathogenic bacteria was below the acceptable daily intake (ADI) mg/kg body weight/day. The MIC of citric acid ascorbic acid has no ADI limit in the body.

Key words: Chemical preservatives, pathogenic bacteria, minimum inhibitory concentration, acceptable daily intake (ADI), inhibition zone.

INTRODUCTION

Food is defined as any chemical substance which when eaten, digested and absorbed by the body, produces energy, promotes growth and repairs the body tissues and regulates these processes (Olunlade et al., 2010). Foods are not only of nutritional value to those who consume them but often are ideal culture media for microbial growth. Chemical reactions that cause offensive and sensory changes in foods are mediated by bacteria that use food as a carbon and energy source. Some of the major bacterial genera which cause food borne infection and intoxication include *Staphylococcus aureus, Klebsiella aerogenes, Proteus mirabilis, Pseudomonas aeruginosus and Escherichia coli* e. t. c. (Pundir and Jain, 2011). Food, despite it supplies nutrients to the body; it can also be responsible for ill health (Adams and Moss, 1999). The ill health occurs as a result of ingestion of

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food borne pathogens (Frazier and Westhoff, 2002). The ingestion of food borne pathogen leads to food borne diseases which are food intoxications and infections (Frazier and Westhoff, 2002). The following micro-organisms (*S. aureus, K. aerogenes, P. mirabilis, P. aeruginosus and E. coli* etc) are normally implicated in food borne diseases (Adeleke and Oladapo, 2010). Based on the type of microorganisms responsible for food borne diseases, these diseases therefore, can be classified as being of bacterial, viral, fungal or protozoal. These diseases cause serious problems on the health of man. There is therefore, the need to control the growth of pathogenic microorganisms in foods. One way by which this is achieved is through the use of chemical preservatives. These chemicals are substances of no nutritional significance (Joshua, 2000). They are added to foods as antimicrobial agents to preserve them from deterioration and extend their shelf life (Jay, 2005). These chemicals should not have toxic effect on human cells (Mahindru, 2000). It has to be economical and should not have an effect on the taste and aroma of the original food, or any substance in food, nor encourage the development were of resistance strains are killed rather than inhibit micro-organisms. Most preservatives are inhibitory at acceptable level (Frazier and Weshtoff, 2002). Chemicals that have been used in the preservation of foods are including sodium chloride, sodium nitrate and nitrite, sodium benzoate, ascorbic acid and propionic acid (Mahindru, 2000). These chemical agents are employed to prevent microbial growth in food (Prescott et al., 2002). Some acids and salts especially benzoic and ascorbic acid and its salts are effective inhibitors of microbial growth and are intentionally added to many foods as preservatives (Dziezak, 1986). Other acids including acetic acid, fumaric acid, proponic acid and lactic acid are added to foods to prevent or delay the growth of pathogenic bacteria (Dziezak, 1986; Greer and Dilts, 1995; Podolak et al., 1996).

This study investigated the inhibitory effect of some chemical preservatives on the growth of selected food-borne bacterial pathogens.

MATERIALS AND METHODS

Collection of chemical preservatives and clinical isolates

Five chemical preservatives were used for the experiments. These were sodium metabisulphite, sodium benzoate, citric acid, ascorbic acid, and potassium sorbate. They were collected from Food Technology Department, Osun State Polytechnic, Iree, Osun State while the clinical isolates of *S. aureus, K. aerogenes, P. mirabilis, P. aeruginosa and E. coli* were collected from Ladoke Akintola University Teaching Hospital Complex, Oshogbo, Nigeria.

Confirmation of the identity of collected organisms

The method described by Cheesbrough (2002) was adopted to confirm the identity of the microorganisms adequately. The isolates were cultured on various selective and differential media to confirm their identity on Grams staining, motility and biochemical tests.

Preparation of different concentrations of chemical preservatives

Five different concentrations ranging from 0.0125 to 1.5 mg/ml of each chemical preservative were weighed and poured into the McCartney bottles containing 10 ml of sterile distilled water. Each concentration prepared was labeled and shaken vigorously (Christian, 1994; Doughari, 2006).

Antibacterial screening of the chemical preservatives

The agar plate diffusion method of Nostro et al. (2000), Xavier et al. (2003), Ajayi (2005), Omoya and Akharaiyi (2010) were used. The medium used was nutrient agar, which supports the growth of bacteria. Then with the aid of sterile needle and syringe pipette, 1.0 ml of each broth culture of standardized bacteria of 10^6 was added into sterilized plate and about 20 ml sterile nutrient agar, which had already cooled to 45°C ± 2, was poured aseptically, mixed and allowed to solidify. With the aid of sterile 6 mm cork borer, well were bored on the agar surface to the edge of the plate and labelled accordingly. Each well was then filled up with the chemical presservative concentration as prepared. The prepared plate cultures were incubated at 37°C ± 2 for 24 h. Clear zones of inhibition around the well indicated the sensitivity of the test bacteria to each of the chemical preservatives and diameter of the clear zones of inhibition was taken as an index of the degree of sensitivity by measuring with caliper. The venial caliper was placed on the transparent meter rule for proper accuracy of inhibition zone measurement (Pundir and Jain, 2011).

RESULTS AND DISCUSSION

Food preservation implies putting microorganisms in a hostile environment in order to cause their death. In this study, different concentrations of different chemical food preservatives were used to inactivate selected food borne pathogenic bacteria by agar diffusion technique. Figures 1 to 5 show the diameter zone of inhibition mediated by different concentrations ranging from 0.0125 to 1.5 mg/ml of food chemical preservatives (potassium metabisulphite, sodium benzoate, citric acid ,ascorbic acid and potasium sorbate) on the five test pathogenic bacteria (*S. aureus, K. aerogenes P. mirabilis, P. aeroginosa* and *E. coli*). The zones of inhibition were observed around the wells, this indicated antibacterial activities of the chemical preservatives. Different organisms have demonstrated different rankings for the inhibiting effects of chemical preservatives (Matsuda et al., 1994). The sensitivity of the different test organisms to different concentrations of chemical food preservatives was shown by zones of inhibition after 24 h of incubation; this is depicted in Figures 1 to 5. The absence of zones of inhibition around each well signified resistance. It was observed that the water used in preparation of these chemical preservatives was used as control which did not inhibit the growth of any of the test bacteria. *The S. aureus* had highest zone of inhibition of 21 mm for potassium sorbate at the

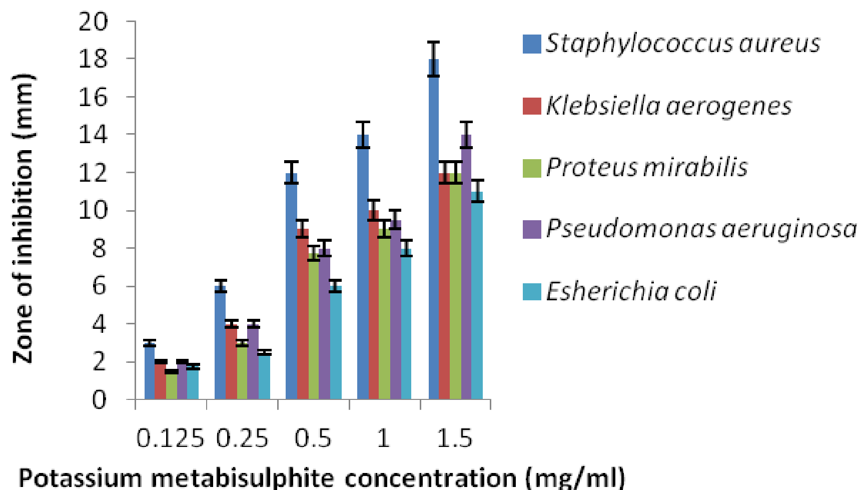

Figure 1. Diameter zone of inhibition (mm) mediated by different concentration (mg/ml) of potassium metabisulphite on selected food borne pathogenic bacteria.

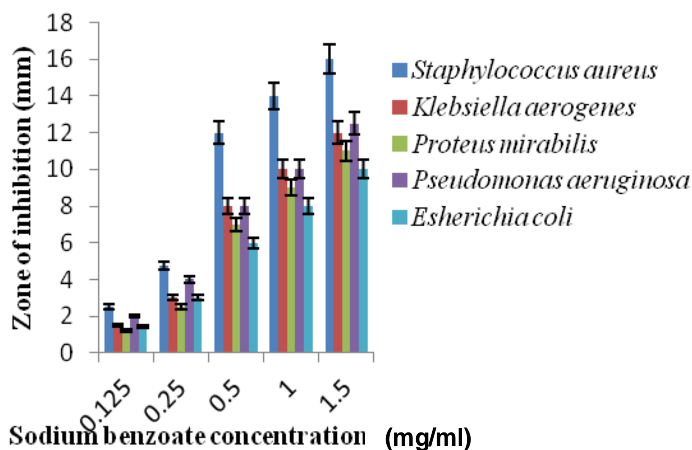

Figure 2. Diameter zone of inhibition (mm) mediated by different concentration (mg/ml) of sodium benzote on selected food borne pathogenic bacteria.

concentration of 1.5 mg/ml. This is an indication that *S. aureus* is sensitive to the chemical preservative as reported by Nanda (2005) that any zone of inhibition lesser than 4.0 mm is resistant, 4.0-12.0 mm are intermediate while any zone of inhibition that is more than 12.0 mm is sensitive to chemical food preservatives. The effect of potassium sorbate at various concentrations namely: 0.0125 to 1.5 mg/ml was tested for the inhibition of growth of the bacterial *S. aureus, K. aerogenes P. mirabilis, P. aeroginosa* and *E. coli* assay method and the results were summarized in Figures 1 to 5. The inhibition zone area increased with increase in the concentration of potassium sorbate for all the five bacterial cultures. Among the five bacterial cultures tested, potassium sorbate highly inhibited the growth of *S. aureus* by showing 21 mm zone of inhibition followed by *P. mirabilis*, (15 mm). The least zone of inhibition was seen in *E. coli* (12 mm) and *P. aerogenosa* (13 mm) and this is in line with the work of Jageethadevi et al. (2012) that reported inhibition zone increased with increase in concentration of chemical food preservatives. Then, higher concentration of chemical preservatives had greater inhibitory power on the microbial growth (Oyawoye et al., 1999). The zone of inhibition depends on Gram reaction of the preservatives. Gram positives bacteria are more sensitive than Gram negative to the chemical preservatives (Adams and Moss, 1999). *S. aureus* is a Gram positive bacteria while the rest test bacteria (*K. aerogenes P. mirabilis, P. aeroginosa* and *E. coli*) are Gram negative. The cell wall of Gram positive bacteria compose of peptidoglycan which is an essential polymer and interference with its synthesis or structure leads to loss of cell

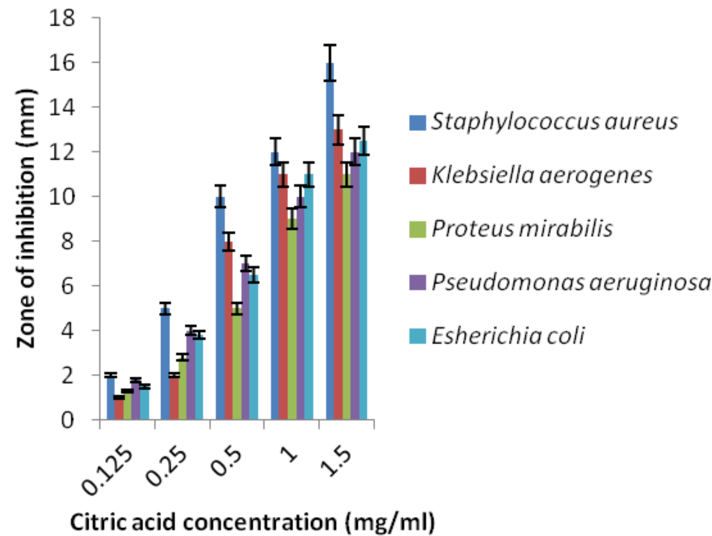

Figure 3. Diameter zone of inhibition (mm) mediated by different concentration (mg/ml) of citric acid on selected food borne pathogenic bacteria.

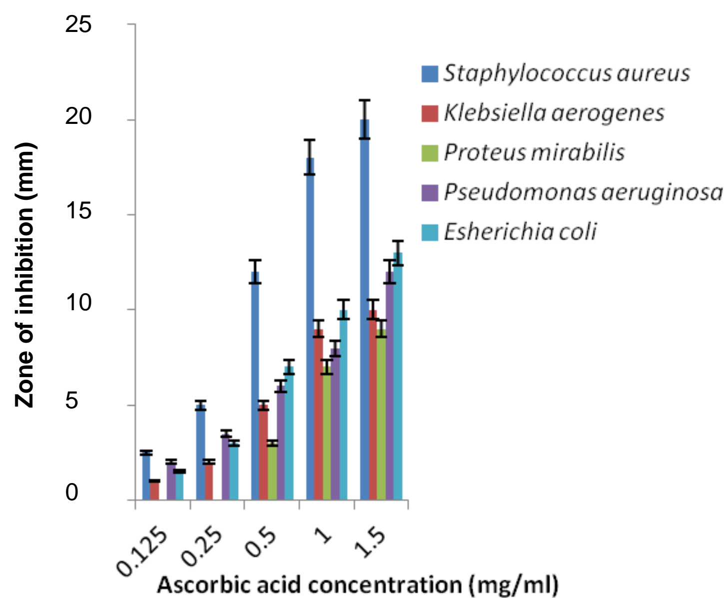

Figure 4. Diameter zone of inhibition (mm) mediated by different concentration (mg/ml) of ascorbic acid on the growth of selected food borne pathogenic bacteria.

shapes and integrity followed by bacterial death (Willey et al., 2011). The Gram negative bacteria are known to possess lipopolysaccharide on the outer membrane. In terms of susceptibility to chemical preservatives, they differ widely between the microorganisms (Mailard, 2002). The complex nature of the outer membrane of Gram negative bacteria has been reported to act as permeability barriers (Nikkado and Vara, 1985; Appleton and Lange, 1994; Willey et al., 2011). The citric acid and ascorbic acid concentration has no limit for the acceptable daily intake in the body. While the prominent zone of inhibition mediated by these chemical preservatives (potassium metabisulphite, sodium benzoate and potassium sorbate) concentrations were below the acceptable daily intake in the body (Mahindru, 2000).

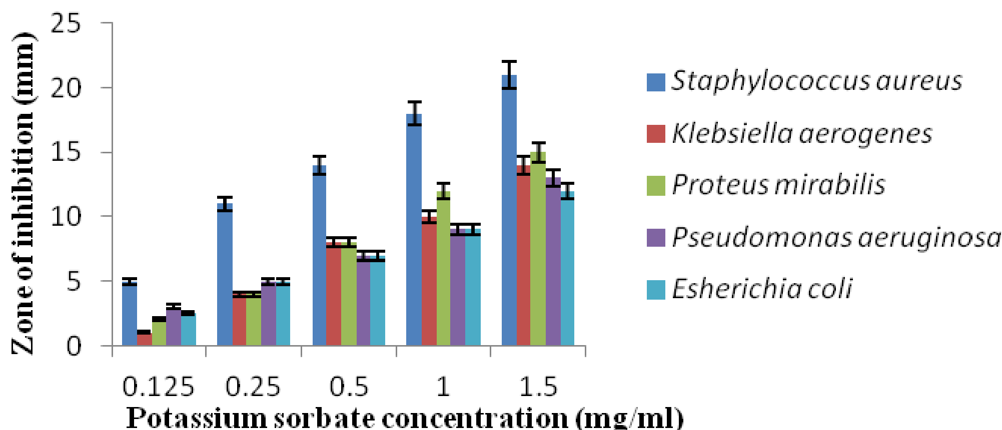

Figure 5. Diameter zone of inhibition (mm) mediated by different concentration (mg/ml) of potassium sorbate on the growth of food borne pathogenic bacteria.

Conclusion

Food preservation implies putting microorganisms in a hostile environment in order to inhibit their growth or shorten their survival or cause their death. The chemical preservatives (potassium metabisulphite, sodium benzoate, citric acid ,ascorbic acid and potasium sorbate) are effective in the control of selected pathogenic bacteria (*S. aureus, K. aerogenes P. mirabilis, P. aeroginosa* and *E. coli*) causing food poisoning and infection. *S. aureus* is highly susceptible to all chemical preservatives used in this study. The minimum inhibitory concentrations (MIC) of the five preservatives were 0.5, 1.5, 1.5, 1.5 and 1.5 mg/ml for potassium metabisulphite, sodium benzoate, citric acid, ascorbic acid and potassium sorbate, respectively.

It can be recommended that the above concentrations of the chemical preservatives could be used to preserve against food borne pathogens. In many cases, a concentration sufficient to result in the lyses of bacterial food poisoning growth may be all that is required to achieve a safe food product. Therefore, employing these chemical preservatives against some food poisoning bacteria provides an exciting potential for the future.

REFERENCES

Adams MR, Moss O (1999). Food Intoxication and Infection In: Food Microbiology,Publ. by Royal Society of Chemistry, New York: 4t Edition . pp. 72–75

Adeleke RO, Oladapo AS (2010). Microorganisms in foods Published in Nigeria By: Omilomo Publishing Firm Osun State. 1st Ed. pp. 44 -45

Ajayi AO (2005). Larvicidal and antimicrobial activity of some medicinal plants commonly grown in Nigeria. J. Res. Biosci. 2(1): 72-76.

Appleton L (1994). Cell wall composition In: Medical Microbiology.3rd Edition.Published by Pretice Hall International U.S.A. pp. 5-8.

Cheesbrough M (2002). Biochemical Tests to Identify Bacteria. In: Laboratory Practice in Tropical Countries, Cheesbrough M (eds). Cambridge edn. pp. 63-70.

Christian GD (1994). Preparation of concentration and Expression of Analytical Results in Analytical Chemistry. Published by Hamiltom Printing company 5th edition; p. 77.

Doughari JH (2006). Antimicrobial activity of *Tamarindus indica* Linn. Trop. J. Pharm. Res. 5(2):592- 603.

Dziezak JD (1986). Preservatives Antimicrobial agents. A means toward products stability. Food Technol. 40: 104-111.

Frazier WC, WestHoff DC (2002). Food intoxication and infection: In Food Microbiology. 13th Edition Tata McGraw – Hill Publishing Company Limited. pp. 144-158.

Greer GG, Dilts BD (1995). Lactic acid inhibition of the growth of spoilage bacteria and cold tolerant pathogens' in pork. Int. J. Food Microbiol. 25: 141-151

Jageethadevi A, Saranraj P, Ramya N (2012). Inhibitory effect of chemical preservatives and organic acids on the growth of bacterial pathogens in poultry chicken, Asian J. Biochem. Pharm. Res.1(2):1-9.

Jay MJ (2005). Chemical preservatives In: Modern Food Microbiology: Chapman & Hall, Inc., New York Fourth ed. pp. 251-279.

Joshua AJ (2000). Chemical preservatives In: Essentials of Food Hygiene Education. Publ. by Spectrum Books Limited, Ibadan 1st Edition. pp. 5-7.

Mahindru SN (2000). Factors influencing the selection of chemical preservatives In: Food Additive, Publ. by Tata McGraw –Hill. Company Ltd. New York. pp. 1-39

Mailard JY (2002). Bacterial target sites for Biocide action. J. Appl. Microbiol. 9 (16-27).

Matsuda T, Yano T, Maruyama A, Kumangai H (1994). Antimicrobial activities organic acids determined by minimum inhibitory concentrations at different Ph ranged from 4.0-7.0 J. Japan Soc. Food SCIand Technol(Nippon Shokuhin Kogyo Gakkaishi). 41(10) 687-701

Nanda M (2005). Determination of zone of inhibition In:Clinical Microbiology1st Ed. Jaype Brothers Medical Publishers, New Delhi.

Nostro A, Germano MP, D'Angelo V, Marino A, Cannatelli MA (2000). Extraction methods an bioauthography for evaluation of medicinal plant antimicrobial activity. Lett. Appl. Microbiol. 30 (5): 379-385

Olunlade BA, Adeleke RO, Oyeleke WA, Akande EA (2010). Basic Food Sci. Hygiene. Graphael Printing and Publ. Company Oshogbo, Nigeria 2nd Ed. p. 1.

Omoya FO, Akharaiyi FC (2010). A pasture honey trial for antibiotic potency on some selected pathogenic bacteria. J. of Natural Products, Vol. 3: pp. 05-11.

Oyawoye OM, Oyawoye EO, Tahir F, Yusuf IZ, Buhari H (1999). Effect of preservatives on the shelf life of locally produce cottage cheese. Sci. J. Pure Appl. Sci. ATIL Press 2(1):52-60.

Podolak RK, Zayas JF, Kastner CL, Fung DYC (1996). Inhibition of Listeria monocytogenes and *E. coli* 0157H7 on beef by application of organic acids. J. Prot. 59:1037-1040

Prescott LM, Harley PP, Klein DA (2002). Uses of chemical agents In:

Microbiology An Introduction.5th ed. McGrawhill Publishing Co Ltd, New York. pp. 504-505.
Pundir RK, Jain P (2011). Evaluation of five chemical food preservatives for their antibacterial activity against bacterial isolates from bakery products and mango pickles Chem. Pharm. Res. 3(1):24-31.
Willey JO, Sherwood LM, Woolverton CJ (2011). Prescott Microbiology:In Food intoxication and infection. McGraw Hill Publish. 8th Ed. pp. 1015-1016.
Xavier TF, Senthilkuma S, Okoh S, Sebastinraj J (2003). Antibacterial activity of Malaxis Rheedii Sw. an ornamental orchid of shvaroy region of Eastern Ghat, S. India. Sci. Focus. 5: 82-88.

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Full Length Research Paper

Bacterial fermentation of *Lemna* sp. as a potential substitute of fish meal in shrimp diets

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***Lemna* sp. flour was fermented with bacteria as a source of protein for the preparation of white leg shrimp diets. *Pediococcus pentosaceus* was isolated from *Litopenaeus vannamei* and *Litopenaeus stylirostris* juveniles and characterized with biochemical (Gram stain, hemolytic activity, extracellular enzymatic activity, hydrophobicity, bacterial growth kinetics, salt tolerance, and cell count) and molecular (16S ribosomal gene) tests. The presumptive bacillus (Ba4), previously isolated and characterized, was identified as *Bacillus pumilus*. *P. pentosaceus* and *B. pumilus* were used in the fermentation of duckweed flour, with different percentages of molasses and humidity. Both bacteria showed high growth in fermented flour. In addition, the fermented flour showed high protein content and a significant decrease in crude fiber, lipids, ashes, and phytic acid. The concentration of tannins remained unchanged. Fermentation of *Lemna* sp. flour improves its nutritional value and suitability as an alternative protein source in formulated diets for white shrimp *L. vannamei*.**

Key words: Fermentation, *Lemna* sp., *Pediococcus pentosaceus*, *Bacillus pumilus*, *Weisella* sp., molasses.

INTRODUCTION

Worldwide aquaculture has been fast growing in the last decades and has become an important industry in many countries, however fish meal used as the major dietary protein source in compounded feed is currently limited, which has resulted in massive research to identify alternative protein sources (Tacon and Metian, 2008; Olsen and Hasan, 2012). In the last years, most researchers have proposed to use an alternative ingredient for fish flour due to its limited supply and high cost. At present, studies focused on the replacement of this protein with lower cost ingredients from vegetable protein are underway (Amaya et al., 2007;

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Gamboa-Delgado et al., 2013).

Duckweed (*Lemna* sp.), as a source of vegetable protein, has a better essential amino acid profile than most vegetable protein sources and, in turn, this profile resembles that of animal protein sources. Duckweed contains from 28 to 43% crude protein, high concentration of trace minerals as potassium and phosphorus, carotenes, and xanthophylls (Chaturvedi et al., 2003). *Lemma* sp. grows in nutrients-rich waters and has been used as a food supplement for fish (He et al., 2013), and as a source of protein to replace fish flour in diets for carp (Yilmaz et al., 2004). However, the presence of antinutritional factors in plant flours affects negatively their nutritional value (Kumaraguru Vasagam et al., 2007). The fermentation process of plants is a simple and cheap method that might decrease considerably the antinutritional factors and crude fiber content, increasing plant digestibility (Bairagi et al., 2002; Nout, 2009). To this regard, fermentation of duckweed flour (*Lemna polyrhiza*) by *Bacillus* sp. decreases fiber and antinutritional factors (Bairagi et al., 2002). Additionally, there are fermentation studies on protein sources with low digestibility, in which microorganisms are used to hydrolyze unusual substrates, such as feathers, to improve their digestibility (Bertsch et al., 2003).

In this study, duckweed was evaluated as a potential substitute of fish meal in shrimp diets (*L. vannamei*) by improving its nutritional quality through a bacterial fermentation process.

MATERIALS AND METHODS

Bacteria

Lactic acid bacteria (LAB) were isolated from the gut and hepatopancreas of healthy juvenile shrimp (*L. vannamei* and *L. stylirostris*). Animals were collected from the Navachiste Bay (Guasave, Sinaloa, Mexico). In the laboratory, guts and hepatopancreas were dissected aseptically and homogenized in Eppendorf tubes with 200 µl of sterile saline solution (2.5% NaCl). One hundred microliters of the homogenate were inoculated on MRS agar (BD Difco, USA) supplemented with 2.5% NaCl and 200 mg/L of aniline blue (Sigma-Aldrich, USA) in duplicate. The plates were kept at 30ºC and their replicates at 37ºC for 24 h. Blue colonies were selected and streaked onto MRS plates and incubated as above. The isolates maintained in pure culture were stored at -70ºC in MRS with 15% (v/v) glycerol and 2.5% NaCl.

The bacillus (Ba4) used in this work was originally isolated from sea water of the Navachiste Bay. The Ba4 isolate was characterized to be used as potential probiont for shrimp by Partida-Arangure et al. (2013).

LAB isolates were characterized using Gram stain, cellular morphology, and DNA sequence analysis. In addition, hemolytic activity (HA), hydrophobicity, extracellular enzymatic activity, salinity tolerance, and kinetics of bacterial growth were studied to be used as criteria to select potential bacteria to ferment duckweed flour.

Bacterial growth kinetics, colony forming unit counts (CFU) and hemolysis assay

The bacillus and LAB were originally isolated and grown at 37 and 30ºC, respectively. In this work, bacteria were grown at different temperatures to obtain the temperature range at which both bacteria have good growth. Bacterial growth was determined by reading the absorbance of cultures in a Thermo Spectronic Genesys 2 Spectrophotometer (Thermo Scientific, USA) at 580 nm for 24 h. Ba4 and LAB grew well at 35ºC and this temperature was used for subsequent cultures. We determined bacterial growth kinetics to determine the log growth phase of each LAB isolate. Additionally, count the colony forming units (CFU) and hemolysis assay were carried out according to Leyva-Madrigal et al. (2011).

The isolates with γ-hemolysis activity (lack of hemolysis) were selected for further analysis; β hemolytic isolates were scrapped for their pathogenic potential.

Extracellular enzymatic activity

Extracellular protease and lipase activities were carried out according to León et al. (2000) with some modifications made by Leyva-Madrigal et al. (2011). Additionally, to determine cellulolytic activity, the isolates were inoculated onto Petri plates with carboxymethyl cellulose agar (1%, w/v, Sigma-Aldrich). The plates were incubated at 35°C for 48-72 h. After the incubation time, 10 ml of a Congo red solution (1%, w/v, Sigma-Aldrich) was added to the plates, after 15 min the excess was removed, and then a saline solution (2 M, NaCl) was added, allowing to stand for 15 min to determine isolates showing hydrolysis halos (Teather and Wood, 1982).

Microbial adhesion to solvents (*p*-xylene)

The microbial adhesion to solvents was measured according to Rosenberg et al. (1980). Additionally, this test was performed on the bacillus (Ba4) isolated by Partida-Arangure et al. (2013). The apolar solvent *p*-xylene was used because bacterial adhesion to this solvent reflects the hydrophobic or hydrophilic nature of the cell surface. LAB were grown in MRS medium and harvested in the stationary growth phase by centrifugation at 5000 *g* for 15 min. The biomass was washed twice and resuspended in PBS buffer (137 mM NaCl, 2.7 mM KCl, 2 mM Na_2HPO_4, pH 7.4). The absorbance of the cell suspension was measured at 600 nm (A0). One milliliter of cell suspension was added to 3 ml of xylene and then incubated for 10 min at room temperature. A system of two phases was obtained after incubation and then the sample was mixed in a vortex for 2 min. The aqueous phase was removed after 20 min of incubation at room temperature and measured for absorbance at 600 nm (A1). The percentage of bacterial adhesion to the xylene solvent was calculated as (1-A1/A0) × 100.

Salinity tolerance

Salinity tolerance of LAB was obtained at different concentrations of NaCl to be used for fermentation. MRS medium supplemented with 0, 0.5, 1.0, 1.5, 2.0, and 2.5% NaCl was placed in 15-ml Falcon tubes, inoculated with 20 µl of LAB stock, and incubated at 35ºC for 24 h. Absorbance was read in a spectrophotometer Thermo Spectronic Genesys 2 for growth. Sterile medium was used as blank.

DNA extraction

DNA extraction of LAB and Ba4 was achieved according to Moretti et al. (1985). Total DNA was isolated to be used as template in the 16S gene amplification. The samples were subjected to further purification by RNAase treatment and phenolization.

16S ribosomal gene (LAB and Ba4)

Amplification of the 16S ribosomal DNA was performed by single PCR using primers 27f and 1525r (Lane, 1991). Purification of the PCR product was performed with the cleaning kit QIAquick PCR Purification Kit (Invitrogen, USA). Purified PCR products were sent for sequencing to the Biotechnology Institute of UNAM (Mexico). Finally, the sequences obtained were compared with the reported nucleotide sequences in the genomic bank (GeneBank database) using the NCBI BLAST software (www.ncbi.nlm.nih.gov/BLAST/).

Phylogenetic analyses

The phylogenetic analyses were performed with the Molecular Evolutionary Genetics Analysis software (MEGA 5 Beta) (Tamura et al., 2011). Evolutionary relationships between sequences were inferred by using the neighbor-joining method (NJ) (Saitou and Nei, 1987). The robustness of the NJ topology was evaluated by bootstrap test using 1000 replicates. The *Thermotoga maritima* sequence was used as out group.

Duckweed flour

Wild duckweed (*Lemna* sp.), identified according to the description of Lot et al. (1999), was collected from a shallow lagoon near a corn-growing area and cultivated in 1000-l plastic tanks with 600 L of freshwater fertilized with 30 mg/L of NPK (nitrogen, phosphorus, and potassium, 1:1:1, w/w). The biomass was harvested every 2 days and sundried for 24 h. Dry plants were passed through a sieve to remove foreign materials and ground in a hammer mill (Thomas Scientific, USA) to a particle size of approximately 450 μm.

Water retention capacity

Water retention capacity was modified from Smith et al. (1973). Distilled water was added to a known amount of flour until saturation and then the weight was obtained to determine the percentage of humidity of the sample.

Fermentation of duckweed flour

The optimum conditions for fermentation of duckweed flour were selected based on the results of four experiments. In experiment I and II, the growth of LAB (BALLvHp2) and bacillus (Ba4) in duckweed flour was evaluated, respectively. Flour without bacteria but with molasses was used as control and treatments with different percentages of molasses (3, 5, 10, and 15%) were inoculated with 1×10^6 CFU/g of flour. Additionally, the pH was measured every 24 h for 144 h. In experiment III, the percentage of molasses in which microorganisms showed better performance was used. The experiment was designed as follows: 1) BALLvHp2; 2) Ba4, 3) Ba4 + BALLvHp2 inoculated 24 h after Ba4; and 4) Ba4 + BALLvHp2 (inoculated simultaneously). Each treatment had six replicates, one replicate was used every 24 h for the analysis of the microbial concentration and pH. In experiment IV, the fermentation conditions were as follows: duckweed flour added with 10% molasses, 1.5% NaCl, BALLvHp2 and Ba4 (1×10^6 CFU/g of flour, inoculated at the same time), and different percentages of moisture (75, 100, and 125%). Each treatment had nine replicates and a replicate was used every 24 h for the analysis of the fermentation process. The fermented samples were diluted in distilled water (1:5) and the pH was measured using an electrode (pH). Microbial growth was determined by counting CFU on MRS and TSA agar plates using the serial dilution method (Shirai et al., 2001).

Chemical composition of ferments

The fermented flour was analyzed at CIBNOR (La Paz, Baja California Sur, Mexico) to determine the content of moisture, protein, crude fat, crude fiber, ash, nitrogen free extract, and gross energy. The amino acid profile was determined at CIAD (Hermosillo, Sonora, Mexico) by liquid chromatography (HPLC) using acid-base digestions (Einarsson et al., 1983).

Determination of tannins and phytic acid

Tannins were determined according to Atanassova and Christova-Bagdassarian (2009), based on the AOAC (1990) and The International Pharmacopoeia. The estimation of phytic acid was according to Wheeler and Ferrel (1971).

Statistical analysis

A factorial analysis of variance (ANOVA) was applied to identify differences among treatments of the fermentation process at 95% interval of confidence ($P < 0.05$). One-way analysis of variance (ANOVA) was applied to examine the differences in proximate composition of the ferments among treatments ($P < 0.05$). Where significant ANOVA differences were found, a Tukey's HSD test was used to identify the nature of these differences at $P < 0.05$.

RESULTS

Bacteria isolation and characterization

Twenty nine presumptive LAB strains were isolated, six isolates from *L. stylirostris* and 23 isolates from *L. vannamei.* Six isolates were γ-hemolytic and 23 isolates were β-hemolytic. Five isolates with γ-hemolysis were selected for successive characterization. The selected isolates were Gram positive, catalase negative and cocci- or cocobacilli-shaped. Selected isolates did not show enzymatic activity (proteases and lipases). However, Ba4 showed cellulolytic activity with a halo of 14.3 ± 0.6 mm (data not shown). Adhesion of selected isolates and Ba4 to the solvent was between 35.52 ± 12.71 and 79.01 ± 5.56%. All isolates showed a log phase between 6 and 12 h. Colony forming units of selected isolates and Ba4 were from 2.315×10^9 to 6.020×10^9 CFU/ml (Table 1).

Molecular identification of bacteria

The molecular analysis of the isolates BALLvHp5 and BALLvHp2 showed identities of 99.5 and 99.9% with *Pediococcus pentosaceus,* respectively. The isolate BALLsI2 showed identity of 98.3% with *Weisella viridescens*. BALLsI6 and BALLsI4 belong to the *Weissella* genus with identities of 97.9 and 93.4%, respectively. The isolate Ba4 belongs to *Bacillus pumilus* with a 100% identity (Figure 1).

Water retention of *Lemna* flour

Results of water retention capacity showed that 1 g of

Table 1. Characterization of LAB isolates from *L. stylirostris* and *L. vannamei.*

Isolate	Gram	CFU/ml	Adhesion to *p*-xylene (%)
BALLvHp2	+	2.645×10^9	79.01 ± 5.56
BALLvHp5	+	2.315×10^9	35.52 ± 12.71
BALLsI2	+	5.680×10^9	69.70 ± 9.72
BALLsI4	+	4.825×10^9	83.46 ± 3.74
BALLsI6	+	3.015×10^9	67.77 ± 14.64
Ba4*	+	95.3×10^6	75.42 ± 2.34

p-Xylene adhesion value is the mean ± standard deviation from three replicates.

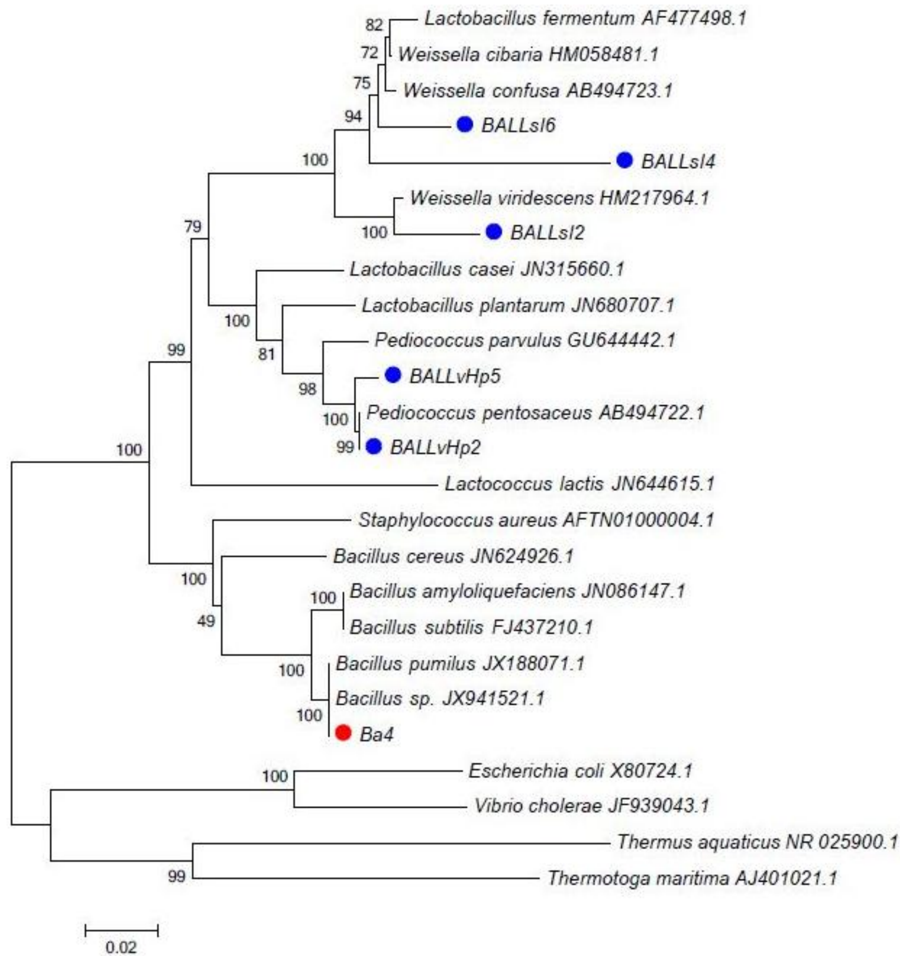

Figure 1. Neighbor-joining tree derived from partial 16S rDNA gene sequences of the five LAB isolates (BALLvHp2, BALLvHp5, BALLsI2, BALLsI4, and BALLsI6, blue marker), a bacillus isolate (Ba4, red marker), and 19 reference strains (sequence accession numbers are given after the species name). *Thermotoga maritima* was chosen as out group. The scale bar shows nucleotide substitution rate per site. Bootstrap probabilities as determined for 1000 replicates are given as percentage.

flour is saturated with 3.97 g of water. This means that the flour has 100% moisture. This result was employed to design the IV fermentation assay with 75, 100, and 125% moisture and the best molasses percentage.

Fermentation assays

In the first fermentation assay, the results showed that the growth of BALLvHp2 was significantly higher in the

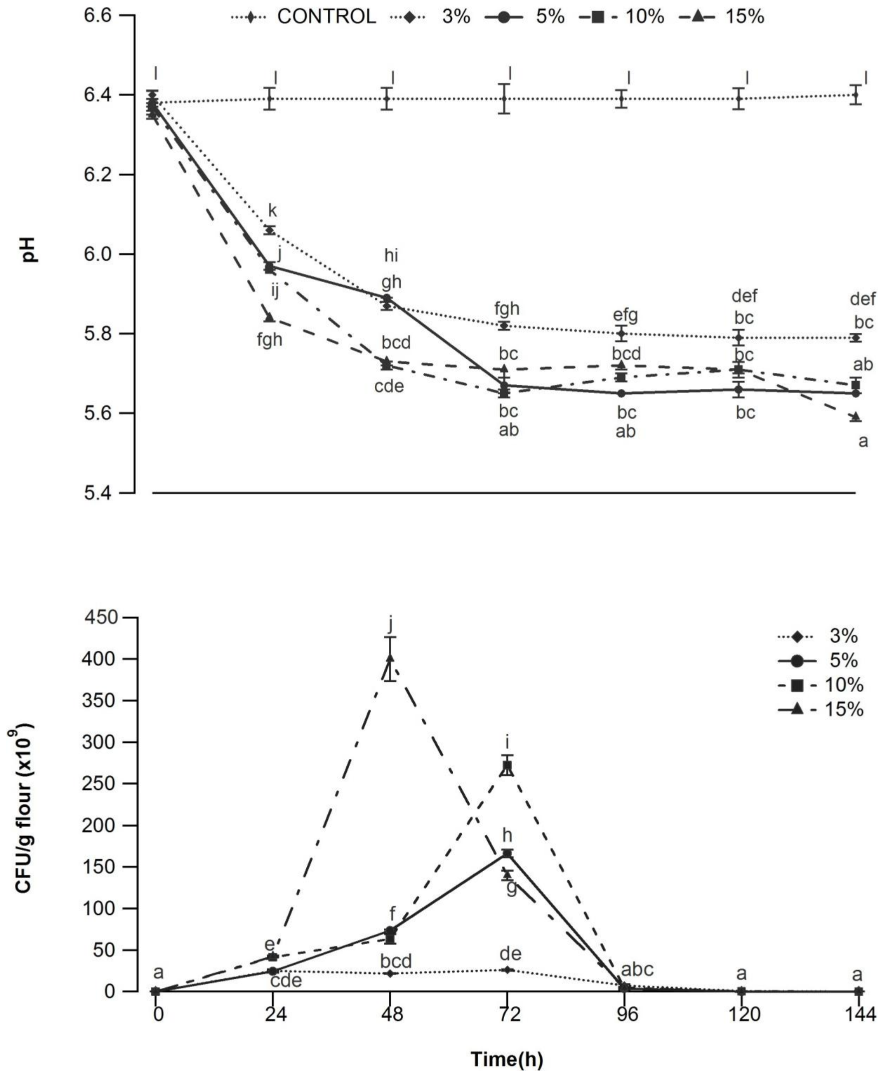

Figure 2. Growth kinetics of BALLvHp2 in *Lemna* flour supplemented with molasses at different percentages (3, 5, 10, and 15%). a) pH values. b) Growth of BALLvHp2 (CFU/g flour) at different fermentation times. Error bars = mean ± SD. Different letters denote significant differences at $P < 0.05$.

treatment with 15% molasses during the first 48 h as compared to 10, 5, and 3% molasses; however, the growth of BALLvHp2 decreased after 48 h. Growth with 5 and 10% molasses at 72 h was significantly higher than with 3 and 15% molasses. Bacterial growth in the treatment with 3% molasses was significantly lower than with 5, 10, and 15% molasses at 48 and 72 h ($F_{(18, 56)} = 512.90$, $P = 0.0001$). During the fermentation process, the pH decreased significantly from 24 to 144 h with all percentages of molasses as compared with the control with molasses but without bacteria. The pH of treatment with 3% molasses was significantly higher than with 5, 10, and 15% molasses ($F = _{(24,\ 133)} = 201.37$, $P = 0.0001$) (Figure 2).

In the second fermentation assay, the results showed that the growth of Ba4 was significantly higher in the treatment with 10% molasses at 48 and 72 h as compared to 5 and 15% molasses. At 96 h, the growth of Ba4 with 5 and 10% molasses was significantly higher than with 15% molasses ($F_{(10,\ 36)} = 158.31$, $P = 0.0000$) (Figure 3).

In the third fermentation assay (Figure 4), 10% molasses and bacteria (Ba4 and BALLvHp2) were added to the flour. When bacteria were inoculated alone, higher growth was obtained, but when both bacteria were inoculated, the growth was affected, specially Ba4 ($F_{(25,\ 72)} = 189.71$, $P = 0.0001$). The pH was significantly lower in treatments II, III, and IV as compared to control ($F_{(20,\ 60)} = 262.67$, $P = 0.0001$).

In the fourth fermentation assay, 10% molasses and

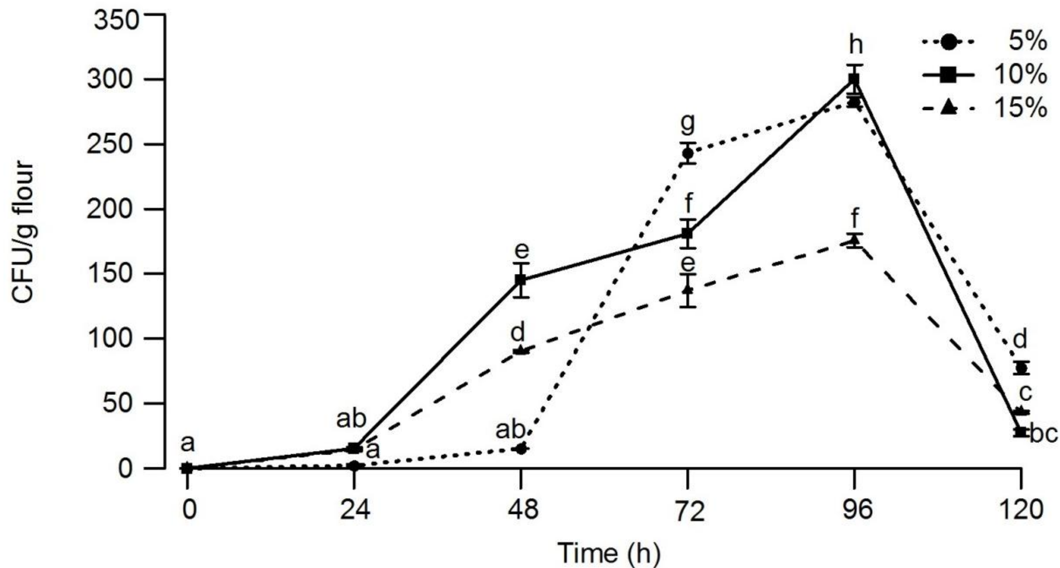

Figure 3. Growth kinetics of isolate Ba4 in *Lemna* flour supplemented with molasses at different percentages (5, 10, and 15%). Growth of bacteria (CFU/g flour) at different fermentation times. Error bars = mean ± SD. Different letters denote significant differences at $P < 0.05$.

bacteria (Ba4 and BALLvHp2), inoculated at the same time, were added to the flour with different moisture percentages (75, 100, and 125%). Ba4 showed a similar trend in growth at different percentages of moisture. The growth of BALLvHp2 was significantly lower at 75% moisture as compared with 100 and 125% moisture ($F_{(16, 54)} = 39.446$, $P = 0.0001$) (Figure 5).

Fermented flour (75, 100, and 125%) showed significantly lower moisture content as compared with the unfermented one ($P < 0.05$). No significant differences were found in protein content of fermented and unfermented flour ($P > 0.05$). Fermented flour showed significantly lower (≈ 50%) ether extract and crude fiber (≈ 50%) as compared to the unfermented one. Ashes in treatments with 100 and 125% moisture were significantly lower as compared to the unfermented flour (12.3 and 21%, respectively) ($P < 0.05$). Samples did not show significant differences in the content of nitrogen-free extract (NFE, $P > 0.05$). Total nitrogen was significantly lower in the fermented flour as compared to the unfermented one ($P > 0.05$). The energy content of fermented flour was significantly lower than in the unfermented one ($P < 0.05$). The percentage of tannins in the unfermented flour was 1.18 ± 0.1, whereas in the fermented flour it was 1.11 ± 0.2, 1.04 ± 0.1, and 1.04 ± 0.2% in treatments with 75, 100, and 125% moisture, respectively. No significant differences were found among samples ($P > 0.05\%$). The percentage of phytic acid in the unfermented flour was 2.24 ± 0.21%, whereas in the fermented flour it was 0.91 ± 0.03, 0.73 ± 0.06, and 0.94 ± 0.05% in treatments with 75, 100, and 125% moisture, respectively. Significant differences were found in the three fermented samples when compared with the unfermented sample ($P < 0.05$). Fermentation reduced phytic acid in 61% (Table 2).

DISCUSSION

Fermentation process of foods contributes to enhance flavor and texture, and to improve shelf-life and digestibility (Nout, 2009). In this work, 29 presumptive LAB were isolated from wild juveniles of *L. stylirostris* and *L. vannamei*. After their characterization, five of these isolates (presumptive LAB) showed potential as fermentative bacteria, including medium and high adhesion capacity, gamma hemolysis, cellulolytic activity (only the bacillus Ba4), and high growth. Lactic acid bacteria are widely used as starter cultures for the production of fermented foods (Ammor and Mayo, 2007).

Molecular identification showed two isolates (BALLvHp5 and BALLvHp2) with high identity with *P. pentosaceus*. This species is a homofermentative lactic acid bacterium that has been used in the fermentation of flours such as wheat and corn (Gerez et al., 2012; Kleinschmit and Kung, 2006). The isolate Ba4 showed high identity with *B. pumilus*. *B. pumilus* ferments *Parkia biglobosa* (Ouoba et al., 2007). *Bacillus* sp. ferments duckweed (*Lemna polyrhiza*) leaf flour and seed flour of *Phaseolus mungo* (Bairagi et al., 2002; Ramachandran and Ray, 2007).

In addition to their role in fermentation, bacteria are an important source of high quality protein with up to 70% (Aas et al., 2006). The growth of bacteria during the

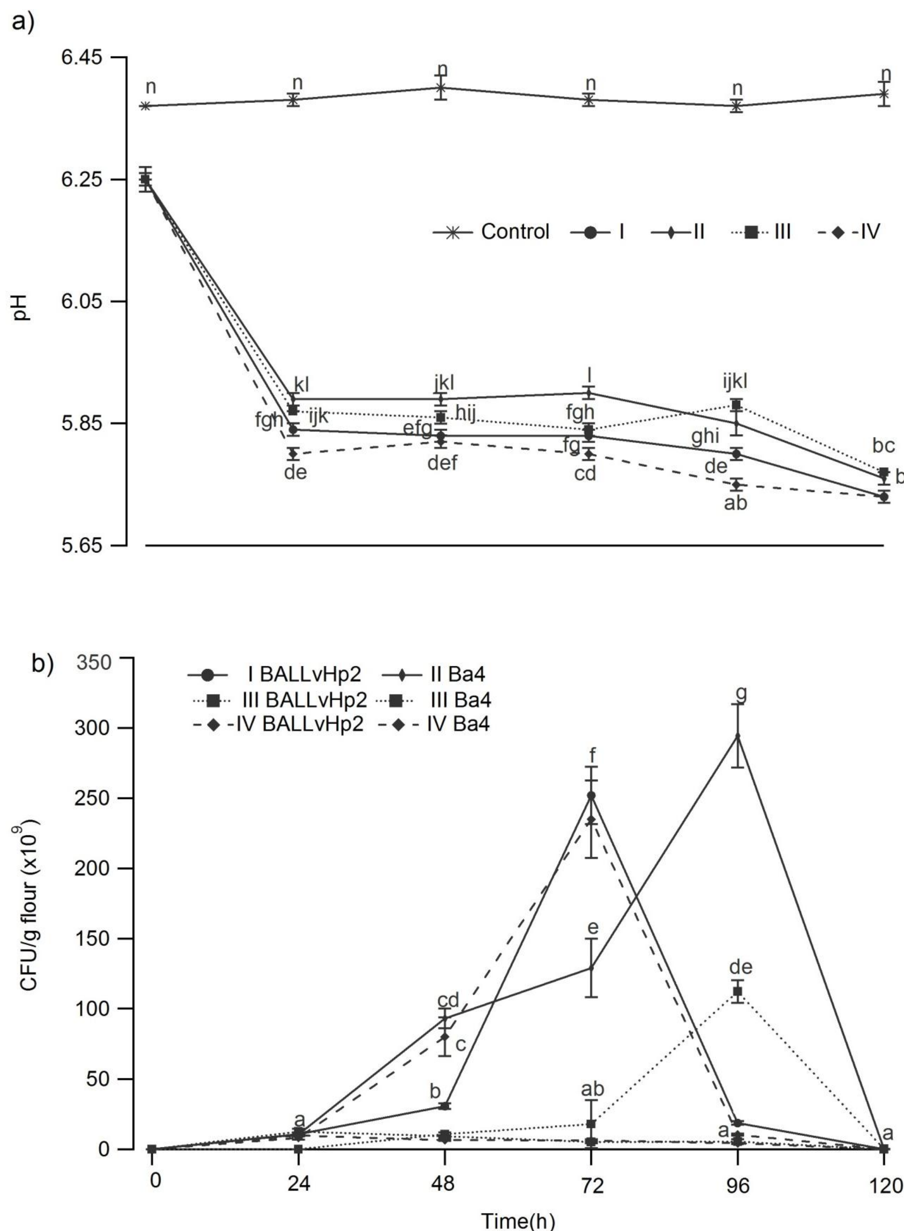

Figure 4. Growth of BALLvHp2 and Ba4 in *Lemna* flour supplemented with 10% molasses. Treatments: I) BALLvHp2; II) Ba4; III) Ba4 + BALLvHp2 (inoculated 24 h later); and IV) Ba4 + BALLvHp2 (inoculated simultaneously). a) pH values. b) Growth of bacteria at different fermentation times. Error bars = mean ± SD. Different letters denote significant differences at $P < 0.05$.

fermentation process was better with 10% molasses and the pH decreased significantly from 24 to 144 h with all percentages of molasses. On the other hand, the growth of bacteria in flour with different percentages of moisture was very similar, differing at specific points of the fermentation time.

In this work, fermentation of *Lemna* flour resulted in a decrease in pH and in the levels of moisture, ether extract (lipids), crude fiber (insoluble carbohydrates), ashes, total nitrogen, and energy. On the other hand, protein, total free amino acid, and nitrogen-free extract (soluble carbohydrates, such as starch and sugar) were similar between unfermented and fermented flours.

The protein content of fermented *Lemna* sp. was similar to results obtained by Cruz et al. (2011) in *Lemna minor* (24.4%) and *Spirodela polyrrhiza* (24.1%). On the other hand, the crude protein found in unfermented *Lemna* sp. was higher than that found by Cruz et al. (2011) (15.7%) and Yilmaz et al. (2004) (18.4%) in unfermented flour of *L. minor*; however, it was lower

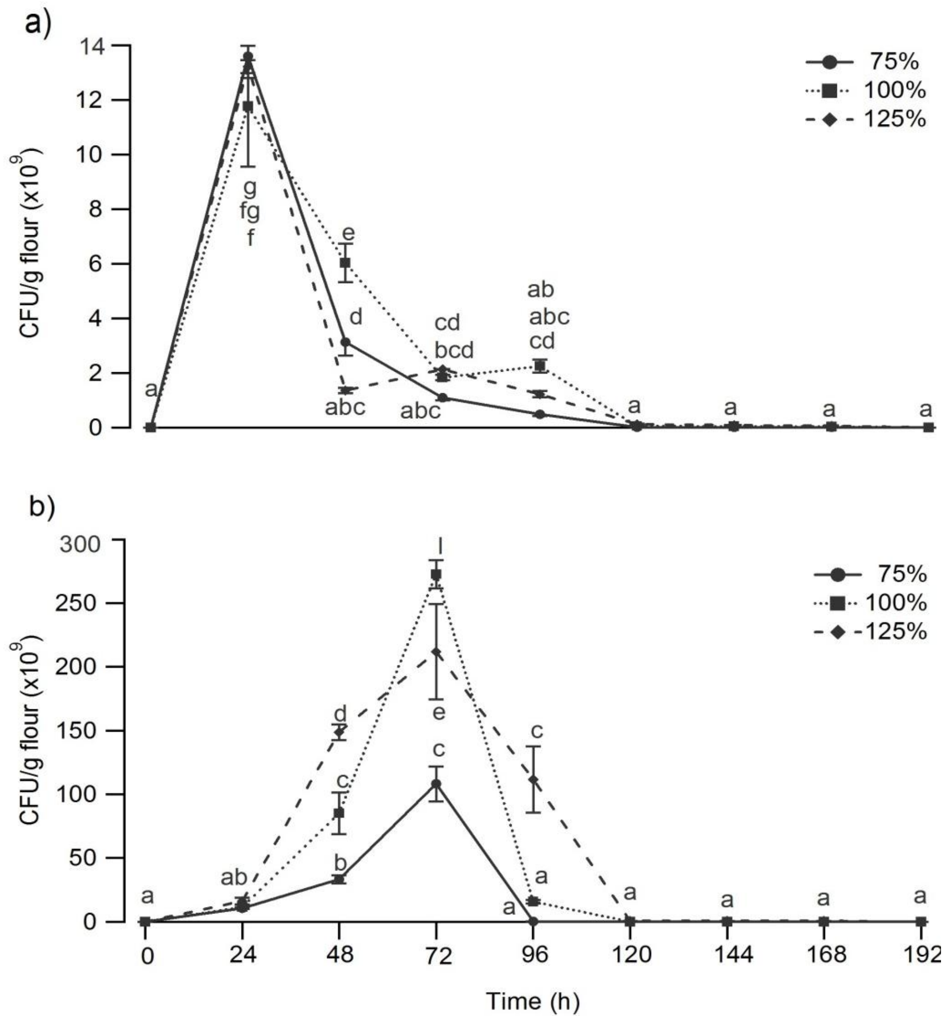

Figure 5. Growth kinetics of isolates (a) Ba4 and (b) BALLvHp2 in *Lemna* flour supplemented with molasses (10%) and with different moisture percentages (75, 100 and 125%). Error bars = mean ± SD. Different letters denote significant differences at $P < 0.05$.

when compared with unfermented *L. minor* studied by Oludayo-Olaniyi and Omoniyi-Oladunjoye (2012) (42.2%), and with *S. polyrrhiza* (29.05%) (Anderson et al., 2011). Although there were not significant differences between fermented and unfermented flour on crude protein content, the observed decrement in fermented flour might be due to possible metabolic utilization by *P. pentosaceus* and *B. pumilus*, which hydrolyze protein from plants to equilibrate the C:N ratio, since molasses has low protein content (4%) and higher (50%) carbohydrate content (Cleasby, 1963). Similarly, Oyarekua (2011) observed a decrement of protein content during fermentation of pigeon pea (*Cajanus cajan*) without molasses or other carbon sources. On the other hand, total free aminoacids were similar between unfermented and fermented flour. Duckweed has a better essential amino acid profile than most vegetable protein sources (Chaturvedy et al., 2003). Moreover, the amino acid composition is similar to that of fish meal, although, it is deficient in lysine (Aas et al., 2006).

In this work, crude fiber (insoluble carbohydrates) of *Lemna* sp. decreased from 6.6% in unfermented flour to 3.4% in fermented flour. Fermentation reduces crude fiber content due to the cellulolytic activity of *B. pulmilus*. Similar results were observed by Saha et al. (2011) when fermenting *Eichhornia crassipes* with *B. subtilis*, *Bacillus megaterium*, and *Lactobacillus acidophilus*. According to Akiyama (1993), the excess of crude fiber affects the palatability and digestibility of diets in shrimp. Therefore, the reduction of crude fiber in fermented flour should be beneficial for shrimp nutrition.

The ash content of fermented flour of *Lemna* sp. was lower than in the unfermented one, but high if we consider the tolerance in diets for *L. vannamei*. Akiyama (1993) observed that the excess of ashes in diets for shrimp can affect their palatability and digestibility.

Table 2. Proximate composition of fermented and non-fermented flour of *Lemna* sp.: moisture, protein, ether extract, crude fiber, ash, nitrogen-free extract (NFE), total nitrogen, energy, and total free amino acids. The flour was fermented with 10% molasses and 75, 100, and 125% moisture.

Treatment	Unfermented flour	75%	100%	125%
Moisture (%)	8.55 ± 0.17[a]	6.96 ± 0.20[c]	7.42 ± 0.25[bc]	7.66 ± 0.27[b]
Protein (%)	27.98 ± 0.06[a]	22.09 ± 0.00[a]	21.24 ± 0.10[a]	21.01 ± 0.13[a]
Ether extract (%)	1.83 ± 0.03[a]	1.15 ± 0.18[b]	1.13 ± 0.08[b]	0.98 ± 0.13[b]
Crude fiber (%)	6.66 ± 0.21[a]	4.35 ± 0.22[b]	3.56 ± 0.11[c]	3.43 ± 0.23[c]
Ashes (%)	36.38 ± 1.83[a]	33.99 ± 1.86[ab]	31.89 ± 1.85[bc]	28.63 ± 1.84[c]
NFE (%)	27.14 ± 0.33[a]	38.43 ± 0.08[a]	42.17 ± 5.52[a]	45.95 ± 0.32[a]
Total nitrogen (%)	4.48 ± 0.01[a]	3.53 ± 0.00[b]	3.40 ± 0.01[c]	3.37 ± 0.02[c]
Energy (cal/g)	3361.37 ± 23.92[a]	3236.93 ± 15.17[b]	3274.35 ± 25.67[b]	3144.77 ± 21.60[c]
Total free amino acid (g/100 g of flour)	0.0175 ± 0.0003	0.0155 ± 0.0011	0.0176 ± 0.0015	0.0164 ± 0.0022
Tannins (%)	1.18 ± 0.1[a]	1.11 ± 0.2[a]	1.04 ± 0.1[a]	1.04 ± 0.2[a]
Phytic acid (%)	2.24 ± 0.21[a]	0.91 ± 0.03[b]	0.73 ± 0.06[b]	0.94 ± 0.05[b]

Results are shown as the mean ± standard deviation. Different letters denote significant differences.

However, it is important to consider that according to results obtained of protein in fermented flour, we might substitute only 22% of fish protein, therefore the excess of ashes will be diluted.

Regarding the lipid content of fermented flour of *Lemna* sp., the present study demonstrates that the fermentation process decreases lipids. However, lipids are low when compared with shrimp tolerance. Nguyen et al. (2012) found that a diet with high lipid content (21.1%) affects survival and weight of *L. vannamei*. In the same way, Akiyama et al. (1992) reported that diets above 10% lipids affect survival and growth of *Penaeus monodon*, with the optimum content between 6.0 and 7.5%. Adebowale and Maliki (2011) found that the fermentation process of *C. cajan* decreased lipids, possibly due to the activities of lipolytic enzymes that hydrolyze lipids. The fermented flour showed lower energy content than the unfermented flour. According to Adebowale and Maliki (2011), the results obtained could be attributed to the decrease in lipids and NFE.

Tannins percentage was similar in fermented and unfermented flour. This anti-nutritional factor can affect shrimp growth. Tannins are a highly diverse class of plant secondary phenolic compounds (Kraus et al., 2003; Norris et al., 2011) and have been found to interfere with the digestibility of proteins, carbohydrates, and the availability of vitamins and minerals (Liener, 1994). The tannin content (all treatments) of *Lemna* sp. was lower when compared with the tannin content of the legumes *Phaseolus vulgaris* (1.7%), *C. cajan* (1.4%) (Sangronis and Machado, 2007), *Cassia rotundifolia* (2.9%), and *Lablab purpureus* (1.7%), but higher when compared with *P. mungo* (0.62%) (Ramachandran and Ray, 2007), *Pisum sativum* (0.92%) (Nikolopoulou et al., 2007), and *Cichorium intybus* (0.83%) (Shad et al., 2012).There are no reports about the effect of tannins on shrimps; however, in fish, these molecules affect digestive enzymes and growth (Bairagi et al., 2002). Phytic acid decreases solubility and functionality of the proteins (El-Adawy et al., 2002). The fermentation process of *Lemna* sp. reduced phytic acid (60%). Ramachandran and Ray (2007) found a phytic acid concentration in *P. mungo* of 12.7 and 8.0% in unfermented and fermented flour, respectively. Saha and Ray (2011) found that fermentation of *E. crassipes* reduces phytic acid from 0.42 to 0.32-0.37%. In *L. vannamei*, 1.5% phytic acid reduces phosphorous and zinc bioavailability (Davis et al.,1993).

Fermentation of *Lemna* flour improves its nutritional value and suitability as an alternative protein source in formulated diets for white shrimp *L. vannamei*. However, further research is needed about the effect in shrimp of diets with fermented *Lemna* sp., especially on growth performance and gene expression of trypsin and chymotrypsin.

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REFERENCES

Aas TS, Hatlen B, Grisdale-Helland B, Terjesen BF, Bakke-McKellep AM, Helland SJ (2006). Effects of diets containing a bacterial protein meal on growth and feed utilisation in rainbow trout (*Oncorhynchus mykiss*). Aquaculture. 261:357-368.

Adebowale OJ, Maliki K (2011). Effect of fermentation period on the chemical composition and functional properties of Pigeon pea (*Cajanus cajans*) seed flour. Int. Food Res. J. 18(4):1329-1333.

Akiyama DM (1993). Semi-extensive shrimp farm management. ASA Technical Bulletin, MITA (P) No.518/12/92, Vol. AQ. 38 1993/3. American Soybean Association, Singapur, Singapur.

Akiyama DM, Dominy WG, Lawrance AL (1992). Penaeid shrimp nutrition. In: Fast AW, Lester LJ (eds) Marine Shrimp Culture: Principles and Practices, Developments in aquaculture and fisheries science. Elsevier. USA, pp. 535-568.

Amaya EA, Davis DA, Rouse DB (2007). Replacement of fish meal in practical diets for the Pacific white shrimp (*Litopenaeus vannamei*) reared under pond conditions. Aquaculture 262:291–298.

Ammor MS, Mayo B (2007). Selection criteria for lactic acid bacteria to be used as functional starter cultures in dry sausage production: An update. Meat Sci. 76(1):138-146.

Anderson KE, Lowman Z, Stomp AM, Chang J (2011). Duckweed as feed ingredient in laying hen diets and its effect on egg production and composition. Int. J. Poult. Sci. 10(1):4-7.

AOAC (1990). Methods of Analysis (15th ed) Association of Official Analytica Chemist, Washington, D.C., USA.

Atanassova M, Christova-Bagdassarian V (2009). Determination of tannin content by titrimetric method for comparison of different plant species. J. Chem. Technol. Metall. 44(4):413-415.

Bairagi A, Sarkar Ghosh K, Sen SK, Ray AK (2002). Duckweed (*Lemna polyrhiza*) leaf meal as a source of feedstuff in formulated diets for rohu (*Labeo rohita* Ham.) fingerlings after fermentation with a fish intestinal bacterium. Bioresour.Technol. 85:17-24.

Bertsch A, Alvarez R, Coello N (2003). Evaluación de la calidad nutricional de la harina de plumas fermentadas por *Kocuria rosea* como fuente alternativa de proteínas en la alimentación de aves. Rev. Cien. Fac. Cien. Vet. LUZ 13(2):139-145.

Chaturvedi KMM, Langote DS, Asolekar RS (2003). Duckweed-fed fisheries for treatment of low strength community waste water. WWWTM Newsletter-Asian Institute of Technology, India.

Cleasby TG (1963). The feeding value of molasses. South Afr. Sugar J. 1:47-360.

Cruz Y, Kijora C, Wedler E, Danier J, Schulz C (2011). Fermentation properties and nutritional quality of selected aquatic macrophytes as alternative fish feed in rural areas of the Neotropics. Livestock Res. Rural Dev. Volume 23, Article #239. Available at: http://www.lrrd.org/lrrd23/11/cruz23239.htm (accessed: May 2013).

Davis DA, Lawrence AL, Gatlin III DM (1993). Evaluation of dietary zinc requirement of *Penaeus vannamei* and effects of phytic acid on zinc and phosphorous bioavailability. J. World Aquacult. Soc. 24(1):40-47.

Einarsson S, Josefsson B, Lagerkvist S (1983). Determination of amino acids with 9-fluorenylmethyl chloroformate and reversed-phase HPLC. J. Chromatogr. 282:609-618.

El-Adawy TA (2002). Nutritional composition and antinutritional factors of chickpeas (*Cicer arietinum* L.) undergoing different cooking methods and germination. Plant Food Hum. Nutr. 57: 83-97.

Gamboa-Delgado J, Rojas-Casas MG, Nieto-López MG, Cruz-Suárez LE (2013). Simultaneous estimation of the nutritional contribution of fish meal, soy protein isolate and corn gluten to the growth of Pacific white shrimp (*Litopenaeus vannamei*) using dual stable isotope analysis. Aquaculture. 380–383:33-40.

Gerez CL, Dallagnol A, Rollán G, Font de Valdez G (2012). A combination of two lactic acid bacteria improves the hydrolysis of gliadin during wheat dough fermentation. Food Microbiol. 32(2):427-430.

He S, Liang XF, Li L, Sun J, Shen D (2013). Differential gut growth, gene expression and digestive enzyme activities in young grass carp (*Ctenopharyngodon idella*) fed with plant and animal diets. Aquaculture. 410–411(0):18-24.

Kleinschmit Kung J, Kung LJ (2006). The Effects of *Lactobacillus buchneri* 40788 and *Pediococcuspentosaceus* R1094 on the Fermentation of Corn Silage. J. Dairy Sci. 89(10):3999-4004.

Kraus TEC, Yu Z, Preston CM, Dahlgren RA, Zasoski RJ (2003). Linking chemical reactivity and protein precipitation to structural characteristics of foliar tannins. J. Chem. Ecol. (29):703-730.

Kumaraguru Vasagam KP, Balasubramanian T, Venkatesan R (2007). Apparent digestibility of differently processed grain legumes, cow pea and mung bean in black tiger shrimp, *Penaeus monodon* Fabricius and associated histological anomalies in hepatopancreas and midgut. Anim. Feed Sci. Technol. 132:250-266.

Lane DJ (1991). 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M, Wiley J (eds), Nucleic acid techniques in bacterial systematics, New York, NY, USA.

León J, Pellón F, Unda V, David J, Anaya C, Mendoza V (2000). Producción de enzimas extracelulares por bacterias aisladas de invertebrados marinos. Rev. Peru de Biol. 7:202-210.

Leyva-Madrigal KY, Luna-González A, Escobedo-Bonilla CM, Fierro-Coronado JA, Maldonado-Mendoza IE (2011). Screening for potential probiotic bacteria to reduce prevalence of WSSV and IHHNV in whiteleg shrimp (*Litopenaeus vannamei)* under experimental conditions. Aquaculture 322-323:16-22.

Liener IE (1994). Implications of antinutritionalcomponets in soybean foods.Crit. Rev. Food Sci. 34:31-67.

Lot A, Novelo-Retana A, Olvera-García M, Ramírez-García P (1999). Catálogo de angiospermas acuáticas de México:hidrófitas estrictas emergentes, sumergidas y flotantes. Cuadernos del Instituto de Biología 33. Universidad Nacional Autónoma de México, México.

Moretti P, Hintermann G, Hutter R (1985). Isolation and characterization of an extrachromosomal element from *Nocardia mediterranei.* Plasmid 14:126-133.

Nguyen HTM, Pérez-Gálvez R, Bergé JP (2012). Effects of diets containing tuna head hydrolysates on the survival and growth of shrimp *Penaeus vannamei*. Aquaculture. 324-325:127-134.

Nikolopoulou D, Grigorakis K, Stasini M, Alexis MN, Iliadis K (2007). Differences in the chemical composition of field pea (*Pisum sativum*) cultivars; Effect of cultivation area and year. Food Chem. 103:847-852.

Norris C, Preston C, Hogg K, Titus B (2011).The influence of condensed tannin structure on rate of microbial mineralization and reactivity to chemical assays. J. Chem. Ecol. 37:311-319.

Nout MJR (2009). Rich nutrition for the poorest–Cereal fermentations in Africa and Asia. Food Microbiol. 26:685-682.

Olsen RL, Hasan MR (2012). Alimited supply of fishmeal: Impact on future increases in global aquaculture production. Trends in Food Sci. Technol. 27:120-128.

Oludayo-Olaniyi C, Omoniyi-Oladunjoye I (2012). Replacement value of duckweed (*Lemna minor)* in nile tilapia (*Oreochromisniloticus*) diet.Transn. J. Sci. Technol. 2(9):54-62.

Ouoba L, Diawara B, Jespersen L, Jakobsen M (2007). Antimicrobial activity of *Bacillus subtilis* and *Bacillus pumilus* during the fermentation of African locust bean (*Parkiabiglobosa*) for Soumbala production. J. Appl. Microbiol.102(4):963-970.

Oyarekua MA (2011). Biochemical and microbioogical changes during the production of the fermented pigeon pea (*Cajanuscajan*) flour. Afr. J. Food Sci. Technol. 2(10):223-231.

Partida-Arangure BO, Luna-González A, Fierro-Coronado JA, Flores-Miranda MC, González-Ocampo HA (2013). Effect of inulin and probiotic bacteria on growth, survival, immune response, and prevalence of WSSV in *Litopenaeus vannamei* cultured under laboratory conditions. Afr. J. Biotechnol. 12(21):3366-3375.

Ramachandran S, Ray AK (2007). Nutritional evaluation of fermented black gram (*Phaseolus mungo*) seed meal in compound diets for rohu, *Labeo rohita* (Hamilton), fingerlings. J. Appl. Ichthyol. 23(1): 74-79.

Rosenberg M, Gutnick D, Rosenberg E (1980). Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. FEMS Microbiol. Lett. 9:29-33.

Saha S, Ray AK (2011). Evaluation of Nutritive Value of Water Hyacinth (*Eichhornia crassipes*) Leaf Meal in Compound Diets for Rohu, *Labeo rohita* (Hamilton, 1822) Fingerlings after Fermentation with Two Bacterial Strains Isolated from Fish Gut. Turk. J. Fish. Aquat. Sci. 11:199-207.

Saitou N, Nei M (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4:406-425.

Sangronis E, Machado CJ (2007). Influence of germination on the nutritional quality of *Phaseolus vulgaris* and *Cajanus cajan.* LWT-Food Sci. Technol. 40: 116-120.

Shad MA, Nawaz H, Rehman T, Ahmad HB, Hussain M (2012). Optimization of extraction efficiency of tannins from *Cichorium intybus* L.: Application of response surface methodology. J. Med. Plants Res. 6(28):4467-4474.

Shirai K, Guerrero I, Huerta S, Saucedo G, Castillo A, González RO,

Hall GM (2001). Effect of initial glucose concentration and inoculation level of lactic acid bacteria in shrimp waste ensilation. Enzyme Microb. Tech. 28:446-452.

Smith GC, Carpenter ZL, Mattil KF, Cater CM (1973). Efficacy of Protein additives as emulsion stabilizers in Frankfurters. J. Food Sci. 38:849-855.

Tacon AGJ, Metian M (2008). Global overview on the use of industrially compounded aquafeeds: trends and future prospects. Aquaculture. 285:146-158.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011). MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28:2731-2739.

Teather RM, Wood PJ (1982). Use of Congo red-polysaccharide interactions in enumeration and characterization of cellulolytic bacteria from the bovine rumen. Appl. Environ. Microbiol. 43:777-780.

Wheeler EL, Ferrel RE (1971). A method for phytic acid determination in wheat and wheat fractions. Cereal Chem. 48:312-320.

Yilmaz E, Akyurt I, Günal G (2004). Use of duckweed, *Lemna minor*, as a protein feedstuff in practical deits for common carp, *Cyprinus carpio*, Fry.Turk. J. Fish. Aquat. Sci. 4:105-109.

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Full Length Research Paper

In-vitro antimicrobial and synergistic properties of water soluble green and black tea extracts

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Green and black teas extracts are known for their antibacterial activity against many pathogenic microorganisms. These studies have been necessitated by the need to combat the recent rise of drug-resistant human pathogens which is becoming a common occurrence in the world making easily manageable infections to become life threatening illnesses. This study evaluated the antimicrobial activity of water soluble green and black tea extracts from a high quality Kenyan tea clone TRFK 6/8 against antibiotic resistant *Escherichia coli* ATCC 25922 and *Staphylococcus aureus*ATCC 25923 using agar well diffusion method. Green and black tea extracts effectively inhibited the growth of both *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 at concentrations of 0.1 and 0.05 mg/ml, respectively after 24 h. Green tea extracts and gentamicin showed greater zone of inhibition compared to penicillin G. In addition, the possible synergistic activity of water soluble green tea extract and antibiotics was also determined using agar well diffusion method. A combination of penicillin G and green tea extract inhibited the growth of *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 compared to penicillin G alone while gentamicin exhibited an additive and antagonistic effect depending on tested bacteria. Green and black tea extracts can be used as an antimicrobial agent and also green tea extract can be used in combination with penicillin G to manage resistant pathogenic bacteria. Furthermore, tea which is a proven safe, cheap and readily available compound can be used in more ambitious trials to test the antimicrobial efficacy and chemo-preventive effects in animal and human models.

Key words: Green tea, black tea, antimicrobial activity, synergistic activity, gentamicin, penicillin G.

INTRODUCTION

Tea which is the most widely consumed beverage in the world is manufactured from the tender leaves of the plant *Camellia sinensis* (Cabrera et al*.,* 2003). The two types of tea products that are widely consumed are green and black tea. Green tea is widely consumed in China, the Middle East, while black tea is consumed in India,

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European countries and regions of Africa (Obanda et al*.,* 2001). The dried leaf of the evergreen tea bush *Camellia sinensis* from which tea is processed, contains a wide variety of biologically active compounds such as polyphenols, methylxanthines, essential oils, proteins, vitamins and amino acid (Bastos et al*.,* 2006; Gallaher et al*.,* 2006). However, most of tea biological activities are ascribed to the polyphenolic fraction, namely: tea catechins (Cabrera et al*.,* 2003). The polyphenols present in tea include: epigallocatechingallate (EGCG), epicatechin (EC), epigallocatechin (EGC) and epicatechingallate (ECG) in green tea, while theaflavins (TFs) and thearubigins (TRs) are present in black tea. Of these polyphenolic components of tea, EGCG is the major constituent and is also the component with the highest bioactivity in green tea due to its high degree of hydroxylation and gallation (Lu et al*.,* 2003). However, many studies have focused on the role of a single catechin EGCG in preventing diseases in human but it is important to point out that the overall protective effect of tea may require a combined action of several components in the beverage. Catechins which belong to the flavan-3-ols family of polyphenols have recently received considerable attention because of their potential therapeutic effects. Emerging scientific data from pharmacological and physiological studies continue to show that tea has beneficial effects on human health (Hu et al*.,* 2004; Vanessa and Williamson, 2004; Yamamoto et al*.,* 2004). Considerable attention is currently being focused on the role of dietary and medicinal phytochemicals to inhibit, reverse or retard diseases mainly due to their radical scavenging properties. Since tea can be consumed over long periods of time without any known side effects, its possible role as an adjunct therapeutic agent in managing bacterial infections deserves consideration.

Recent reports have indicated antimicrobial properties of tea catechins on resistant bacteria and fungi (Hiroshi, 2012). Studies have reported that catechin from green tea inhibit *Staphylocococcus aureus*, *Vibrio parahemolyticus, Clostidium perfringens, Bacillus cereus, Pleisomonas shigelloides, Aeromonas sobria, Acinetobacter baumannii* and other food borne pathogens (Friedman et al*.,* 2006; Hiroshi*,* 2012, Osterburg et al*.,* 2012). Japanese green tea extracts have also been reported to inhibit the growth of *Vibrio cholerae*and *Shigella dysentriae* (Das, 1962). Theaflavins and thearubigins in black tea have also been shown to have antibacterial properties both *in vivo* and *in vitro* (Bandyopadhyayet et al., 2005). However limited research has been done on antimicrobial properties of Kenyan black tea and synergism of green tea extract in combination with some of conventional antibiotics. The purpose of this study was to establish whether black and green teas have antimicrobial activity and the level of synergistic or antagonistic effects of green tea extracts with antibiotics.

MATERIALS AND METHODS

Bacteria

The test bacteria of the American Type Culture Collection (ATCC) were obtained from the Department of Biochemistry, Egerton University (Njoro) and included *Escherichia coli* ATCC 25922 and *Staphylococcus aureus*ATCC 25923.

Tea samples

The tea sample were sourced from the Tea Research Foundation of Kenya's Kangaita substation in Kirinyaga (0° 26'S and 37° 15'E, elevation 2020 m a.m.s.l) and processed into green and black tea products at Tea Research Foundation of Kenya miniature factory in Kericho as described by Karori et al. (2007).

Antimicrobial assays

Determination of antimicrobial activity using Welldiffusion Method

Antimicrobial activity was measured using a well diffusion method according to the National Committee for Clinical Laboratory Standard (NCLSI, 2011). Briefly, Petri plates containing approximately 25-30 ml of Mueller Hinton agar medium were inoculated using a cotton swab with a 4-6 h old culture of the bacterial strains. Wells (10 mm diameter) were punched in the agar and filled with 100µl of tea extracts (green and black) and 25 µl of penicillin G and gentamicin, respectively. Replicate of each plate was done in triplicates. The plates were incubated at 37°C for 18-24 h. The antimicrobial activity (mm) was assessed by measuring the inhibition zone diameters around the well. The average of three replicates for green and black tea extracts was calculated.

Determination of the combined activity using Welldiffusion Method

Combination of green tea extract with penicillin G and gentamicin was determined according to the National Committee for Clinical Laboratory Standard (NCLSI, 2011). A volume of 75 µl of green tea extracts from a stock solution was combined with 25 µl of penicillin G and gentamicin that already been prepared from stock solution and added to each well. The plates were incubated at 37°C for 18-24 h. The average of three replicates for green tea extracts and in combination with antibiotics was calculated. Synergistic effect was considered when combinations of green tea extracts with penicillin G or gentamicin exhibited enlargement of combined inhibition zone diameter size by 0.5mm (Ahmad and Aqil, 2007).

Statistical analysis

The results were presented in the form of tables and plates. The test was done in triplicates and the average and standard deviation results were computed using SAS statistical package 9.1 version.

RESULTS

Antimicrobial activity for penicillin G and gentamicin

Penicillin G showed the highest inhibition zone diameters

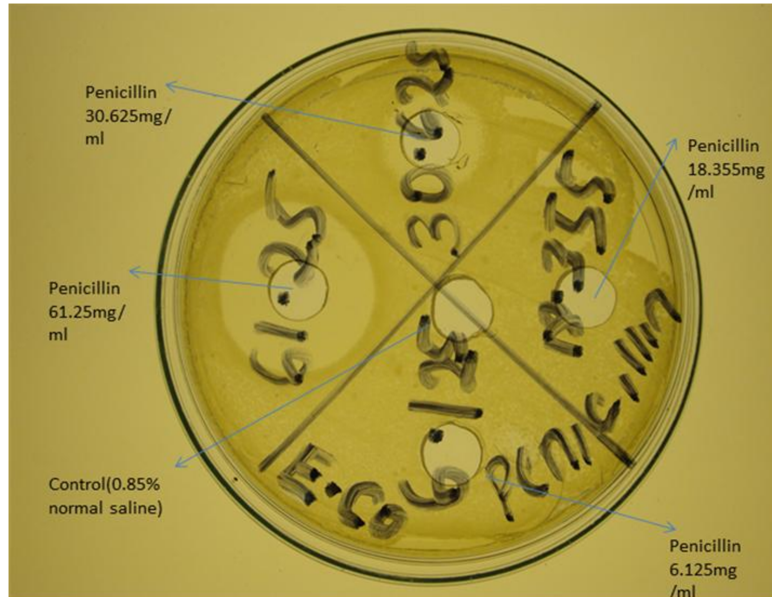

Figure 1. A representative image showing dose determination of penicillin G on *E. coli.*

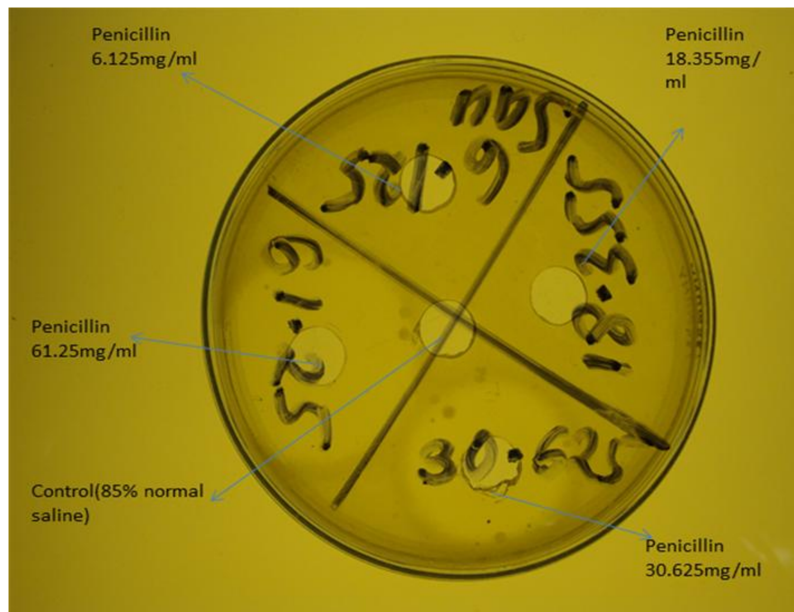

Figure 2. A representative image showing the dose determination of penicillin on *S. aureus.*

against both *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 at high concentrations of 1.53 mg/ml (Figure 1) and 0.77 mg/ml (Figure 2). However, penicillin G at a lower concentrations of 0.46 and 0.15 mg/ml did not show any inhibition against *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 (Table 1).

In addition, gentamicin inhibited *E. coli* ATCC 25922 (Figure 3) and *S. aureus* ATCC 25923 (Figure 4) but at lower concentration compared to penicillin G. The highest inhibition zone diameter exhibited by gentamicin was at a concentration of 0.25 mg/ml (Table 1). Furthermore, lower concentrations of 0.13 and 0.08 mg/ml did inhibit *E. coli* ATCC 25922 and *S. aureus* ATCC 25923. However, gentamicin at a concentration of 0.03 mg/ml did not

Table 1. Antimicrobial activity for penicillin G and gentamicin.

Antibiotic	Concentration (mg/ml)	Inhibition zone diameters (mm)	
		E. coli ATCC 25922	*S. aureus* ATCC 25923
Penicillin G	1.53	25.3 ± 0.50	37.3 ± 0.58
	0.77	17.3 ± 0.58	26.5 ± 0.50
	0.46	0 ± 0.00	0 ± 0.00
	0.15	0 ± 0.00	0 ± 0.00
Gentamicin	0.25	27.3 ± 0.29	26.2 ± 0.29
	0.13	25.7 ± 0.58	23.8 ± 0.29
	0.08	21.3 ± 0.58	22.7 ± 0.58
	0.03	0 ± 0.00	0 ± 0.00

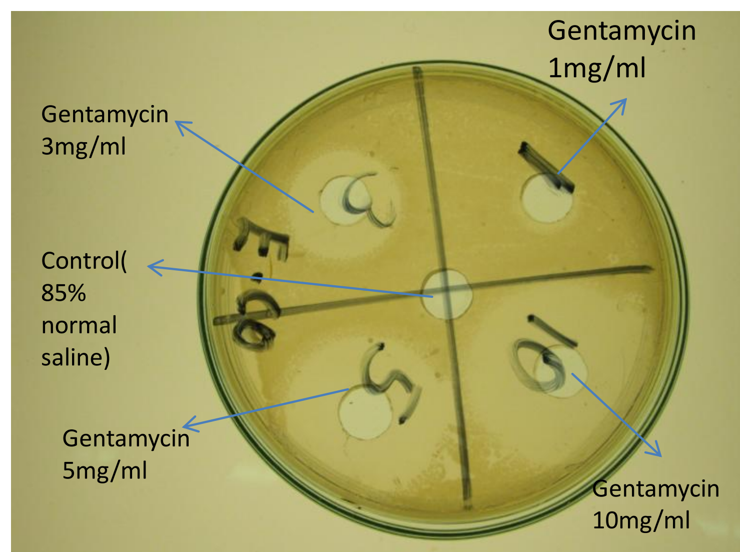

Figure 3. Results for antimicrobial activity for gentamicin in *E. coli.*

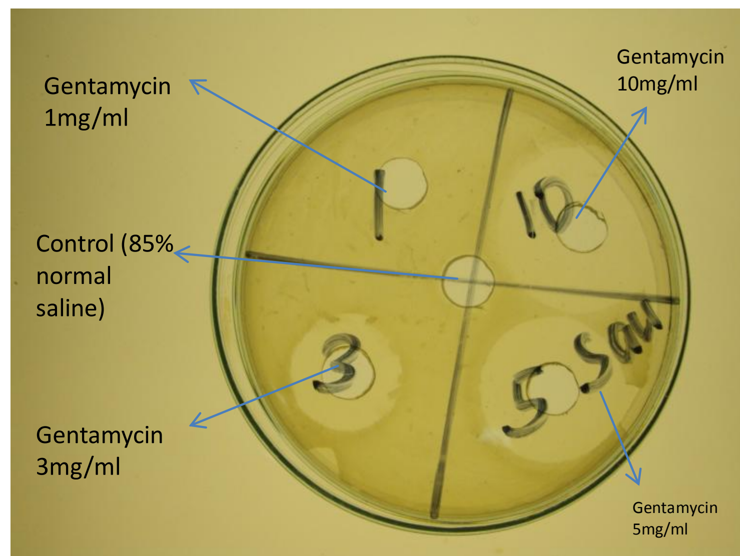

Figure 4. Results for antimicrobial activity for gentamicin in *S. aureus.*

Table 2. Antimicrobial activity for green and black tea extracts.

Tea sample	Concentration (mg/ml)	Inhibition zone Diameters (mm)	
		***E. coli* ATCC 25922**	***S. aureus* ATCC 25923**
Green tea TRFK 6/8	0.05	0 ± 0.00	0 ± 0.00
	0.10	21.3 ± 0.29	22.7 ± 0.58
	0.20	0 ± 0.00	0 ± 0.00
	1.00	0 ± 0.00	0 ± 0.00
Black tea TRFK 6/8	0.05	27.3 ± 0.58	27.7 ± 0.58
	0.10	0 ± 0.00	0 ± 0.00
	0.20	0 ± 0.00	0 ± 0.00
	1.00	0 ± 0.00	0 ± 0.00

inhibit both *E. coli* ATCC 25922 and *S. aureus* ATCC 25923.

In this study therefore, both *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 were more resistant to penicillin G as compared to gentamicin which exhibited high inhibition zone diameters at even lower concentrations (Table 1).

Antimicrobial activity for green and black tea extracts

The results on the antimicrobial activity of TRFK 6/8 processed as green and black tea products are presented in Table 2. Green tea product processed from TRFK 6/8 exhibited an inhibition against both *E. coli* ATCC 25922 and *S. aureus* ATCC25923 at concentration of 0.1 mg/ml. However, at alower concentration of 0.05 mg/ml, green tea extract did not exhibit any inhibition against the tested antibiotic resistant bacteria. Furthermore, on increasing the concentration of green tea extract, there was no inhibition against antibiotic resistant *E. coli* ATCC 25922 and *S. aureus* ATCC 26923. This could be attributed to the inability of higher concentration of tea extracts to diffuse through the Mueller Hinton agar medium.

Black tea extract processed from TRFK 6/8 tea cultivar exhibited different inhibitory effects compared to green tea processed from the same tea cultivar. Black tea extracts inhibited antibiotic resistant *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 at lower concentration of 0.05 mg/ml compared to green tea of the same cultivar. However, increasing concentration of black tea extracts did not exhibit any inhibition observed earlier in green tea extracts. This may also be as a result of the inability of higher concentration of black tea extract to diffuse through the Mueller Hinton agar medium. From this study, black tea extract exhibited a higher inhibitory effect at lower concentration compared to green tea extracts. In addition, there was no significant difference in the inhibitory effects of black tea extract against antibiotic resistant *E. coli* ATCC 25922 and *S. aureus* ATCC 25923.

Evaluation of green tea extracts interactions with some antibiotics

In this study, green tea extract from the TRFK 6/8 cultivar, exhibited an antagonistic effect against antibiotic resistant *E. coli* ATCC 25923 when combined with gentamicin (Figure 5). However, a combination of green tea extract with penicillin G exhibited a synergistic effect. Similar results were also observed when green tea extracts from TRFK 6/8 was combined with penicillin G against antibiotic resistant *S. aureus* ATCC 25923 as shown in Figure 6. Green tea extracts in combination with gentamicin exhibited an additive effect against antibiotic resistant *S. aureus* ATCC 25923. In addition, it was also observed that the green tea extract from TRFK 6/8 cultivar exhibited a higher inhibition zone diameters as compared to penicillin G and its activity was comparable to that of gentamicin (Table 3).

DISCUSSION

Tea is one of the most popular non-alcoholic beverages with health enhancing effects. The inhibitory effect of green and black tea extracts on antibiotic resistant bacteria has been reported (Bandyopadhyayet et al., 2005). The results of the present study show that green tea and black tea processed from TRFK 6/8 have antimicrobial activity on antibiotic resistant *E. coli* ATCC 25922 and *S. aureus* ATCC 25923. These results are in agreement with the finding of Koech et al. (2013b). Although black and green tea both contain similar amount of flavonoids, their chemical structure is different. Green tea contains more catechins (simple flavonoids) but during black tea processing, leaf oxidation converts these simple flavonoids to more complex theaflavins and thearubigins (Cabrera et al., 2006). These catechins, theaflavins and thearubigins are the antimicrobial agents present in tea (Hamilton-Miller, 1995; Luczaj and Skrzydlewska, 2005).

The findings in this study also indicated that the

Figure 5. Antibacterial and synergistic activity of water soluble tea extracts with antibiotics against *E. coli*ATCC 25922 and *S. aureus*ATCC 25923. TP- tea and penicillin, TG- tea and gentamicin, GENTA- gentamicin.

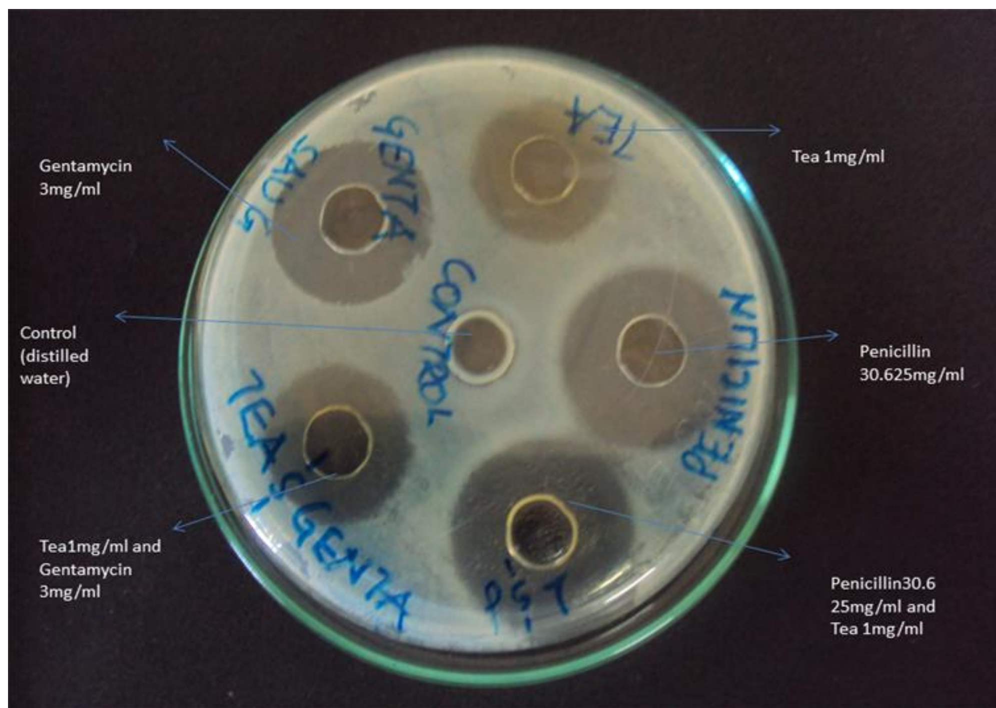

Figure 6. Antibacterial and synergistic activity of water soluble tea extracts with antibiotics against *E. coli*ATCC 25922 and *S. aureus*ATCC 25923. TP- tea and penicillin, TG- tea and gentamicin, GENTA- gentamicin.

Table 3. Evaluation of green tea extracts interactions with some antibiotics.

Tested bacteria	Gentamicin (0.08 mg/ml)	Penicillin G (0.77 mg/ml)	Tea (Green) (0.1 mg/ml)	Tea + Gentamicin (0.1 + 0.08 mg/ml)	Tea + Penicillin G (0.1 + 0.77 mg/ml)
E. coli ATCC 25922	22.3 ± 0.50	18.5 ± 0.58	21.3 ± 0.33	19.2 ± 0.33	25.3 ± 0.50
S. aureus ATCC 25923	23.2 ± 0.28	27.3 ± 0.28	23.7 ± 0.33	23.2 ± 0.29	28.7 ± 0.29

antimicrobial effects of assayed tea extracts differed depending on the type and the concentration of the tea extract and also the type of test organism. The inhibitory effect of black tea extract on *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 was lower in concentrations than green tea extract. This finding is in agreement with the previous studies in which black tea extracts exhibited high antimicrobial activity against *Streptococcus mutans* more than green tea extracts (Hamdi et al., 2008).

In this study, increasing concentration of tea extracts (green and black) resulted in no inhibition activity. This could be attributed to the inability of higher concentrations of the tea extract to diffuse through the Muller Hinton agar medium. This impairment in drug diffusion is a major limitation in the evaluation of the antimicrobial effects of plant extracts using agar diffusion method (Esiome et al., 2006). The conclusion by Taguri et al. (2006) that the antimicrobial potency of tea polyphenols is dependent upon bacterial species is consistent with the findings of this study, which showed that, the green and black tea extracts were more active against the Gram-positive, *S. aureus* ATCC 25923 as compared to *E. coli* ATCC 25923 which is Gram-negative.

The antimicrobial results of this study showed a marked increase in the inhibition zone diameters on combination of green tea extracts with penicillin G. This is in agreement with results by (Hu et al., 2002) who reported enhanced effect of tea extracts on inhibitory activities with β-lactams antibiotics against antibiotic resistant *S. aureus*ATCC 25923. Synergistic inhibition by green tea extracts and penicillin G could be attributed to the presence of dual binding sites on the bacterial surface for antibiotic and tea extract (Tiwari et al., 2005). The tea extracts-induced damage of the bacterial cell wall and the possible interference with its biosynthesis through direct binding with peptidoglycan may be the major reasons for the synergism against antibiotic resistant *S. aureus*ATCC 25923. When green tea extract was combined with gentamicin against *S. aureus* ATCC 25923, an additive effects was observed which could be hypothesized that gentamicin also directly or indirectly attacks the same site. The double attack of both agents on different target sites of the bacteria could theoretically lead to an additive effect (Esiome et al., 2006). However, antagonistic interaction between green tea extracts and gentamicin was observed against *E. coli* ATCC 25922. This could be because green tea extract and gentamicin have different mode of action against tested bacteria.

Conclusion

In conclusion, green and black tea exhibited antimicrobial properties against antibiotic resistant *E. coli* ATCC 25922 and *S. aureus* ATCC 25923. In addition, green tea extracts did not impair the antimicrobial properties of penicillin G but rather it enhanced its activity in synergistic manner. The combination of green tea extract and penicillin G could be useful in management of emerging antibiotic resistant bacteria.

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Conflict of Interests

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

REFERENCES

Ahmad I, Aqil F (2007).*In vitro* efficacy of bioactive extracts of 15 Medicinal plants against ESL-producing multidrug-resistant enteric bacteria. Microbiol. Res. 162: 264-275.

Bandyopadhyayet D, Chatterjee TK, Dasgupta A, Lourduraja J, Dastidar SG (2005). *In vitro* and *in vivo* antimicrobial action of tea: The commonest beverage of Asia. Biol. Pharm. Bull. 28: 2125-2127.

Bastos DM, Ishimoto EY, Marcia UM, Ferri AF, Torres EA.(2006). Essential oil and antioxidant activity of green mate tea (*Ilex paraguariensis*) infusions. J. Food Comp. Anal. 19: 538-543.

Cabrera C, Artacho R, Gimenez R (2006). Beneficial effects of green tea–a review. J. Am. Coll. Nutr. 25 (2):79-99.

Cabrera C, Gimenez ME, Lopez CM (2003). Determination of tea components with antioxidant activity. J. Agric. Food Chem. 51: 4427-4435.

Das DN (1962). Studies on antibiotic activity of tea.J. Indian Chem. Soc. 39: 849-854.

Esiome CO, Iroha IR, Ibezim EC, Okeh CO, Okpana EM (2006).In vitro evaluation of the interaction between tea extracts and penicillin G against *Staphylococcus aureus.* Afr. J. Biotechnol. 5: 1082-1086.

Friedman M, Henika PR, Levin CE, Mandrell RE, Kozukue N (2006). Antimicrobial activities of tea catechins and theaflavins and tea extracts against *Bacillus cereus*. J. Food Prot. 69: 354-361.

Gallaher RN, Gallaher K, Marshall AJ, Marshall AC (2006). Mineral analysis of ten types of commercially available tea. J. Food Comp. Anal.19: 53-57.

Hamdi K, ShoaHasani AR, Ordouzadeh N, Ghaemi A (2008). The effect of black and green tea extracts on dental-plaque forming *Streptococci.* Shahrekord Univ. Med. Sci J. Fall. 10(3): 1-8.

Hamilton-Miller JM (1995). Antimicrobial properties of tea (*Camellia sinensis L.).* Antimicrob. Agents Chemother. 39: 2375- 2377.

Hiroshi Y (2012). Tea Catechins and S*taphylococcus* aureus. In: Preedy (eds) Tea in health and disease prevention. Elsevier, London, pp. 1207-1213.

Hu ZQ, Zhao WH, Asano N, Yoda Y, Hara Y, Shimamura T (2002). Epigallocatechingallate synergistically enhances the activity of carbapenems against methicillin- resistant *Staphylocccusaureus.* Antimicrob. Agents Chemother. 46: 558-560.

Hu ZQ, Zhao WH, Shimamura T (2004). Different susceptibilities of Staphylococcusand gram negative rods to EGCG: In Proceedings of International Conference of Tea Culture and Science. Shizuoka Japan. pp. 578 -581.

Karori SM, Wachira FN, Wanyoko JK, Ngure RM (2007). Antioxidant capacity of different types of tea products. Afr. J. Biotechnol. 6: 2287-2296.

Koech KR, Wachira FN, Ngure RM, Wanyoko JK, Bii C, Karori SM (2013b). Antibacterial and synergistic activity of different tea crude extracts against antibiotic resistant *S. aureus, E. coli* and a clinical isolate of *S. typhi.* Sci. J. Microbiol. 3: 2276-626X

Lu H, Meng X, Li C, Sang S, Patten C, Sheng S, Hung J, Bai N, Winnik B, Ho TC, Yang CS (2003).Glucoronides of tea catechins: Enzymology of biosynthesis and biological activities. Drug Metab. Dispos. 31:452-461.

Luczaj W, Skrzydlewska E (2005). Antioxidative properties of black tea. Prev. Med. 40: 910-918.

National Clinical and Laboratory Standards Institute (NCLSI) (2011).M100-S21, Performance standards for antimicrobial susceptibility testing; Twenty-First informational supplement Wayne, PA.

Obanda M, Owuor PO, Mang'oka R (2001). Changes in the chemical and sensory quality parameters of black tea due to variations of fermentation time and temperature. J. Food Chem. 75: 395-404.

Osterburg AR, Karen LM, George FB (2012). Antibacterial-*Acinetobacter baumannii*- Activity of Epigallocatechin-3-Gallate (EGCG) In: Preedy (eds). Tea in health and disease prevention. Elsevier, London, pp. 1195-1206.

Taguri T, Tanaka T, Kouno I (2006). Antibacterial spectrum of plant polyphenols and extracts depending upon hydroxyphenyl structure. Biol. Pharm. Bull. 29: 2226-2235.

Tiwari RP, Bharti SK, Kaur RP, Dikshit RP, Hoondal GS (2005). Synergistic antimicrobial activity of tea and antibiotics. Indian J. Med. Res.122: 80-84.

Vanessa C, Williamson G (2004). A review of the health effects of green tea catechins in in-vivo animal models. J. Nutr. 134: 3431-3440.

Yamamoto MM, Inagaki N, Kitaura J, Chikumoto T, Kawahara H, Kawakami Y, Kawakami T, Nagai H (2004). O-Methylated catechins from tea leaves inhibit multiple protein kinases in mast cells. J. Immunol. 172: 4486-4492.

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Full Length Research Paper

Effect of the endophyte *Ceratobasidium stevensii* on 4-HBA degradation and watermelon seed germination

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A preliminary trial indicated that the replanting obstacle of watermelon could be mitigated by the addition of the endophytic fungus *Ceratobasidium stevensii* B6 to the soil. The mechanism by which this treatment reduced the obstacles to continuous cropping was not clear, although the phenolic acids secreted by watermelon are a known problem. Consequently, we investigated the degradation of phenolic acids by *C. stevensii*, using 4-hydroxybenzoic acid (4-HBA) as a model. When the concentration of 4-HBA rose to 500 mg/L, it completely inhibited the germination of watermelon seeds. We therefore investigated the optimal conditions for 4-HBA degradation. When active *C. stevensii* broth was inoculated into degradation culture broth at a density of 5% (v/v), the 4-HBA concentration decreased to a level below the level of detection after 6 days. *C. stevensii* could also degrade 4-HBA when it was used as a sole carbon source. Glucose, sucrose, starch, and fructose stimulated the degradation of 4-HBA. High concentrations of glucose enhanced 4-HBA degradation. The optimal conditions for the degradation of 4-HBA by *C. stevensii* were 28°C, initial pH 5.5, and a shaking speed of 150 rpm. The addition of 500 mg/kg of 4-HBA to dried soil lasted for 2 months, followed by addition of *C. stevensii* which reduced the concentration of 4-HBA in soil below the level of detection after 7 days. Watermelon seedlings cultured in treated soil had a significantly increased number of stems and greater leaf length compared to controls. Therefore, *C. stevensii* has great potential in effectively lessening the obstacles to watermelon continuous cropping caused by 4-HBA accumulation.

Key words: *Ceratobasidum stevensii*, 4-hydroxybenzoic acid, watermelon, continuous cropping obstacles, degradation.

INTRODUCTION

Watermelon (*Citrullus lanatus*) is widely cultivated in China for its short growth period, high yield, and high monetary return. The current planting area of this crop is approximately 1.81×10^6 ha (Ministry of Agriculture of the People's Republic of China, 2011). However, once watermelon has been planted for a year, the land

Table 1. Basic physical and chemical properties of soil.

Soil	pH (H_2O)	Organic matter (g/kg)	Total nitrogen (g/kg)	Available nitrogen (mg/kg)	Available phosphorus (mg/kg)	Available potassim (mg/kg)	Clay (<0.002 mm) (%)
Soil from Jiangxi (Agri-Udic Ferralosols)	4.45	11.8	0.56	62.71	7.20	168.20	39.35
Soil from Hunan (Haplic-Udic Ferralosols)	4.82	11.6	0.89	77.32	25.17	588.31	32.99
Soil from Nanjing (Ferri-Udic Argosols)	7.58	11.8	0.59	124.2	40.06	111.10	25.86

becomes unsuitable for planting ordinary watermelon seed under normal circumstances for 5-6 years (Wu, 2008). One of the most important causes of this obstacle to continuous cropping is the toxic effect of phenolic allelochemicals in the soil (Hao et al., 2010). 4-HBA is a common and typical phenolic allelochemical that is released into the environment by plants through foliar leaching and residue decomposition. Nie et al. (2007) reported that the addition of 4-HBA significantly impaired watermelon seed germination and seedling growth. Previous studies have shown that 4-HBA can reduce the ability of plant roots to absorb ions, induce depolarization of plant cell membranes, and lead to increased membrane permeability to cations and anions (Baziramakenga et al., 1995; Yu and Matsui, 1997). In addition, 4-HBA also affects the activity of plant defense enzymes and the synthesis of hormones and proteins (Wang et al., 2009). Therefore, the removal of this compound from soil is likely very important for crop growth.

Endophytic fungi live within the inner tissues of plants without causing visible symptoms of disease (Aly et al., 2010). In recent years, an increasing number of studies have shifted from examining the ecological functions of endophytic fungi *in vivo* toward *in vitro* experiments (Borges et al., 2009; Chen et al., 2013; Russell et al., 2011). The recently characterized novel fungal endophyte strain *C. stevensii* B6, which was isolated from the inner bark of the stem of *Bischofia polycarpa*, could decompose 89.51% of phenanthrene (a polycyclic aromatic hydrocarbon) within 10 days (Dai et al., 2010). Thus, it may be assumed that these endophytic fungi have the potential to degrade single cyclic molecules such as 4-HBA, which have simpler structures than PAH.

Therefore, the objectives of the present study were to investigate the ability of *C. stevensii* B6 to degrade 4-HBA as a sole carbon source *in vitro,* determine the optimal conditions of 4-HBA degradation, and study the effect of inoculation with *C. stevensii* B6 on the germination of watermelon seeds.

MATERIALS AND METHODS

Strain, materials and reagents

Ceratobasidium stevensii B6 was isolated from the stem endothelium of *Bischofia polycarpa* at Jiangsu Key Laboratory for Microbes and Functional Genomics (Dai et al., 2006). The endophyte was maintained on potato dextrose agar (PDA) slants at 4°C.

Soil samples were collected from layers 0 to 20 cm in depth from monocropped watermelon fields in Yongzhou, Hunan province (N26°45′, E111°53′); Yingtan, Jiangxi province (N28°04′, E117°09′); and Nanjing, Jiangsu province (N32°16′, E118°79′). Table 1 shows the physical and chemical properties of the soils. Soil pH was measured in a 1:2.5 (v/v) soil to water ratio using a DMP-2 mV/pH detector (Quark Ltd, Nanjing, China). Soil organic matter was analyzed by wet digestion with H_2SO_4–$K_2Cr_2O_7$, and total nitrogen was determined by the semi-micro Kjeldahl method. Available nitrogen in soil was determined using an alkaline hydrolysis method. The available phosphorous in soil was extracted with 0.05 M $NaHCO_3$, and the absorbance at 700 nm was detected on a spectrophotometer. The available potassium in soil was extracted with 1.0 mol/L ammonium acetate (NH_4OAc) and measured using emission flame spectrometry. Soil texture was determined by the pipette method (Lu, 2000).

4-hydroxybenzoic acid (4-HBA) was obtained from Sigma-Aldrich. Methanol, acetonitrile, and glacial acetic acid were all HPLC-grade. All other reagents used were of analytical grade. The variety of watermelon seed (*Citrullus lanatus*) was Black Beauty.

Effect of different 4-HBA concentrations on the germination of watermelon seeds

Five concentrations of 4-HBA, 0, 125, 250, 375, and 500 mg/L, were used in the present experiment. Watermelon seeds were sterilized for 10 min in 4% NaOCl and rinsed three times with sterile distilled water. For each treatment, twenty seeds were placed in Petri dishes containing filter paper, with 3 replicates. Seed germination was recorded after 3 days in the dark at 28°C.

Degradation of 4-HBA by B6

C. stevensii was first activated in potato dextrose broth (PDB: peeled potato extract 200 g/L, glucose 20 g/L, pH 7.0) in a shaker at 180 rpm and 28°C for five days. Five milliliters of the active

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fungal suspension (containing dry fungal hyphae at approximately 3.77 g/L) was then used to prepare fungal inoculum by culturing for 2 days at 28°C at 180 rpm in 100 mL of 4-HBA degradation medium (peeled potato extract 10 g/L, glucose 15 g/L, $NaNO_3$ 1.5 g/L, $K_2HPO_4 \cdot 3H_2O$ 1.3 g/L, KCl 0.5 g/L, $MgSO_4 \cdot 7H_2O$ 0.5 g/L, $FeSO_4 \cdot 7H_2O$ 0.02 g/L, 4-HBA 500 mg/L, pH 7.5), using 250 mL Erlenmeyer conical flasks.

Five milliliters of inoculum containing fungal mycelium at 0.57 g/L was added to the 4-HBA degradation test medium described above; kept at 28°C; and shaken at 180 rpm for 0, 1, 2, 3, 4, 5, 6, and 7 days. The fermentation broth was then filtered through filter paper. The fungal mycelia were extracted by stirring with 20 mL of methanol (25°C at 150 rpm) for 1 h and then filtered. The 4-HBA adsorbed on the mycelium was dissolved in methanol, and extracts were evaporated at 30°C to dryness and redissolved in 1 mL of water: methanol (80:20 v/v). The residue was filtered through a 0.45 μm membrane. The amount of 4-HBA adsorbed on the mycelium (w1) was determined by high-performance liquid chromatography (HPLC) (Reis et al., 2011). At the same time, 1 mL of broth (without fungal hyphae) was filtered through a 0.45 μm membrane, and the amount of 4-HBA remaining in the broth was determined (w2). The total amount of 4-HBA remained was calculated as w1 + w2. Another bottle of fermentation broth was also filtered, and the mycelium was washed 3 times with double-distilled water (ddH_2O). The mycelium was dried at 60°C to constant weight to measure the biomass of *C. stevensii* B6. Blank control cultures without the fungus and inactivated controls with inactivated B6 fungal mycelium were processed in an identical manner. Each treatment was repeated 3 times.

4-HBA degradation test

Several ecological factors were used to investigate the influence of culturing conditions on 4-HBA degradation. We used temperatures of 18, 23, 28 and 33°C. Shaking speeds were 120, 150, 180 and 210 rpm. The initial pH values of the culture solutions were adjusted to 4.5, 5.5, 6.5, 7.5 and 8.5 with HCl (0.2 M) or NaOH (0.2 M). Starch, fructose, glucose, cellulose, and sucrose were used to test different carbon sources. Glucose was utilized as the carbon source to explore the effects of different concentrations of carbon on the degradation of 4-HBA. Finally, inoculation quantities of 5, 10, 15, 20 and 25% (v/v) were applied to test the effect of inoculation concentration.

Simulation of conditions under which *C. stevensii* B6 degrades 4-HBA in soil

Soil samples were dried in an electric dryer at 40°C for 12 h and then passed through a 0.85 mm sieve. 4-HBA, dissolved in water, was added to the dried soil (500 mg/kg). This mixture was placed in pots in the botanical garden at Nanjing Normal University and aged for 2 months (Hu et al., 2011). This period is long enough to allow natural equilibration of the various sorptions in the soil. During the equilibration, soil water was maintained 80% maximum water holding capacity by adding water and weighing the pots on a weekly basis. After undergoing fermentation for 7 days, broth containing *C. stevensii* B6 mycelium was passed through 4 layers of gauze. Mycelia were washed 3 times with ddH_2O. Wet fungal mycelia (20.0 g, equivalent to 0.7 g dry fungal weight) were suspended in 20 mL ddH_2O and mixed with 200 g of dry soil containing residual 4-HBA. The treated soil samples from Jiangxi, Hunan, and Jiangsu were identified as J-B6, H-B6, and Z-B6, respectively. Twenty grams of wet inactivated mycelia (121°C, 20 min) and 20 ml of ddH_2O were mixed with 200 g of dried soil as the inactive control (MB6) and blank control (CK), respectively. Therefore, nine treatments were designed: J-CK, J-MB6, J-B6, H-CK, H-MB6, H-B6, Z-CK, Z-MB6, and Z-B6. Mycelia and soil mixtures were put into 250 ml Erlenmeyer flasks. Each treatment was repeated 3 times. Soils were kept in an incubator in the dark at 33°C, and ddH_2O was added to keep the soil moist (Zhang et al., 2010).

Twenty-five fresh soil samples were taken from each flask to determine the amount of residual 4-HBA using HPLC at 0, 1, 2, 3, 4, 5, 6 and 7 d. Soil phenolic compounds were extracted from soils based on the method described by Macias et al. (2004) and determined by HPLC.

Determination of the amount of residual 4-HBA using HPLC

The amount of residual 4-HBA was analyzed using high-performance liquid chromatography (HPLC) (Agilent 1100). The chromatography conditions were as follows: ultraviolet detector, 260 nm; flow rate, 1 mL/min; mobile phase, acetonitrile:1.3% glacial acetic acid solution (17:83). The chromatography column was a Venusil MP C18 with an internal diameter of 4.6 mm and length of 250 mm (Agela Technologies Inc.). The injection volume was 20 μL. The column temperature was 35°C. Samples were filtered through a 0.45 μm membrane prior to HPLC analysis and were diluted to concentrations proper for interpolation within their corresponding standard curves. The levels of 4-HBA were determined using the corresponding peak area. 4-HBA in culture solution and soil extracts was identified by its retention time and co-injection tests with 4-HBA standard compounds. The recovery rate of 4-HBA was 97.6% in pure solution.

Seedling growth test

Watermelon seeds were germinated in ddH_2O for 48 h. Uniform germinated seeds were transferred to soil to simulate the conditions of *C. stevensii* B6 degradation of 4-HBA in soil over 7 days. Soil from Hunan with no added 4-HBA or *C. stevensii* B6 was used as a control (CK) (the background value of 4-HBA was 2.53 ± 0.01 mg/kg). The lengths of leaves and roots were measured after one month's growth in an artificial climate chamber (temperature 24 ± 1°C, light intensity 2000–2500 LX, illumination time 16 h/day).

Statistical analysis

Data were analyzed using Microsoft Excel. Values are the means of 3 replicates (mean ± SD) for each treatment. One-way ANOVA was used to detect significant differences between treatments, and multiple comparisons were performed using Duncan's multiple range tests with SPSS version 13.0 (Chicago, IL, USA). Differences at P=0.05 were considered significant.

RESULTS

With increased concentration of 4-HBA, the germination rate of watermelon seeds decreased significantly (Figure 1). When the concentration of 4-HBA reached 500 mg/L, the seed germination of watermelon was 100% inhibited. Therefore, the initial concentration of 4-HBA in the liquid media was set to 500 mg/L.

As shown in Figure 2, CK and sterilized *C. stevensii* B6 could hardly utilize 4-HBA, while residual 4-HBA could hardly be detected in active B6 culture solution after 6 days (Figure 3). We therefore chose to detect 4-HBA

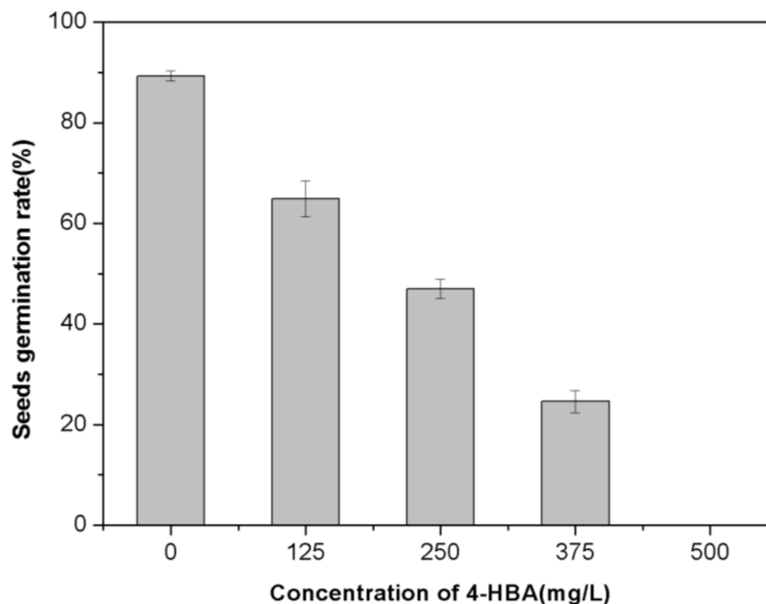

Figure 1. Effect of different 4-HBA concentrations on the germination of watermelon seeds. Each value is the mean ±SD of three replicates.

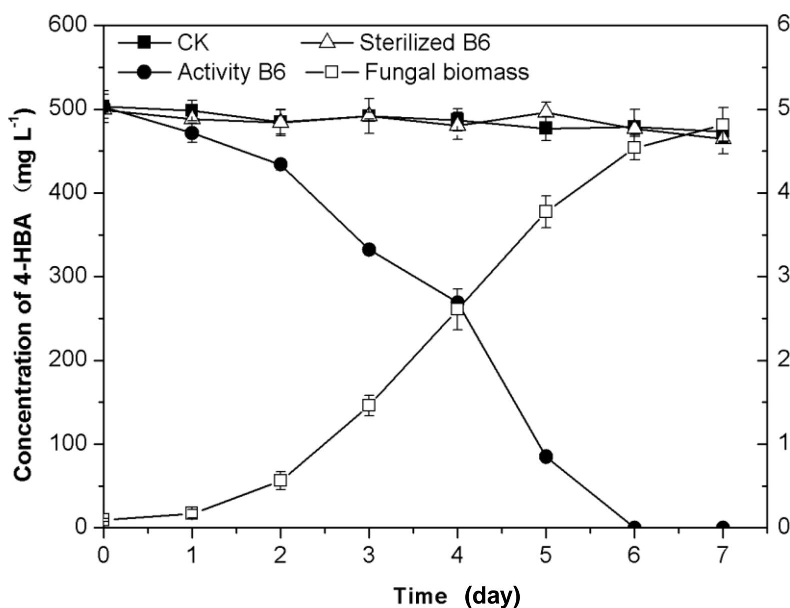

Figure 2. Effect of time on the degradation of 4-HBA. The initial concentration of 4-HBA was 500 mg/L. CK: five milliliters of sterile water was added to 100 mL of culture solution. Sterilized B6: five milliliters of inactive seed culture solution (121°C, 20 min) was added to 100 mL of culture solution. B6: five milliliters of active seed culture solution was added to 100 mL of culture solution. Each value is the mean ± SD of three replicates.

within one week in subsequent 4-HBA degradation experiments.

4-HBA was decomposed by B6 in all temperature tests, and its degradation efficiency increased as the temperature increased from 18 to 28°C. The degradation of 4-HBA was slower at 33°C than that at other temperatures, but the concentration of 4-HBA was still below the level of detection (1 mg/L) on the fifth day (Figure 4a). Initial pH significantly affected the degradation of 4-HBA. Degradation efficiency increased

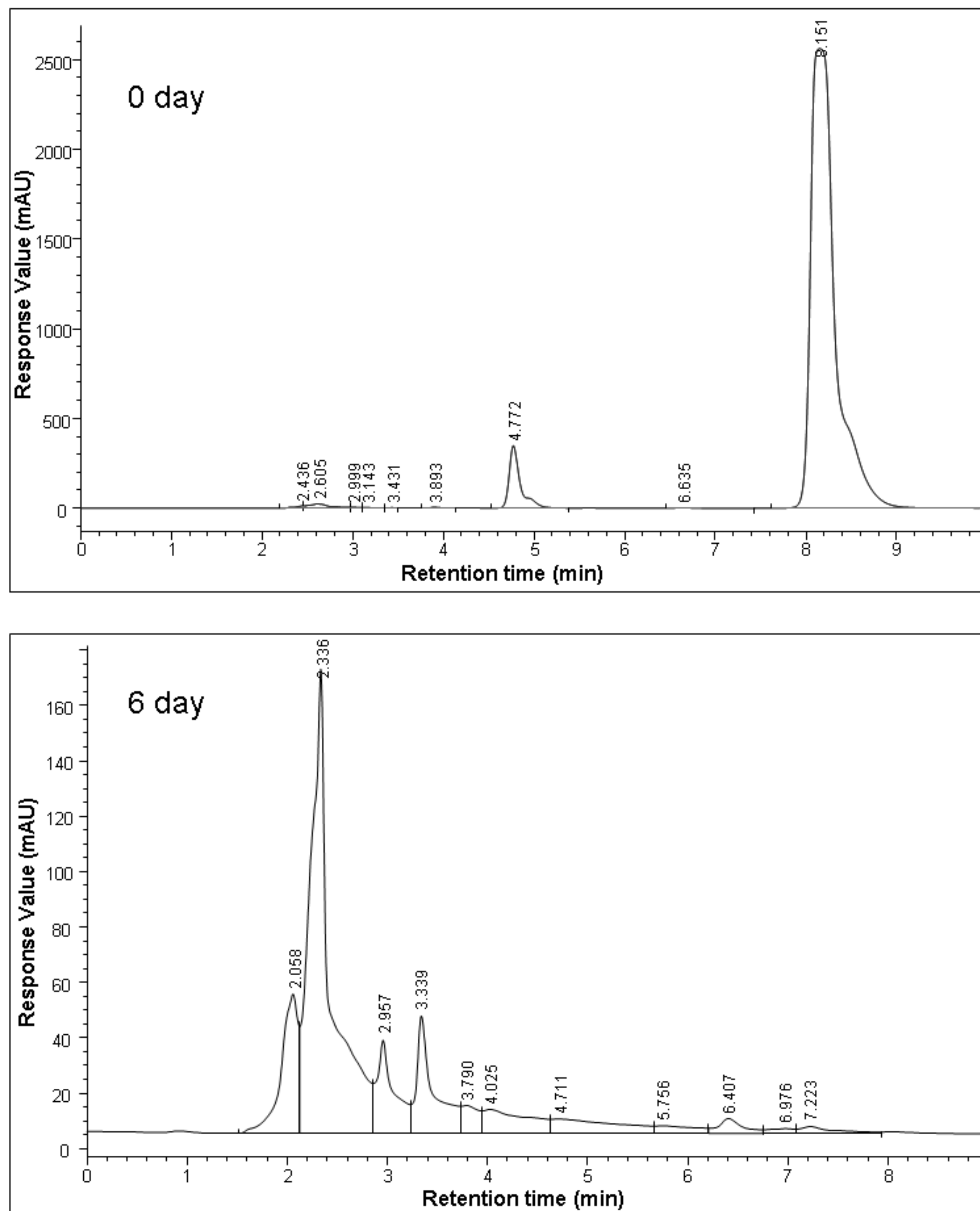

Figure 3. HPLC chromatogram of 4-HBA degradation. The retention time of 4-HBA was 8.151 min. Samples for each culture solution were collected 0, 1, 2, 3, 4, 5, 6, and 7 days after inoculation. Chromatograms at 0 and 6 days are presented here.

as the initial pH decreased from 8.5 to 5.5 (Figure 4b). pH below 5.5 was found to adversely affect degradation. When the shaking speed was 150 rpm, the degradation efficiency was higher than at 120, 180, or 210 rpm (Figure 4c). *C. stevensii* B6 could not utilize cellulose, and 4-HBA reductions over the first 3 days may have resulted from the residual glucose in seed culture solution. Other carbon sources, and glucose in particular, were suitable for 4-HBA degradation. With glucose as a carbon source, the mass of residual 4-HBA was only 153.58 mg, which was the fastest degradation among all treatments over 5 days (Figure 4d). The degradation efficiency depended on glucose concentration. A higher glucose concentration corresponded to faster decomposition of 4-HBA (Figure 4e). The inoculation quantity was an important factor in the degradation of 4-HBA. 4-HBA decomposed best when the inoculation density was 20 or 25% (v/v). When the inoculum was

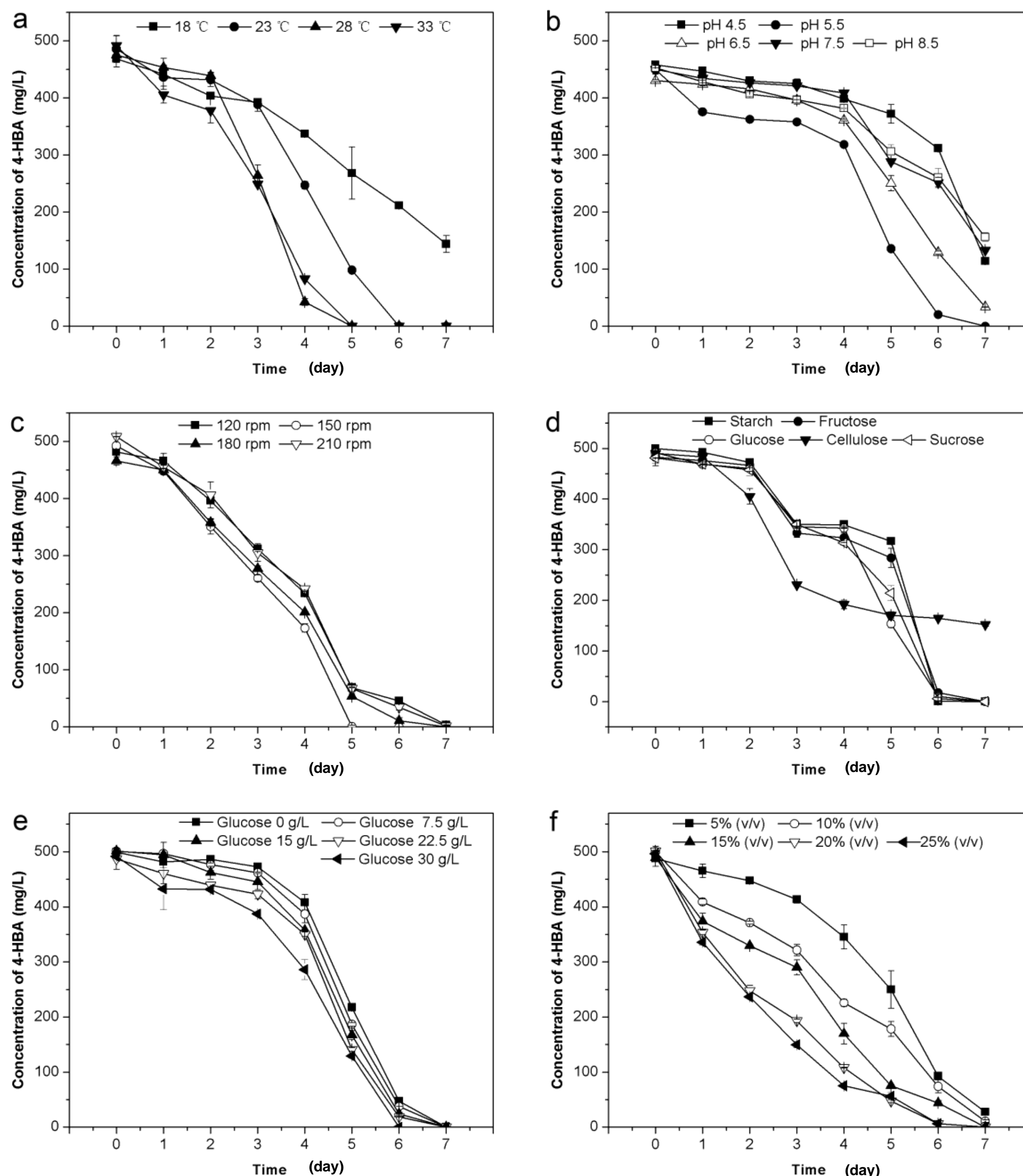

Figure 4 Effect of single factors on the degradation of 4-HBA. The initial concentration of 4-HBA was 500 mg/L. (a) Temperature; (b) Initial pH; (c) Shaking speed; (d) Carbon source; (e) Concentration of glucose; (f) Inoculation amount. Each value is the mean ± SD of three replicates.

below 15%, the degradation rate was relatively slow for the first 6 days; however, by the 7th day, the differences between treatments were small (Figure 4f).

The 4-HBA content in the soil decreased from 500 to 4-5 mg/kg in dried soil after it was aged for 2 months. This decrease may have been due to soil adsorption and transformation by indigenous microorganisms after 2 months in the natural environment (Hu et al., 2011). The 4-HBA levels in the control (CK) and MB6 were barely reduced in 3 soil tests (Figure 5a-c). However, 4-HBA could not be detected (≤ 1 mg/kg dry soil) following *C. stevensii* B6 treatment over 7 days.

The inhibition of stems and leaf elongation by 4-HBA was reversed by *C. stevensii* B6 treatments. The stem lengths in H-B6, J-B6, and Z-B6 increased by 62.6, 23.7 and 66.2%, respectively, compared with the control. The leaf lengths in H-B6, J-B6, and Z-B6 increased by 30.0, 10.0, and 50.0%, respectively, compared with the control. Compared with CK, H-B6 significantly reversed the 4-HBA inhibition of watermelon growth, and root, stem, and

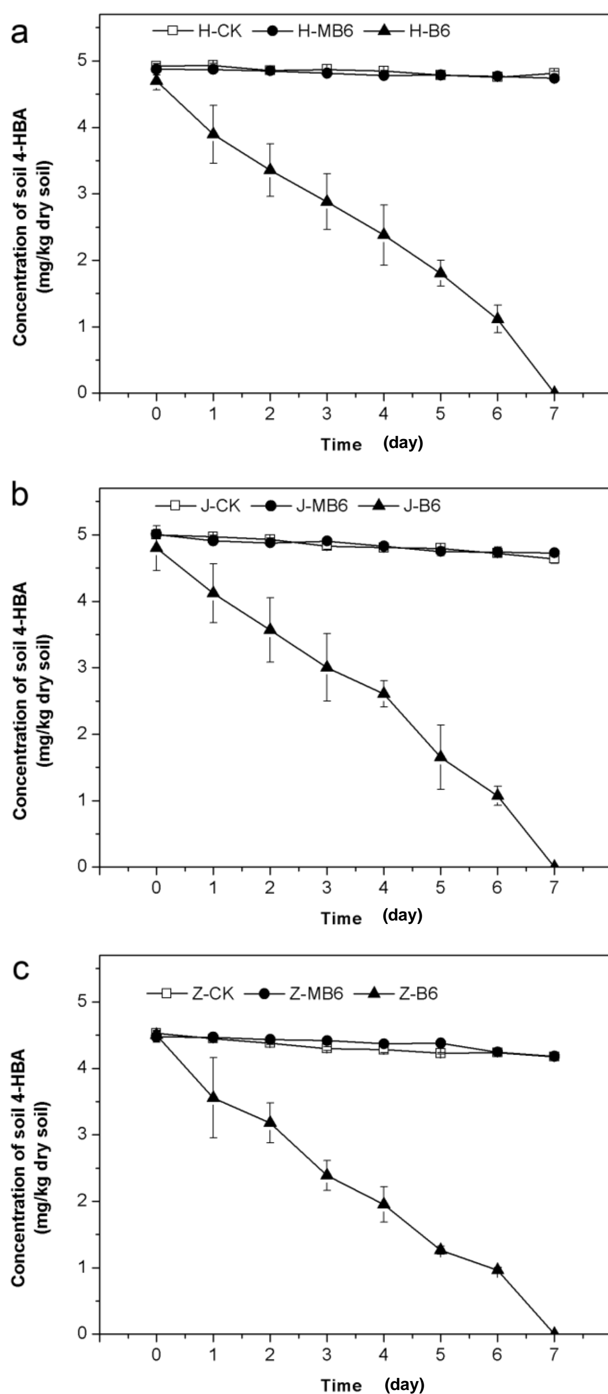

Figure 5. Effect of soil type on the degradation of 4-HBA. Each soil sample contained 500 mg/kg of 4-HBA. (a) Soil from Hunan Province; (b) Soil from Jiangxi Province; (c) Soil from Jiangsu Province. CK: control; MB6: inactivated B6; B6: activated B6.

leaf growth increased by 19.2, 62.6, and 30.0%, respectively. With the addition of sterilized B6 mycelium, the growth states were the same as in the controls (Figure 6).

DISCUSSION

Phenolic acids are typical self-toxic allelochemicals secreted by plant roots. 4-HBA is a phytotoxic allelochemical in plant root exudates that accumulates in the cytosol and cell walls of plants and in root exudates and plant residues released into soils (Sircar et al., 2007; Chen et al., 2008; Sircar and Mitra, 2008). Some phenolic acids used as carbon sources are able to stimulate the production of spores in *F. oxysporum* (Wu et al., 2009). We found that addition of *C. stevensii* B6 to the soil decreased the number of *F. oxysporum* colony forming units (CFUs), and the DNA of B6 could be detected in soil after 4 weeks (Xiao et al., 2012 a,b). Disease caused by *F. oxysporum* was the main factor leading to cropping obstacles (Wu et al., 2008; Wu et al., 2010 a,b). We observed in this study that C. *stevensii* B6 effectively degraded 4-HBA and mitigated its toxicity to watermelon. This degradation is the probable mechanism by which *C. stevensii* lessens the obstacles to continuous cropping.

In all three soils, *C. stevensii* B6 decomposed nearly 100% of the available 4-HBA within 7 days, much more efficiently than *Trichoderma harzianum,* which was shown to result in only 88% degradation within 8 days (Chen et al., 2011), suggesting that the endophytic fungus *C. stevensii* B6 could adapt to the soil environment and promote the degradation of 4-HBA. The concentration of 4-HBA in the control soil also decreased, indicating that other microbes in soil had the potential to utilize 4-HBA. Middelhoven et al. (1992) and Zhang et al. (2010) have screened *Pseudomonas* sp. and ascomycetous yeast strains from soil that effectively degrades phenolic compounds. However, in our study, the indigenous soil microorganisms did not completely degrade 4-HBA after 2 months, and the residual concentration of 4-HBA in the non-bioaugmented soil microcosms remained relatively high.

Creating conditions appropriate for the growth of *C. stevensii* in agricultural settings is very important. The degradation rate is likely to accelerate as temperatures rise. The maintenance of soil pH at approximately 5.5 would be suitable for the growth of *C. stevensii*, similar to the ideal pH for the rot fungus *Phanerochaete chrysosporium* (Bhattacharya et al., 2013). Therefore, if B6 were applied to acidic soil in subtropical China, the use of an alkaline fertilizer would be beneficial to adjust soil pH. In addition, as this is an aerobic fungus, moderate tillage during watermelon planting is also advisable to maintain soil oxygen. *C. stevensii* would be able to take advantage of starch, fructose, glucose, and sucrose as carbon sources. Watermelon root exudates are rich in carbohydrates (Hao et al., 2010) and conducive to the survival of *C. stevensii* in the soil. *C. stevensii* colonization of soil may be used to resolve 4-HBA. Increasing soil organic matter by the application of organic fertilizers would enhance the degradation of 4–HBA by *C. stevensii*. Inoculum size is best maintained at

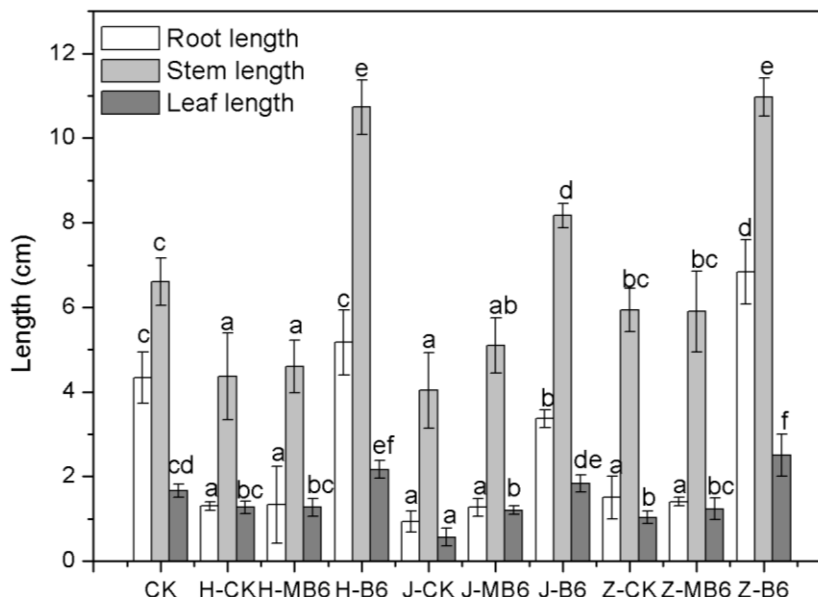

Figure 6. Effect of *C. stevensii* B6 on seedling growth inhibition by 4-HBA. The different letters represent the significance between pairs of mean values at P≤0.05 according to a Duncan test.

15% (v/v, volume of inoculation seed liquid to volume of the soil). In terms of cost, it should be noted that a 5% inoculum was sufficient after one week.

Following placement in the natural environment for 2 months, the original 4-HBA content in the soil decreased from 500 mg/kg to 4.92 mg/kg. This decrease may have been due to soil adsorption and transformation by indigenous microorganisms (Middelhoven et al., 1992; Zhang et al., 2010). Even after monocropping peanuts for 15 years, the 4-HBA content in the soil was only 2.76 mg/kg (Li et al., 2010). When the 4-HBA concentration reaches 4.92 mg/kg, the allelopathic effects on crop growth are likely to be very serious (Li et al., 2010). In nature, phenolic acids usually mix together, and the toxicity of the mixed chemicals is more significant than that of single chemicals such as 4-HBA (Li et al., 2010). In addition, although the level of phenolic acids in soils is not very high, it may be high in partial rhizosphere soil and may thus be toxic for plant growth. The degradation of 4-HBA from 4.92 mg/kg to zero is of key importance to agriculture.

In our experiment, the dry mycelial biomass applied to the soil reached 3.55 g/kg dry soil. In the field conditions, watermelons are planted in holes, and each hole contains approximately 15 kg of soil. If the watermelon line spacing is 2 m and the plant spacing is 1 m, the amount of dry mycelium needed is approximately 266 kg/ha. In the future, strategies to improve the adaptation of *C. stevensii* to soil conditions should be considered to further improve degradation efficiency and reduce microbial dosage. In addition, the efficiency of a *C. stevensii* symbiotic host in facilitating the degradation of 4-HBA warrants future investigation.

Conflict of interests

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

ACKNOWLEDGEMENTS

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REFERENCES

Aly AH, Debbab A, Kjer J, Proksch P (2010). Fungal endophytes from higher plants: A prolific source of phytochemicals and other bioactive natural products. Fungal Divers. 41(1):1-16.

Baziramakenga R, Leroux GD, Simard RR (1995). Effects of benzoic and cinnamic acids on membrane permeability of soybean roots. J. Chem. Ecol. 21:1271-1285.

Bhattacharya SS, Syed K, Shann J, Yadav JS (2013). A novel P450-initiated biphasic process for sustainable biodegradation of benzo [a] pyrene in soil under nutrient-sufficient conditions by the white rot fungus *Phanerochaete chrysosporium*. J. Hazard. Mater. 261:675-683.

Borges WS, Borges KB, Bonato PS, Said S, Pupo MT (2009). Endophytic fungi: natural products, enzymes and biotransformation reactions. Curr. Org. Chem. 13(12):1137-1163.

Chen LH, Yang XM, Raza W, Li JH, Liu YX, Qiu MH, Zhang F, Shen QR (2011). *Trichoderma harzianum* SQR-T037 rapidly degrades

allelochemicals in rhizospheres of continuously cropped cucumbers. Appl. Microbiol. Biotechnol. 89:1653-1663.

Chen X, Zhang J, Liu JH, Yu BY (2008). Biotransformation of p-, m-, and o-hydroxybenzoic acids by *Panax ginseng* hairy root cultures. J. Mol. Catal. B: Enzym. 54:72-75.

Chen Y, Xie XG, Ren CG, Dai CC (2013). Degradation of N-heterocyclic indole by a novel endophytic fungus *Phomopsis liquidambari*. Bioresour. Technol. 129(4):568-574.

Dai CC, Tian LS, ZhaoYT, Chen Y, Xie H (2010). Degradation of phenanthrene by the endophytic fungus *Ceratobasidum stevensii* found in *Bischofia polycarpa*. Biodegradation 21(2):245-255.

Dai CC, Yu BY, Wang XF, Jiang JH, Shi Y (2006). Identification of endophytic fungi killing pieris rapae and primary determination of its chemical matter. J. Anhui Agric. Sci. 34(4):694-697.

Hao WY, Ren LX, Ran W, Shen QR (2010). Allelopathic effects of root exudates from watermelon and rice plants on *Fusarium oxysporum* f.sp. niveum. Plant Soil 336:485-497.

Hu SL, Jia LY, Gao HP, Gao B, Xie J, Xu L (2011). The influences of different soil organic fractions on the sorption and bioavailability of phenanthrene. Acta Scientiae Circumstantiae 31(2):341-348.

Li PD, Wang XX, Li YL, Wang HW, Liang FY, Dai CC (2010). The contents of phenolic acids in continuous cropping peanut and their allelopathy. Acta Ecologica Sinica 30:2128-2134.

Lu RK (2000). Analytical methods of soil and agricultural chemistry. Beijing: China Agricultural Science and Technology Press.

Macias FA, Bastidas AO, Marin D, Castellano D, Simonet AM, Molinillo J (2004). Degradation studies on benzoxazinoids: Soil degradation dynamics of 2, 4-dihydroxy-7-methoxy-(2H)-1, 4-benzoxazin-3(4H)-one (DIMBOA) and its degradation products, phytotoxic allelochemicals from Gramineae. J. Agric. Food Chem. 52:6402-6413.

Middelhoven WJ, Coenen A, Kraakman B, Gelpke MDS (1992). Degradation of some phenols and hydroxybenzoates by the imperfect ascomycetous yeasts Candida parapsilosis and *Arxula adeninivorans*: evidence for an operative gentisate pathway. Antonie van Leeuwenhoek 62:181-187.

Ministry of Agriculture of the People's Republic of China (2011). Agriculture Statistical Yearbook of China (in Chinese) .China Agricultural Press, Beijing, p. 303.

Nie LC, Feng ZZ, Zhou Z (2007). Effects of benzoic acid and p-hydroxybenzoic acid on watermelon seed germination and seedling growth. Chin. Agric. Sci. Bull. 23:237-239.

Reis FS, Ferreira I, Barros L, Buelga CS, Martins A (2011). Mycorrhizal induction of phenolic compounds and antioxidant properties of fungi and seedlings during the early steps of symbiosis. Chemoecology 21:151-159.

Russell JR, Huang J, Anand P, Kucera K, Sandoval AG, Dantzler KW, Hickman D, Jee J, Kimovec FM, Koppstein D (2011). Biodegradation of polyester polyurethane by endophytic fungi. Appl. Environ. Microb. 77(17):6076-6084.

Sircar D, Mitra A (2008). Evidence for p-hydroxybenzoate formation involving enzymatic phenylpropanoid side-chain cleavage in hairy roots of *Daucus carota*. J. Plant Physiol. 165:407-414.

Sircar D, Roychowdhury A, Mitra A (2007). Accumulation of p-hydroxybenzoic acid in hairy roots of *Daucus carota*. J. Plant Physiol. 164:1358-1366.

Wang C, Xu GY, Ge CC, Mao ZQ (2009). Progress on the phenolic acid substances and plant soil sickness. North. Hortic. 3:134-137.

Wu H, Liu D, Ling N, Bao W,Ying R, Shen Q (2009). Influence of root eaudates of watermelon on *Fusarium oxysporum* f. sp. niveum. Soil Boil. Biochem. 73(4):1150-1156.

Wu HS (2008). Microbial ecology mechanisms on Fusarium wilt of watermelon and its biological control in fusarium-infested soil under long-term monoculture system. Ph.D. thesis, University of Nanjing Agricultural, Nanjing, China.

Wu HS, Luo J, Raza W, Liu YX, Gu M, Chen G, Hu XF, Wang JH, Mao ZS, Shen QR (2010a). Effect of exogenously added ferulic acid on *in vitro Fusarium oxysporum* f. sp. niveum. Sci. Hortic. 124:448-453.

Wu HS, Raza W, Liu DY, Wu CL, Mao ZS, Xu YC, Shen QL (2008). Allelopathic impact of artificially applied coumarin on *Fusarium oxysporum* f.sp. niveum. World J. Microbiol. Biotechnol. 24:1297-1304.

Wu HS, Shen SH, Han JM, Liu YD, Liu SD (2010b). The effect *in vitro* of exogenously applied p-hydroxybenzoic acid on *Fusarium oxysporum* f. sp. niveum. Phytopathol. Mediterr. 48:439-446.

Xiao Y, Dai CC,Wang XX, Liu FY,Wang HW. (2012b). The effect of endophytic fungus Ceratobasidum stenvensii B6 on *Fusarium oxysporum* in a continuously cropped watermelon field. Acta Ecologica Sinica 32(15):4784-4792.

Xiao Y, Wang XX, Wang HW, Liu FY, Dai CC (2012a). The effects of the addition of *Ceratobasidum stevensii* B6 and its growth on the soil microflora at a continuously cropped watermelon (*Citrullus lanatus*) site in China. Acta Ecologica Sinica, 32(4):1185-1192.

Yu JQ, Matsui Y (1997). Effects of root exudates of cucumber (*Cucumis sativus*) and allelochemicals on ion uptake by cucumber seedlings. J. Chem. Ecol. 3:817-827.

Zhang ZY, Pan LP, Li HH (2010). Isolation, identification and characterization of soil microbes which degrade phenolic allelochemicals. J. Appl. Microbiol. 108:1839-1849.

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