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

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

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Induction of chitinase, β -glucanase, and xylanase taken from *Trichoderma* sp. on different sources: A review

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Biological control by antagonistic organisms is a potential nonchemical tool for crop protection against phytopathogenic fungi. *Trichoderma* spp. are the most commonly used biocontrol agent that are being used against many soil borne pathogens. The biocontrol mechanism of *Trichoderma* involves mycoparasitism, antibiosis and competition for key nutrients. Chitinase, xylanase and β -glucanase are the major cell wall-degrading enzymes (CWDEs) that are involved in the mycoparasitic action. CWDEs improve the biocontrol activity of these strains. Different carbon sources are supplemented in the growth medium for the induction of these CWDEs.

Key words: Cell wall-degrading enzymes (CWDEs), mycoparasitic, phytopathogenic, *Trichoderma*.

INTRODUCTION

Traditional methods of crop protection include use of chemical pesticides. But the use of chemical pesticides has drastic effects on environment and consumers. Chemical pesticides are not beneficial for long term use as they pollute the environment, leave harmful residues in the soil and they also lead to the development of resistant strains. Use of biocontrol agents (BCAs) is the solution for this problem. The term biocontrol agent includes use of beneficial microbes and their products against pathogens. In present era there are number of BCAs that are available and commercialized in agriculture field against plant pathogens. To date, a number of BCAs are available as commercial products, as *Agrobacterium*, *Pseudomonas*, *Streptomyces* and

Bacillus, and fungal genera such as *Gliocladium*, *Trichoderma*, *Ampelomyces*, *Candida* and *Coniothyrium*. *Trichoderma* spp. are the most common soil fungi (Harman et al., 2004). These fungi are opportunistic, avirulent plant symbionts, and act as parasites and antagonists of many phytopathogenic fungi, such as *Sclerotonia*, *Pythium* etc. Thus, protecting plants from disease. *Trichoderma* spp. are among the most studied fungal BCAs and commercially marketed as biopesticides, biofertilizers and soil amendments (Harman, 2000; Harman et al., 2004; Lorito et al., 2004). *Trichoderma* provides numerous advantages: (i) colonization of the rhizosphere by the BCA ("rhizosphere competence") allowing rapid establishment within the

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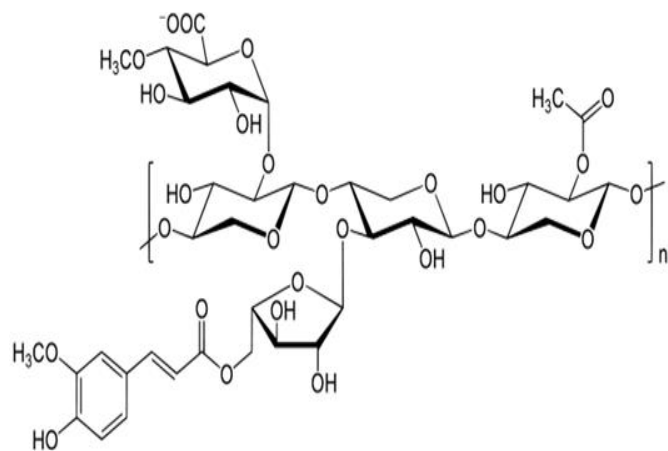


Figure 1. Structure of Xylan.

stable microbial communities in the rhizosphere; (ii) control of pathogenic and competitive/deleterious microflora by using a variety of mechanisms; (iii) improvement of the plant health and (iv) stimulation of root growth (Harman et al., 2004; Papavizas et al., 1985).

Several strains from the genus *Trichoderma* have been described as antagonistic fungi able to control a wide range of phytopathogenic fungi. The antifungal activity of *Trichoderma* involves production of antibiotics, including competition for key nutrients, and production of fungal cell wall-degrading enzymes (CWDEs) (Hjeljord and Tronsmo, 1998; Elad et al., 1982).

MYCOPARASITIC ACTION AND LYTIC ENZYMES

Process of mycoparasitic action includes several steps: Recognition of the host, attack and subsequent penetration and killing. During this process *Trichoderma* secretes CWDEs that hydrolyze the cell wall of the host fungus, subsequently releasing the cell wall oligomers (Kubicek et al., 2001; Howell, 2003; Woo et al., 2006).

The antifungal action of *Trichoderma* spp. includes a great variety of lytic enzymes such as proteases, xylanase, chitinase, glucanases (Lorito, 1998; Lorito et al., 1994a, 1996a), most of which play a great role in biocontrol (Harman and Kubicek, 1998; Baek et al., 1999; Carsolio et al., 1999; Woo et al., 1999; Zeilinger et al., 1999; Kullnig et al., 2000; Kubicek et al., 2001). These CWDEs isolated from the different strains of *Trichoderma* have been purified and characterized (Lorito, 1998). Interestingly, when tested alone or in combinations, the purified proteins showed antifungal activity against a variety of pathogens (that is species of *Rhizoctonia*, *Fusarium*, *Alternaria*, *Ustilago*, *Venturia* and *Colletotrichum*, as well as fungus like organisms such as the *Oomycetes Pythium* and *Phytophthora* which lack chitin in their cell walls (Tronsmo, 1991; Lorito et al.,

1993, 1994a).

The direct application of these secreted CWDEs produced by fungal BCAs, instead of using the whole "live" organisms, has numerous advantages in industry and agriculture. The selective production of these enzymes active can be increased by modifying the growth conditions, that is type and composition of culture medium, carbon sources, temperature of incubation and pH, etc. (Lorito and Scala, 1999; Lorito et al., 2006). The presence of different carbon sources, such as mono- or polysaccharides, colloidal chitin, or fungal tissues, has been shown to increase the secretion of CWDEs (Mach et al., 1999). Moreover, purified mixture of CWDEs showed improved antifungal effects against various plant pathogens (Lorito et al., 1994b, 1996a; Baek et al., 1999; Carsolio et al., 1999).

Xylanases

Xylanases (β -1,4-xylan xylanohydrolase, E.C.3.2.1.8) (Figure 1) are the major components of xylanolytic enzymes and randomly cleave the β -1,4-glycosidic bond of xylan backbone. Xylan is the second most abundant natural polysaccharide. Xylan is a heteropolysaccharide consisting of β -1,4-linked D-xylose monomers in connection with side branches of arabinosyl, glucuronosyl, acetyl, uronyl, and mannosyl residues (Tsujiibo et al., 1997). Complete degradation of xylan structures requires the concerted and synergistic function of several enzymes including endo- β -1,4-xylanases (EC 3.2.1.8) (Jun et al., 2009). Due to the broad applications in biopulping and biobleaching in paper industry, xylanase has attracted the attention of many researchers (Tsujiibo et al., 1997).

Xylanase production

Solid state fermentation (SSF) was carried out at 30°C with 50 ml of Vogel's medium with some modifications and 1% of birchwood xylan in 250 ml Erlenmeyer flasks in a BOD incubator. After eight days, the contents of the flasks were filtered through filter paper and the obtained culture broths were centrifuged 10 min at 3000 \times g supernatants were then assayed for extracellular xylanase activity.

For maximum xylanase production of, various culture conditions viz., carbon and nitrogen source, inoculum size, pH, temperature and incubation period were optimized by conventional "one variable at a time" approach which involves varying a single independent variable at a time while maintaining the others at a constant level. Fungal isolates were grown in different media containing 1% birchwood xylan, at pH 6.0 for 192 h. Medium giving maximum growth was further used to standardize the inoculum size by inoculating different

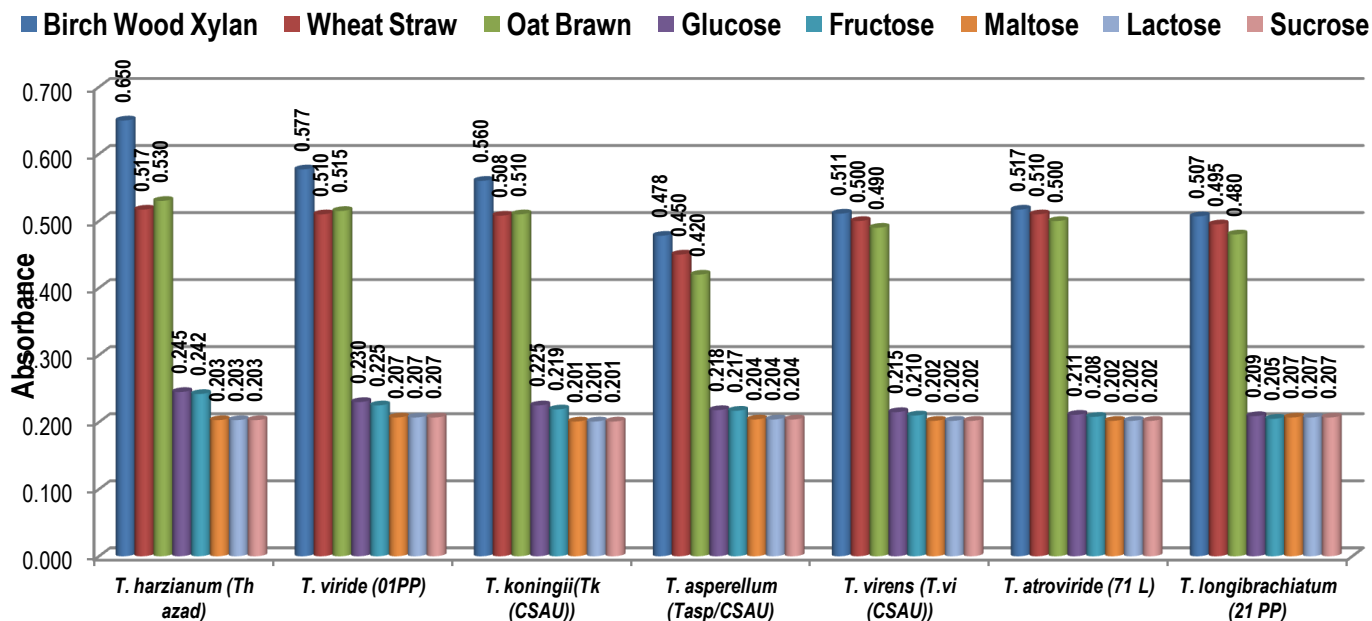


Figure 1. Effect of different carbon source on xylanase production by *Trichoderma* spp.

concentrations (1-5 ml) of inoculums. Similarly, the temperature and time of incubation were optimized by growing the fungal isolate at different temperatures (25-65°C) for different time periods (96-192 h). Different organic viz., yeast extract (YE), beef extract (BE), peptone (PPT), soybean residue (SR) and corn powder (CP) were supplemented separately to a final concentration of 0.3% (w/v) to study the microbial growth and xylanase activity. Glucose, maltose and sucrose at a final concentration of 0.1% and wheat bran (WB), corn cob (CC) birchwood xylan (BW) and carboxy methyl cellulose (CMC) were used as the carbon source at a final concentration of 1.0% (Figure 2). After 7 days of growth, the fungal broth was filtered through filter paper and centrifuged at 3000 × g for 15 min at 4°C in a refrigerated centrifuge. The supernatant taken as enzyme extract containing extracellular xylanase was used to assay the enzyme activity. The highest xylanase activity was shown by Birch wood xylan (Sonika et al., 2014)

Applications of xylanases

Besides the use of xylanase enzymes in antagonistic mechanism, the potential applications of xylanases also include the bioconversion of lignocellulosic material and agro-wastes into fermentative products, the clarification of juices, the improvement of the consistency of beer and the digestibility of animal feed stocks. The incorporation of xylanase from *Trichoderma longibrachiatum* into the rye-based diet of broiler chickens reduced intestinal viscosity, thus, improving both the weight gain of the chicks and their feed conversion efficiency. One of the most important biotechnological applications of xylanase

is its use in pulp bleaching.

Glucanase enzyme

β -1,3-glucanases are hydrolytic enzymes, widely distributed among bacteria, fungi and higher plants. β -1,3(1,6)-glucans are major components in cell wall of yeasts and fungi. In most yeast, especially *Saccharomyces*. *Trichoderma* spp. secretes β -1,3-glucanases when it is grown on polysaccharides, fungal cell walls.

Cellulose is the most abundant organic polymer on earth. Cellulases hydrolyze β -1,4- glycosidic bond in the cellulose structure and operate as a multicomponent enzyme system which consists of three classes of enzyme; Endoglucanases (endo- β -1,4-glucanase, CMCCase, EC 3.2.1.4), cellobiohydrolases (exoglucanase, avicelase, EC 3.2.1.91) and β -glucosidase (cellobiase, EC 3.2.1.21) (Dincer and Telefoncu, 2006). The first two enzymes are called as "real cellulose" and produce cellobiose and glucose by acting directly on cellulose. The cellobiose is then hydrolyzed into glucose by β -glucosidase (Dincer and Telefoncu, 2006; Andersen, 2007). Endoglucanases cleave the internal glycosidic bonds of cellulose chains and act synergistically with exoglucanase and β -glucosidase during the hydrolysis of crystalline cellulose. Also, endoglucanases are used in increasing the yield of fruit juices, beer filtration, and oil extraction, improving the nutritive quality of bakery products and animal feed, and enhancing the brightness, smoothness, and over all quality of cellulosic garments. Therefore, new fungal

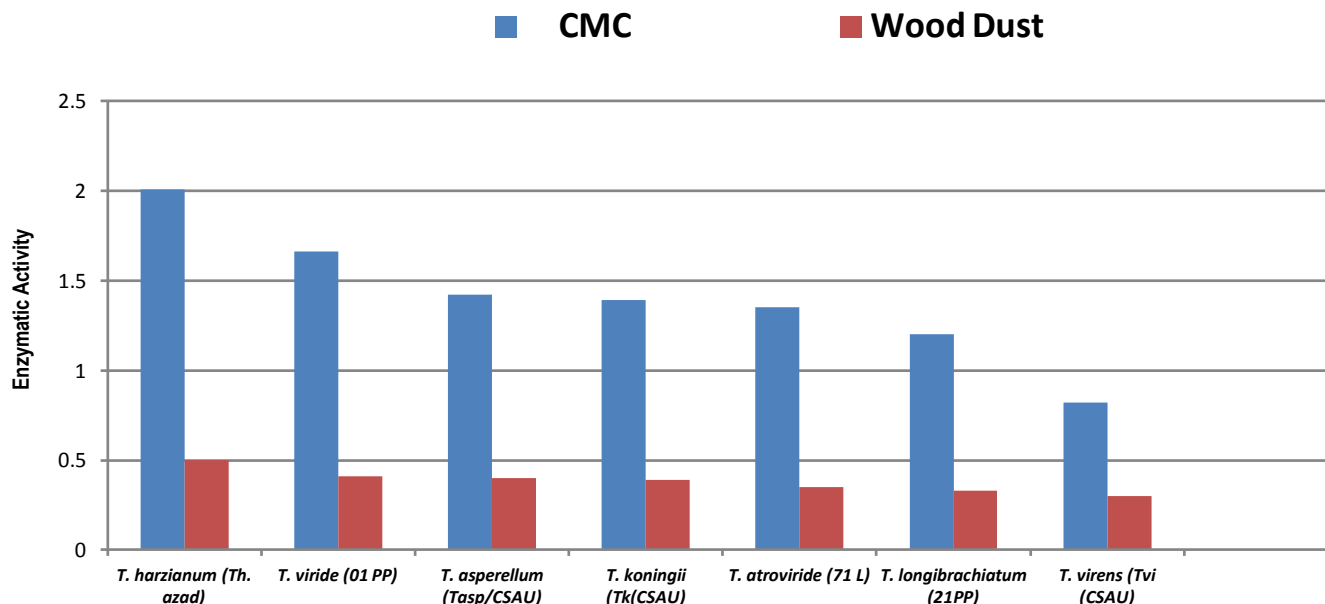


Figure 3. The effect of carbon sources on glucanase enzyme induction.

sources and properties (optimal pH and temperature) of EG must be investigated (Naika et al., 2007).

Glucanase enzyme production

Trichoderma sp. previously isolated from the different states of Uttar Pradesh were cultivated on Czapek Dox Medium containing CMC and wood dust as sole carbon source (1%) (Figure 3). Cultures were incubated for 10-14 days on orbital shaker at 150 rpm. At the end of the incubation time contents of conical flasks were filtered and the filtrate was centrifuged at 5000 rpm for 10 min. The clear supernatant was considered as the source of crude enzymes. The clear supernatant used as a source of crude enzyme was purified by the slow addition of Ammonium Persulfate with continuous stirring till 80% saturation. The obtained precipitate was dissolved in citrate phosphate buffer pH 5.0 and used for enzyme activity determination (Pandey et al., 2014).

Chitinase enzyme

Trichoderma are well known producer of chitinolytic enzymes and used commercially as a source of these proteins. Due to the importance of chitinolytic enzymes in insect, nematode, and fungal growth and development, they are receiving attention in regard to their development as biopesticides or chemical defense proteins in transgenic plants and microbial biocontrol agents. In this sense, biological control of some soil-borne fungal diseases has been correlated with chitinase production.

Fungi and bacteria producing chitinases exhibit antagonism against fungi, and inhibition of fungal growth by plant chitinases has been demonstrated. Insect pathogenic fungi have considerable potential for the biological control of insect pests.

Chitin, a homopolymer of β -(1, 4)-N-acetylglucosamine (GlcNAc) is the second most abundant source of nutrients and energy after cellulose (Agrawal and Kotasthane, 2012; Lorito et al., 2004; Tronsmo, 1991; Dincer and Telefoncu, 2006). It is widely distributed in the nature as the integuments of insects and crustaceans and as a component of fungi and algae (Baek et al., 1999; Hjeljord and Tronsmo, 1998). Chitinases are a group of enzymes that decompose chitin into a variety of products that include the deacylated oligomer chitosan, the disaccharide chitobiose and the monomer N-acetyl glucosamine (Dincer and Telefoncu, 2006; Pandey et al., 2014). The present study was aimed for screening the different *Trichoderma* species to select potent isolate, production and purification of chitinase followed by determination of its molecular weight (Saraswathi and Jaya, 2014).

For the evaluation of chitinases activity, two different insoluble chitin sources (colloidal chitin derived from commercial chitin and Seashells) were used. Chitinase detection medium consisted of a basal medium comprising (per liter) 0.3 g of $MgSO_4 \cdot 7H_2O$, 3.0 g of $(NH_4)_2SO_4$, 2.0 g of KH_2PO_4 , 1.0 g of citric acid monohydrate, 15 g of agar, 200 μ l of Tween-80, 4.5 g of chitin source and 0.15 g of bromocresol purple; pH used was adjusted to 4.7. Lukewarm medium was poured in Petri plates and allowed to solidify. Fresh culture plugs of the isolates to be tested for chitinase activity were inocu-

lated on the medium and incubated at 25±2°C and were observed for colored zone formation (Agrawal and Kotasthane, 2012). In our study, it was found that during the study the colloidal chitin derived from the commercial chitin was more potent in inducing chitinase enzyme activity as compared to the colloidal chitin derived from the sea- shells.

Conflict of Interests

The authors have declared that they have no conflict of interests.

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Full Length Research Paper

Scanning electron microscopy of biofilm formation by *Staphylococcus aureus* on stainless steel and polypropylene surfaces

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Attachment of bacteria to food processing equipment surfaces can lead to product contamination, spoilage, and surface destruction. Most of bacteria in their habitat live in communities of different degrees of complexity, in general composing biofilms. Once installed the biofilm, the resistance of these sessile organisms increases, making it difficult thus the action of sanitizers. This multidisciplinary study led to the description regarding the effect of antimicrobial solutions of essential oils of *Syzygium aromaticum* and *Thymus vulgaris* and their combination on biofilm formed by *Staphylococcus aureus* ATCC 25923 on AISI 304 stainless steel and polypropylene surfaces and evaluate the possible effects of these oils on *Staphylococcus aureus* cells after treatment with sanitizing solutions based on these natural compounds. All sanitizing solutions showed antibacterial potential, being effective in reducing bacterial biofilms on these surfaces. The solution containing the combination of essential oils was the most efficient by reducing 7.38 and 6.58 Log CFU.cm⁻² of cells adhered on the surfaces of AISI 304 stainless steel and polypropylene, respectively, after 5 min of contact.

Key words: Antimicrobial effect, microorganism, *Syzygium aromaticum*, *Thymus vulgaris*.

INTRODUCTION

Biofilms are surface-attached microbial communities found ubiquitously in almost any kind of environment. In the process of building a biofilm, bacterial cells form

aggregated microcolonies surrounded by thick extracellular polymeric substances (EPS) that include nucleic acids, proteins, lipids and polysaccharides.

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The EPS serves as the structural scaffold and offers protective shielding of biofilm bacteria, which dramatically increases bacterial resistance to sanitizers and antimicrobial agents. Biofilm formation is a dynamic process which in a sequential manner involves attachment, microcolony formation and maturation as well as dispersal (Tan et al., 2014).

Bacteria communicate via a phenomenon termed quorum-sensing (QS) in which they secrete and detect chemical signal molecules in their surrounding environment (Fuqua et al., 1994). Quorum-sensing allows a bacterial community to activate a coordinated cellular response such as virulence factor production, biofilm development and shielding (Alhede et al., 2009). Quorum-sensing has been implicated in food spoilage (Skandamis et al., 2012). As such, QS is a potential target for the prevention/eradication of biofilms in the food industry.

In the food industry, biofilm formation leads to serious health and economic losses due to food contamination and equipment damage. Biofilms can develop the most varied surfaces, and those most used in food production plants is the AISI 304 stainless steel (American Iron and Steel Institute) and polypropylene. The microorganisms present in the biofilm catalyze chemical and biological reactions causing corrosion of metal pipes and tanks, reducing heat transfer due to the thickness of the biofilm, among others (Shafahi and Vafai, 2009).

Among these microorganisms, *Escherichia coli* and *Staphylococcus aureus* are usually found in nature, their presence in food being the consequence of the often precarious sanitary conditions of food production, due to contaminated handling or surfaces. In the food industry, biofilm formation leads to serious hygiene problems and economic losses, mainly due to food contamination, spoilage and damage to equipment. Once established, biofilms act as points of constant contamination, releasing pathogenic and / or spoilage microorganisms (Boari et al., 2009).

Surveys have been documented involving food contact surfaces and various microorganisms, such as *Listeria monocytogenes*, *Yersinia enterocolitica*, *Campylobacter jejuni*, *E. coli* O157: H7, *S. aureus* and *Pseudomonas aeruginosa*, among others (Shi and Zhu, 2009).

In the quest for better understanding of the biofilm formation process on different surfaces, the search for, and research on sanitizing agents and antimicrobial alternatives should be generated. In this context, it has been observed that the essential oils found in condiment plant extracts have antibacterial, antifungal and antioxidant properties, which has aroused the interest of food industries (Di Pasqua et al., 2007; Kalemba and Kunicka, 2003; Lambert et al., 2001).

The inhibitory effect of these oils on microorganisms is an alternative to reduce the use of chemical additives in food and for the formulation of new sanitizing agents. Various studies have shown that essential oils extracted

from leaves and different parts of plant species have high antimicrobial activity (Bakkali et al., 2008; Gill and Holley, 2006; Burt, 2004; Lambert et al., 2001).

Given the above, the objective of this study was to evaluate the action of sanitizing solutions formulated with essential oils of clove (*Syzygium aromaticum*) and thyme (*Thymus vulgaris*) and their combinations, on bacterial biofilms formed by *Staphylococcus aureus* on AISI 304 stainless steel and polypropylene surface and evaluate the possible effects of these oils on *S. aureus* cells after treatment with sanitizing solutions based on these natural compounds.

MATERIALS AND METHODS

Microbiological analyzes were performed in the Food Microbiology Laboratory, Food Science Department, Universidade Federal de Lavras (UFLA), MG. The electron micrographs of the structure of mature biofilms and sanitized were obtained from scanning electron microscopy, from the Laboratory of Electron Microscopy and Ultrastructural Analysis, Plant Pathology Department, Universidade Federal de Lavras (UFLA), MG.

Microorganism used, standardization, inoculum preparation and storage

The microorganism used in the development of this study was *S. aureus* ATCC 25923. The culture of *S. aureus* was maintained at -18°C in microcentrifuge tubes with freezing medium [glycerol (150 mL), peptone (5 g), yeast extract (3 g), NaCl (5 g), H₂O (1.000 mL), pH 7.2 ± 7.4]. During the experiment, subcultures were made for the maintenance of cultures. Aliquots were transferred to the microcentrifuge tubes containing tryptic soy broth (TSB) and incubated at 37°C/24 h. After culturing, 1 mL of the culture was dispensed into sterile microcentrifuge tubes and centrifuged at 6,000 x g for 8 min in a microcentrifuge. After removing the supernatant, the content was again coated with freezing medium and stored at -18°C.

For reactivation and use of the strain, 10 µL of the culture was inoculated in tubes containing 3 mL of TSB and incubated at 37°C/24 h. After incubation, 20 µL of the inoculum was removed and transferred to 200 mL of TSB. The number of cells per millilitre was quantified using standard curve and the growth monitored by spectrophotometry at 600 nm and then counting on plates. The bacterial culture was standardized to a concentration of 10⁸ CFU/mL.

Experimental model of biofilm formation

Preparation and cleaning of coupons

The bacterial adhesion was conducted on AISI 304 stainless steel and polypropylene coupons with 1 mm thickness and dimensions of 10x20 mm.

The AISI 304 stainless steel coupons were cleaned individually with 100% acetone, submerged in detergent, rinsed with sterile distilled water, dried and cleaned with 70% ethanol (v/v). After cleaning, the coupons were again washed with sterile distilled water, dried for 2 h in oven at 60°C and autoclaved at 121°C/15 min (Rossoni and Gaylarde, 2000). As for the polypropylene coupons, they were initially immersed in a solution of commercial 0.3% peracetic acid for 30 min under stirring at 50 rpm at 50°C. They were then soaked in sterile distilled water at 80°C for 5 min and at

Table 1. Composition of the sanitizing solutions.

Sanitizing solution	Composition (mL)		
	Saline solution	Ethanol	Essential oil
Control	8.00	2.00	0.00
<i>S. aromaticum</i>	8.00	1.99	0.01
<i>T. vulgaris</i>	8.00	1.99	0.01
Combination*	8.00	1.99	0.005

*Combination of oils of *S. aromaticum* and *T. vulgaris* at a 1:1 proportion.

room temperature for 1 min under agitation of 50 rpm. The coupons were dried at 40°C for 2 h and autoclaved for 15 min at 115°C (Oulahal et al., 2008).

Adhesion of bacterial cells to surfaces

In two Petri dishes (140 mm diameter) 45 AISI 304 stainless steel coupons and 80 mL TSB were added and inoculated with 10^8 CFU/mL of culture. In two other Petri dishes the same procedures were employed, however, with 45 polypropylene coupons, with the aim of promoting the formation of biofilms on these surfaces. The plates were incubated at 37°C with orbital agitation of 50 rpm. At 48-h intervals, the coupons were collected, washed with peptone water (0.1% w/v) five times and immersed in TSB contained in sterile plates. This procedure was performed five times in order to complete the formation of the biofilm after 10 days of incubation (Joseph et al., 2001).

Enumeration of adhered cells

For enumerating the adherent cells, one AISI 304 stainless steel and one polypropylene coupon was removed from each Petri dish every two days of incubation. These were washed with peptone water (0.1% w/v) five times to remove planktonic cells and the sessile cells were collected using a standard sterile cotton swab. The swabs were transferred to tubes containing peptone water (0.1% w/v) and then agitated in a vortex for 2 min. After this procedure, serial dilution was carried out in which 0.1 mL aliquots were plated and the number of viable cells quantified in Tryptic Soy Agar (TSA), using the surface seeding technique. The plates were incubated at 37°C/24 h, conducting a standard plate count at the end of this period, results expressed in CFU/cm² (Joseph et al., 2001).

Obtention of essential oils

The essential oils of *S. aromaticum* and *T. vulgaris* were purchased through the Ferquima Ind. e Com. Ltda Company (Vargem Grande Paulista, São Paulo, Brazil); their physical and chemical parameters being described by the supplier, which produces and sells essential oils on an industrial scale.

Preparation of sanitizing solutions

To perform the sessile cell sensitivity test, four sanitizing solutions

were formulated containing saline (NaCl 0.85% w/v), ethanol (p.a. 95% v/v) and essential oil as shown in Table 1.

All sanitizing solutions contained a total volume of 10 mL and the amount of essential oils used in the formulation of the sanitizing solutions was defined from the Minimum Inhibitory Concentration (MIC) test results previously performed by disk diffusion technique (NCCLS, 2000 with modifications).

Treatment of biofilms with sanitizing solutions containing essential oils at different contact times

On the tenth day of cultivation, polypropylene and steel coupons were taken from each Petri dish, rinsed in 0.1% peptone water (v/v) five times to eliminate non-adherent cells, and immersed in the above sanitizing solutions for durations of 5 and 10 min at room temperature. After the sanitizing, the coupons were rubbed with standardized sterile swabs. The swabs were transferred to tubes containing 0.1% peptone water (v/v) and then agitated in a vortex for 2 min. After this procedure, serial dilution was conducted, 0.1 mL aliquots were plated and the number of viable cells determined in TSA medium, using the surface seeding technique. The plates were incubated at 37°C for approximately 24 h, conducting the standard plate count at the end of this period with, results expressed in CFU/cm² (Joseph et al., 2001; with adaptations).

View the surfaces of coupons by scanning electron microscopy

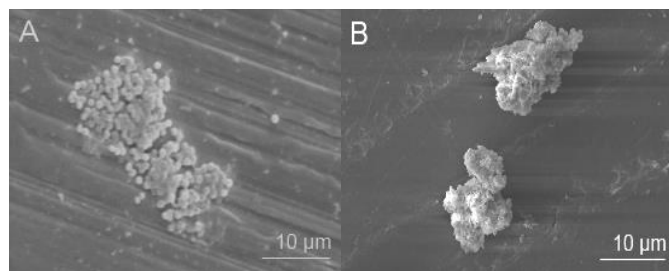
The coupons of stainless steel and polypropylene containing biofilm and sanitizers were immersed in fixative solution (modified Karnovsky), pH 7.2, for 48 h. The coupons were cleaned by soaking for 10 mins in cacodylate buffer, and this procedure was repeated three times and posteriorly were fixed in 1% osmium tetroxide in water for three hours. After this process, were washed three times in distilled water and dehydrated in ethanol gradient (25, 50, 70, 90, 95%, 10 min and 100% three times for 10 min). Next was the drying of this material in a critical point apparatus (Bal-Tec CPD 030), mounting stubs and covered with a thin gold layer (Bal-Tec SCD sputter 050). Electron micrographs of the bacteria adhered to the surfaces of stainless steel and polypropylene were obtained before and after treatment with sanitizing solutions, and a scanning electron microscope Leo 040 Evo (Alves, 2004).

Experimental design and statistical analysis

A completely randomized design (CRD) was used in a 2 x 5 factorial outline with 3 replicates, the surface factor having 2 levels: stainless steel and polypropylene, the time factor with five quantitative levels: 48, 96, 144, 192 and 240 h. The enumeration of adhered cells on the stainless steel and polypropylene coupons after treatment with these sanitizing solutions at different contact times, used the CRD in a factorial scheme (4 x 2 x 2) with three replicates with the factor agents at four qualitative levels: control, *S. aromaticum*, *T. vulgaris* and combination, the factor surfaces with two qualitative levels: stainless steel and polypropylene, and the time factor with two quantitative levels: 5 and 10 min. Statistical analyzes were performed using the SISVAR (Ferreira, 2003) and R Development Core Team programs (R Development Core Team, 2004).

Table 2. Identification and quantification of chemical constituents

Peak	Constituent ID	%
<i>Syzygium aromaticum</i>		
1	Eugenol	92.3
2	Acetileugenol	0.2
3	Caryophyllene	5.5
4	α -Humulene	0.9
<i>Thymus vulgaris</i>		
1	α -Pinene	2.2
2	Camphene	0.8
3	Myrcene	1.4
4	ρ -Cymene	26.8
5	1.8-Cineol	1.3
6	γ -Terpinene	6.0
7	Linalool	5.2
8	Camphor	1.5
9	Borneol	0.9
10	Thymol	47.3
11	Carvacrol	3.1
12	β -Caryophyllene	0.8

**Figure 1.** Scanning electron micrographs demonstrating adherence of *Staphylococcus aureus* after 240 h of biofilm formation, the surfaces stainless steel of AISI 304 (A) and polypropylene (B).

RESULTS AND DISCUSSION

Table 2 shows the counts of sessile cells adhered to the surfaces of the AISI 304 stainless steel and polypropylene coupons during the biofilm formation process.

The adhesion of bacterial cells depends on factors such as physiology and cell morphology and physico-chemical properties of the contact surface. Gram negative microorganisms have greater ease of adhesion on surfaces when compared to Gram positive, as they have pili, flagella and fimbriae, as well as an outer membrane. Microorganisms electrically charged with

negative charges have more difficulty to link directly to surfaces. The participation of a conditioning film formed by various compounds and molecules from the aqueous phase, will be decisive.

It can be observed that the microbial cells adhered similarly to both surfaces up to 192 h and differed significantly only at the end of the biofilm formation process that is at 240 h. The electron micrographs in Figure 1 illustrate cell adhesion of *S. aureus* on surfaces stainless steel of AISI 304 and polypropylene.

The adhesion of bacterial cells depends on factors such as physiology and cell morphology and physico-chemical properties of the contact surface.

The ability of *S. aureus* to adhere to solid surfaces producing compounds by multilayered cells embedded in a exopolysaccharide matrix is considered one of the relevant aspects of the epidemiology of this bacterium (Cucarella et al., 2001; Flach and Karnopp, 2005). This organization is extremely beneficial to all species of microorganisms, because it provides protection against adversity such as dehydration, colonization by bacteriophages and antimicrobial resistance (Gilbert et al., 2003).

By the phenomenon known as passivation, chromium, due to its high affinity for oxygen, tends to combine with it, forming a thin layer of chromium oxide with an approximate thickness of 40Å. This passive layer is responsible for the corrosion resistance and the hydrophobicity of stainless steel.

In this context, in the case of initial adhesion, the more hydrophobic the bacterial cell, the greater its ability to bind directly to this surface. Similar considerations were observed by Meylheuc et al. (2006) and Sheng et al. (2007).

Thus, surfaces considered hydrophobic, such as stainless steel, allow the adhesion to occur more easily than less hydrophobic or hydrophilic surfaces, which is evidenced by counts and electron micrographs, which show improved adhesion of the cells on the surface of stainless steel at the end of 240 h incubation compared to the polypropylene surface.

In studies conducted by Boari et al. (2009), that consisted of evaluating *S. aureus* biofilm formation on stainless steel using milk as substrate and different growing conditions, biofilm formation by *S. aureus* was observed by scanning electron micrographs in all conditions tested, revealing the adhesion ability of this bacterium mainly to the stainless steel surface, which was also observed in electron micrographs of the present study.

In a review by Chmielewski and Frank (2003) it is shown that a layer of organic matter on the surface can promote and facilitate bacterial adhesion. Moreover, these authors state that the time of contact between cells and surfaces also influence the bacterial adhesion. The

Table 3. Number of sessile cells (Log CFU.cm⁻²) of *Staphylococcus aureus*, quantified during biofilm formation on of AISI 304 stainless steel and polypropylene surfaces.

Time (h)	Stainless steel (Log CFU.cm ⁻²)	Polypropylene (Log CFU.cm ⁻²)
48	6.13 ± 0.42 ^a	6.13 ± 0.54 ^a
96	6.17 ± 0.33 ^a	6.12 ± 0.48 ^a
144	6.57 ± 0.44 ^a	6.56 ± 0.49 ^a
192	6.89 ± 0.17 ^a	6.64 ± 0.49 ^a
240	7.38 ± 0.12 ^b	6.58 ± 0.05 ^a

**Results are expressed as mean ± standard deviation. Means followed by different letters in the same line differ by the Tukey test at 5% probability.

irreversible cell adhesion to surfaces occurs between 20 min and a maximum of 4 h of contact. After this period the removal of these cells requires the application of physical force, chemicals or heat. In this present study it is possible to observe that the bacterial cells have obtained adhesion to the stainless steel and polypropylene surfaces from 48 hand increasing, to a small extent, up to 240 h.

The probability of cells remaining irreversibly attached after sanitation procedures is high and corresponds to one of the main reasons for the formation of biofilms on surfaces that come into contact with food, becoming a constant source of contamination.

Table 3 presents the counts of sessile cells adhered to the surfaces of the AISI 304 stainless steel and polypropylene coupons after treatment with the control sanitizing solution and the essential oil-based sanitizing solutions. Table 4 shows the reduction percentage of the number of sessile cells after treatment with the sanitizing solutions.

The effectiveness of the sanitizing solutions containing essential oils can be observed by the counts obtained after treatment of coupons on both surfaces and the reduction percentage of these cells. A significant difference in the counts and reduction percentage of adhered sessile cells can be noted among the different treatments (Table 5). All sanitizing solutions based on essential oils showed more superior antimicrobial activity than the control sanitizing solution.

The effectiveness of the sanitizing solutions based on *S. aromaticum*, *T. vulgaris*, and their combination differ significantly from each other, their combination being the most effective to reduce the number of sessile cells adhered to the surfaces. It can be observed that the 5 min exposure of the coupons containing the biofilm to the sanitizing solution based on the combination of oils was effective to promote non-recovery of viable cells adhered to both surfaces. Scanning electron micrographs of

Figure 2 show a reduction of *S. aureus* cells on surfaces stainless steel of AISI 304 and polypropylene.

The sanitizing solution based on *T. vulgaris* was more effective compared to *S. aromaticum*. This solution allowed the non-recovery of viable cells after exposure for 10 min to both surfaces. The sanitizing solution based on the essential oil of *S. aromaticum* was less effective, presenting a reduction in the number of sessile cells, but after 10 min of stainless steel and polypropylene coupon exposure to this solution, viable cells were still recovered.

As *S. aureus* is Gram positive, it is concluded that the cell wall does not serve as a barrier to the entrance of such antibacterial compounds through the cytoplasmic membrane. Since the cell wall of these bacteria is permeable, usually it does not restrict the penetration of these sanitizing agents (Schaffer and Messner, 2005).

The difference between the performance of the sanitizing solutions within each phase of biofilm formation analyzed can be attributed to environmental and growth factors that are related to the concentration and nature of the chemical constituents, such as composition, functional groups and the structural configuration of the essential oil components (Chang et al., 2001).

Marques et al. (2007) show in their work that *S. aureus*, when in the form of biofilm, becomes more resistant to sanitizing agents.

The effects of colonization of surfaces where food is processed can result in various problems, been of an economic or public health nature. On the economic front, spoilage bacteria can contaminate food by changing its characteristics and resulting in economic losses. The risk to public health is the most serious problem, because the biofilm can transport pathogenic microorganisms and be a source of chronic contamination (Ribeiro-Furtini, 2005).

This multidisciplinary study led to the description of the sanitizing solutions essential oils of *S. aromaticum* and *T. vulgaris* and their combination on biofilm formed by *S. aureus* (ATCC 25923) on AISI 304 stainless steel and polypropylene surfaces. All solutions showed potential antibacterial sanitizers, being effective in reducing bacterial biofilms on these surfaces. The solution containing the combination of essential oils was more efficient by reducing 7.38 and 6.58 Log CFU.cm⁻² cells adhered on the surfaces of AISI 304 stainless steel and polypropylene respectively, after 5 min of contact.

For the analysis of scanning electron micrographs obtained, were observed structural changes on the cell surface. Probably, these changes were caused by the action of essential oils in the cytoplasmic membrane, resulting in increased permeability and extravasation of intracellular constituents.

Studies by Oliveira et al. (2010) revealed, by means of electron micrographs of transmission electron microscopy, alterations in the cell wall of *Clostridium perfringens*,

Table 4. Number of *Staphylococcus aureus* sessile cells (Log CFU.cm⁻²) quantified on of AISI 304 stainless steel and polypropylene surfaces at 240 h of biofilm formation after treatment with the control sanitizing solution and the essential oil-based sanitizing solutions.

Sanitizing agent	Surfaces and exposure times			
	Stainless steel (Log CFU.cm ⁻²)		Polypropylene (Log CFU.cm ⁻²)	
	5 min	10 min	5 min	10 min
Control	7.09±0.06 ^{dB}	6.68± 0.27 ^{cα}	6.32± 0.0 ^{dA}	6.23± 0.03 ^{cα}
<i>S. aromaticum</i>	5.25± 0.50 ^{CB}	4.23± 0.36 ^{bα}	4.58± 0.71 ^{cA}	4.27± 0.57 ^{bα}
<i>T. vulgaris</i>	3.89± 0.62 ^{bA}	0± 0.00 ^{aα}	3.47± 0.01 ^{bA}	0± 0.00 ^{aα}
Combination	0± 0.00 ^{aA}	0± 0.00 ^{aα}	0± 0.00 ^{aA}	0± 0.00 ^{aα}

*Results expressed as mean sessile cell counts (Log CFU.cm⁻²). Means followed by different lowercase latin letters in the same column differ by Tukey test at 5% probability, means followed by different uppercase latin letters on the same line differ by Tukey test at 5% probability; means followed by different Greek letters on the same line differ by Tukey test at 5% probability.

Table 5. Reduction of the number of *Staphylococcus aureus* cells (Log CFU.cm⁻²) and in percentage, quantified on the AISI 304 stainless steel and polypropylene surfaces, at 240 h of biofilm formation after treatment with the control sanitizing solution and essential oil-based sanitizing solutions.

Sanitizing agent	Surfaces and Exposure time			
	Stainless steel (Log CFU.cm ⁻²) (%)		Polypropylene (Log CFU.cm ⁻²) (%)	
	5 min	10 min	5 min	10 min
Control	0.29 - 3.9 ^{aA}	0.70 - 9.4 ^{aα}	0.26 - 3.9 ^{aA}	0.35 - 5.3 ^{aα}
<i>S. aromaticum</i>	2.13 - 28. ^{bA}	3.15 - 42.6 ^{bβ}	2.00 - 30.3 ^{bA}	2.31 - 35.1 ^{bα}
<i>T. vulgaris</i>	3.49 - 47.2 ^{cA}	7.38 - 100 ^{cβ}	3.11 - 47.2 ^{cA}	6.58 - 100 ^{cα}
Combination	7.38 - 100 ^{dB}	7.38 - 100 ^{cβ}	6.58 - 100 ^{dA}	6.58 - 100 ^{cα}

*Values obtained from subtracting the number of adhered cells without any sanitizing treatment of stainless steel (7.38 Log CFU.cm⁻²) and polypropylene coupons (6.58 Log CFU.cm⁻²), at 240 h of biofilm formation after treatment with the control sanitizing solution and the essential oil-based sanitizing solutions. Means followed by different lowercase Latin letters in the same column differ by Tukey test at 5% probability, means followed by different uppercase Latin letters on the same line differ by the Tukey test at 5% probability; means followed by different Greek letters in the same line differ by the Tukey test at 5% probability.

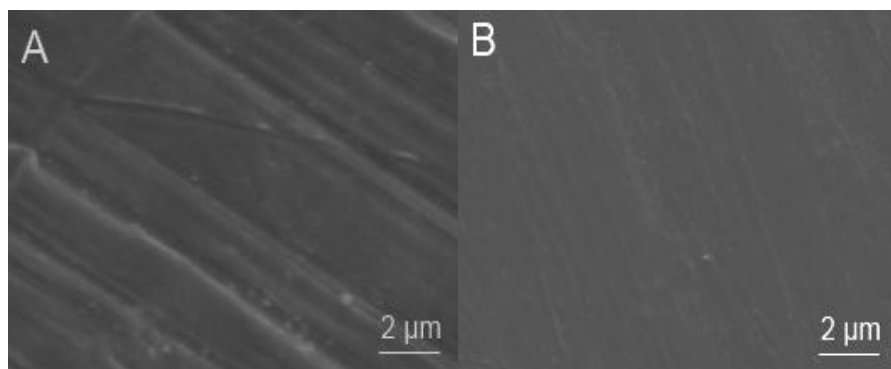


Figure 2. Scanning electron micrographs showing a reduction of *Staphylococcus aureus* cells on the surfaces of AISI 304 (A) stainless steel and polypropylene (B).

when it was treated with essential oil of *Satureja montana*. The electron micrographs of cells not exposed to the culture that oil had continuous, smooth and thin cell

walls and cell structures were defined. Already electron micrographs of cells that were exposed to treatment with the oil showed morphological changes in the wall as

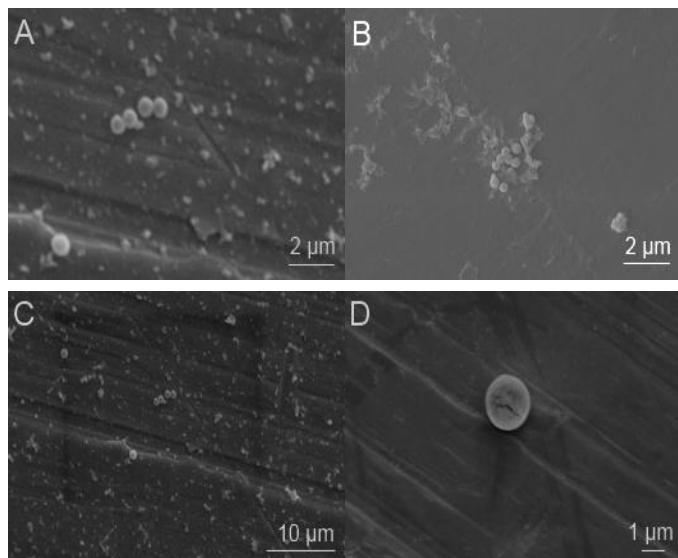


Figure 3. Electron micrographs scan demonstrating structural changes caused by the action of essential oils of *Syzygium aromaticum* (A and C) and *Thymus vulgaris* (B and D) in the cells of *Staphylococcus aureus* on the surfaces stainless steel of AISI 304 (A, C and D) and polypropylene (B).

roughness and irregularities, leading to rupture of the wall and subsequent cell lysis.

Furthermore, binding and clustering of intracellular material in the cytoplasm of the cells were observed because the electron micrographs revealed that these cells lacked cytoplasm in certain regions due to functional loss of the membrane caused by the action of the chemical components of *S. montana*.

Damage in cells of *C. perfringens* were also observed by Si et al. (2009), evaluated the effect of different essential oils in the morphology of bacterial cells by scanning electron microscopy, where occurred formation of cracks in the cell walls of the cultures treated with essential oils, which can also be observed in this study (Figure 3D).

The scanning electron micrographs below (Figure 3) reveal structural alterations probably caused by the action of essential oils *S. aromaticum* and *T. vulgaris* cells of *S. aureus* on surfaces stainless steel of AISI 304 and polypropylene.

Conflict of Interests

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

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Full Length Research Paper

Effect of N-acyl homoserine lactones (AHLs) quorum sensing signal molecules on *Enterococcus faecalis* biofilm formation

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The Gram-positive bacterium *Enterococcus faecalis* is an etiological agent of endodontic infections in oral biofilms. In the present study, we analyzed the effect of N-acyl homoserine lactones (AHLs) quorum sensing signal molecules on *E. faecalis* biofilm formation ability. Our results provide important effects of N-Hexanoyl-L-homoserine lactone (C6-HSL) and N-octanoyl-L-homoserine lactone (C8-HSL) molecules on *E. faecalis* biofilm formation. These AHLs molecules promoted the biofilm formation of *E. faecalis*.

Key words: N-acyl homoserine lactones (AHLs), bacterial communication, biofilm, *Enterococcus faecalis*, quorum sensing.

INTRODUCTION

Several species of bacteria employ a mechanism of intercellular communication known as quorum sensing (QS). This signaling process allows the cells comprising a bacterial colony to coordinate their gene expression in a cell-density dependent manner (Fuqua et al., 2001; Bassler and Losick, 2006). Thus, quorum sensing enables the cells within a bacterial colony to act cooperatively, facilitating population-dependent adaptive behavior (Atkinson and Williams, 2009). Quorum sensing has been shown to play a critical role in pathogenic

bacteria-host interactions (Boyer and Wisniewski-Dyé, 2009).

Bacterial intercellular communication relies on autoinducers (AI), endogenous chemical compounds which are synthesized intracellularly throughout the growth of the bacteria and secreted into the environment. Some AI are bound to the membrane receptors located on the cell surface, while others may penetrate the membrane and bind to the intracellular receptors. After binding with the receptors, AI activate a broad spectrum

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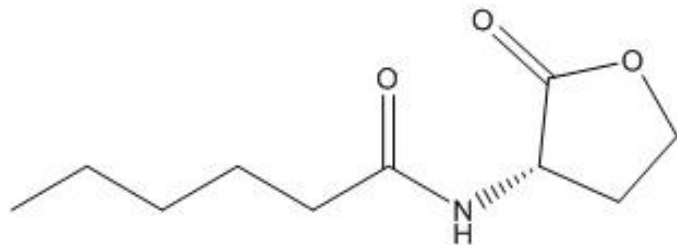


Figure 1. The biochemical structure of C6-HSL.

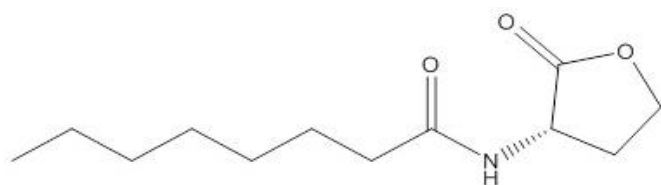


Figure 2. The biochemical structure of C8-HSL.

of effector systems affecting the regulation of expression of a number of genes, including those encoding the synthesis and secretion of antibiotics, virulent factors, and formation of cell aggregates or surface biofilms (Whitehead et al., 2001; Sun et al., 2004; Waters and Bassler, 2005; Bassler and Losick, 2006; Camilli and Bassler, 2006; Steindler and Venturi, 2007).

The most widespread and important autoinducers are lactones of *N*-acylated homoserine (AI-1, AHL) (Galloway et al., 2011). AHLs are the most common class of autoinducer used by Gram-negative bacteria; indeed quorum sensing mediated by AHLs represents one of the best-understood bacterial systems at the molecular level (Geske et al., 2008).

Cyclic peptides are the major class of cell-cell signaling molecules in Gram-positive bacteria (Duan et al., 2003; Xavier and Bassler, 2003; Vendeville et al., 2005; De Keersmaecker et al., 2006). Furthermore, small-molecule effects can be controlled by altering the concentration of the small molecule, thereby allowing the generation of dose-response data (Geske et al., 2008; Galloway et al., 2011).

E. faecalis is a Gram-positive cocci; facultative anaerobic bacteria that is known to be the most frequently detected species in root canals with failed endodontic treatment (Wang et al., 2011). It has been reported that the quorum sensing system in *E. faecalis* is closely related to the biofilm formation (Lu et al., 2013). However, the effect of quorum sensing signal molecules *N*-Acyl homoserine lactones (AHLs) produced only by Gram-negative bacteria on biofilm formation of Gram-positive oral bacteria is not yet investigated and not well understood. The objective of this study was to investigate the effect of a quorum sensing signal molecules AHLs

produced by Gram-negative bacteria on biofilm formation of a Gram-positive bacterium which is *E. faecalis* recovered from root canals of patients with endodontic infections.

MATERIALS AND METHODS

Bacterial strain, growth conditions and identification

E. faecalis strain was isolated from clinical samples of teeth of patients with endodontic infections in the dental surgery service of the University Hospital of Annaba (Algeria). *E. faecalis* was grown anaerobically (BD Gas Pak Anaerobe Generating Container System) at 37°C on M17 agar plates. M17 broth medium (Sigma-Aldrich) supplemented with 1% glucose was used for broth cultures (Kristich et al., 2004). Isolates, which were suspected as *Enterococcus* were immediately Gram stained to verify that they were Gram-positive, and analyzed for catalase-negative test (it is generally agreed that the genus *Enterococcus* comprises Gram-positive cocci that are catalase negative, usually facultative, anaerobic bacteria that grow in 6.5% NaCl, 40% bile salts. They grow at 10 and 45°C and can resist 30 min at 60°C) (Schleifer and Kilpper-Bälz, 1984; Schleifer and Kilpper-Bälz, 1987; Leclerc et al., 1996; Manero and Blanch, 1999). Isolates were identified to species level according to the standard biochemical tests using the API 20Strep system (bioMérieux, USA) for the identification of *E. faecalis* strain (Winston et al., 2004; Zoletti et al., 2006). The identification of *E. faecalis* strain was also confirmed using the mass spectrometry (MALDI-TOF) analysis.

AHLs molecules

The AHLs quorum sensing signal molecules used in this study were purchased from Sigma-Aldrich (Spain); they are commercially available. In the present study, we will investigate the effect of two AHLs molecules: *N*-hexanoyl-L-homoserine lactone (C6-HSL) (Figure 1) and *N*-octanoyl-L-homoserine lactone (C8-HSL) (Figure 2) on biofilm formation of the Gram-positive bacterium *E. faecalis*. The concentration of each AHL molecule added to the M17 liquid culture of *E. faecalis* was 1 µL.

Biofilm formation

An automatic microplate reader was used to investigate the biofilm formation ability of *E. faecalis* strain. The microplates for biofilm cultivation were sterile 96-well polystyrene (Sterilin Limited, U.K). The inoculum was prepared from *E. faecalis* grown in M17 broth supplemented with 1% glucose (weight/volume), and 200 µL is poured into the well (Christensen et al., 1985; Kennedy and O'Gara, 2004; Stepanović et al., 2007). The negative control wells (NC) (without AHLs) contain only 100 µL of *E. faecalis* culture in M17 broth supplemented with 1% glucose (initial optical density OD₆₀₀ at 600 nm was OD₆₀₀ = 0.2 nm) + 100 µL of fresh and sterile M17 broth supplemented with 1% glucose per well. The positive control wells (PC1 and PC2) (with AHLs) contained 100 µL of *E. faecalis* culture in M17 broth supplemented with 1% glucose (OD₆₀₀ = 0.2 nm) + 99 µL of fresh and sterile M17 broth supplemented with 1% glucose + 1 µL of the AHL molecule used (C6-HSL and C8-HSL) respectively per well (the positive control PC1 with C6-HSL; and the positive control PC2 with C8-HSL). The inoculated microplate was covered with a lid and incubated for 48 h of growth at 37°C, anaerobically using BD Gas Pak Anaerobe Generating Container System. After incubation, the wells in the microplate were emptied

from the liquid culture of *E. faecalis*, then washed three times with a sterile saline solution of (NaCl 0.9 % weight/volume).

After washing, the remaining attached bacteria should be heat-fixed (drying completely) by exposing them to hot air at 56°C for 30 min; then the adherent biofilm layer formed on the bottom and walls of the well was stained; 200 µL of crystal violet 0.1% per well for 30 min at room temperature (Seneviratne et al., 2013). After staining, the wells were rinsed off by placing the microplate under running water. Washing was continued until the washings were free of the stain. Then, the microplate was air dried at room temperature. Then, the dye bound to the cells were resolubilized, that is eluted from attached cells with 95% ethanol (200 µL per well); ethanol was gently added and thereafter the microplate was covered with the lid (to minimize evaporation) and left at room temperature for at least 30 min without shaking (Branda et al., 2005). Finally, the optical density (OD) of each well stained with crystal violet was measured at 570 nm using the automatic microplatereader. Optical density at 570 nm (OD₅₇₀) was measured to assess biofilm formation ability by the *E. faecalis* strain in each well. Then, the average in each lane (8 wells) of the microplate was calculated. This protocol was repeated three times to confirm the effect of AHLs used on *E. faecalis* biofilm formation.

RESULTS

Bacterial strain, growth conditions and identification

Mass spectrometry (MALDI-TOF) allowed the identification of *E. faecalis* strain by analysis of its protein content and study of ionic species moving in electromagnetic fields. The ions generated from the bacterial colony of *E. faecalis* spread on the steel metallic target, were separated according to their time of flight (TOF). It means the measurement of the time that ions took when they were subjected to an accelerating voltage to travel the length of the flight tube. The separation of ions depends mainly on their mass. For each group of ions that have the same ratio *m/z* (mass/charge), a signal was recorded in the detector as a time/intensity function. Thus, the set of peaks constituted a recorded mass spectrum. The spectra generated from the whole bacteria were then compared to reference spectra present in the data base of an expert system. Comparison of the spectrum of our strain *E. faecalis* (previously identified by the standard biochemical test using API 20 Strep system) was obtained from the mass spectrometer with thousands of reference spectra. The name of the bacterium displayed on the screen was: *E. faecalis*. The spectrum of *E. faecalis* was obtained after placing the plate containing the whole bacterial cells in the spectrometer.

Quantification of *E. faecalis* biofilm formed on polystyrene

The results of the average of optical density at 570 nm (OD₅₇₀) obtained were: In the negative control wells NC (without AHLs) OD₅₇₀ = 1.1 nm; in the positive control wells PC1 (with 1 µL of C6-HSL molecule) OD₅₇₀ = 1.9 nm; in the positive control wells PC2 (with 1 µL of C8-

HSL molecule) OD₅₇₀ = 1.7 nm.

The results mentioned above have shown that the addition of 1 µL of the AHLs molecules tested in this study (C6-HSL or C8-HSL) to the culture of *E. faecalis* in M17 broth supplemented with glucose 1% promoted the biofilm formation ability of *E. faecalis* strain (Figure 3). The values of the optical density at 570 nm (OD₅₇₀) obtained in the negative and positive controls were the same in the three repetitions.

DISCUSSION

The *E. faecalis* bacteria have been identified as the most commonly recovered species from teeth with persistent endodontic infections (Martos et al., 2013). *E. faecalis* has also been implicated in persistent root canal infections (Preethee et al., 2012). Anaerobic bacteria, especially Gram-negative species of *Prevotella* and *Porphyromonas*, are frequently associated with signs and symptoms of endodontic origin (Haapasalo, 1989; Sundqvist et al., 1989; Hashioka et al., 1992), and facultative species such as *E. faecalis* have been isolated in high frequency from refractory cases (Hancock et al., 2001). The cell-to-cell communication of microorganisms is known to be via exertion of certain chemical compounds (signal molecules) and is referred to as quorum sensing. QS phenomenon is widespread in microbial communities (Safari et al., 2014). It is known that bacteria utilize a quorum sensing system to coordinate gene expression by monitoring the concentration of molecules known as AI (Shao et al., 2012). Bacteria use the QS mechanism to regulate a variety of phenotype, such as biofilm formation which is essential for the successful establishment of a symbiotic or pathogenic relationship with eukaryotic host cell (Myszka and Czaczyk, 2012). AHL-QS systems produce and respond to AHLs using two proteins that mediate signal production and response, LuxI and LuxR-like proteins, respectively (Nealson et al., 1970; Ruby, 1996). LuxR-like proteins are response regulators that mediate the expression of genes required for communal behavior in response to intracellular concentrations of cognate AHLs molecules (Fuqua and Winans, 1994; Fuqua et al., 1996; Cude and Buchan, 2013). Some bacterial pathogens are capable of colonizing infection sites and employing N-acyl homoserine lactones (AHLs) based quorum-sensing systems to co-ordinate biofilm formation (Bhargava et al., 2012). In the present study, we confirmed that the AHLs quorum sensing signal molecules (C6-HSL and C8-HSL) promoted the biofilm formation of the Gram-positive bacterium *E. faecalis* isolated from endodontic infections.

In 2012, a study realized in Beijing (China) at the institute of disease control and prevention, academy of military medical sciences, reported that the quorum sensing system in *E. faecalis* is based on AI-2 molecule.

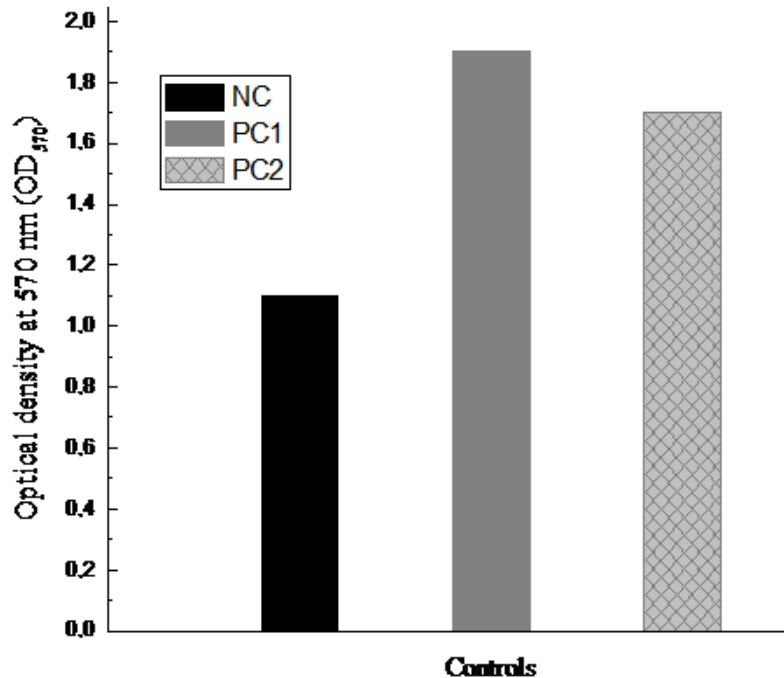


Figure 3. Values of optical density at 570 nm (OD₅₇₀) for *E. faecalis* biofilm formation on polystyrene obtained in negative and positive controls. NC, negative control (without AHLs). PC 1, positive control with 1 µL of C6-HSL; PC 2, positive control with 1 µL of C8-HSL.

Further, the role of AI-2 in biofilm formation by *E. faecalis* was investigated, showing that the addition of AI-2 molecule to *E. faecalis* V583 cultures resulted in increased biofilm formation (Shao et al., 2012). Thus, the quorum sensing system in *E. faecalis* is closely related to the biofilm formation of this bacterium (Lu et al., 2013). From the results obtained in the present study, we suggest that the *N*-Acyl Homoserine Lactones AHLs molecules produced by Gram-negative bacteria can interfere with the quorum sensing system of a Gram-positive bacterium which is *E. faecalis* and consequently regulate the biofilm formation ability in *E. faecalis* strain. This suggestion confirms the possible interspecies communication between Gram-positive and Gram-negative bacteria.

Mature dental biofilms consist of towering microcolonies in which the resident bacterial cells interact with one another and exchange messages in the form of signaling molecules and metabolites. It is evident that mutually beneficial interactions between microbial cells are essential to the development of biofilms in the oral cavity (Jakubovics, 2010). Thus, the presence of Gram-negative bacteria in oral biofilms can influence the biofilm formation and growth of Gram-positive bacteria like *E. faecalis* by producing quorum sensing signal molecules (AHLs) and consequently promoting the development of endodontic infections.

In 2010, a study realized in Osaka (Japan) at the

Department of Restorative Dentistry and Endodontology, Osaka University graduate school of dentistry, reported that three synthetic *N*-acyl homoserine lactones analogues (*N*-acyl HSL) inhibited the biofilm formation in the Gram-negative bacterium *Porphyromonas gingivalis* which is a primary agent of periodontal disease in oral biofilms (Asahi et al., 2010). Thus, the effects of *N*-acyl homoserine lactones molecules on bacterial biofilm formation are different depending on the Gram-positive or Gram-negative bacteria (Figure 4).

These different effects of the autoinducer molecules named AHLs on bacterial biofilm formation of the Gram-positive strain *E. faecalis* and the Gram-negative strain *P. gingivalis* confirms that the difference in biochemical composition of the bacterial cell wall between Gram-positive and Gram-negative bacteria plays a critical role in recognition and binding of the autoinducer molecule and also in the resulting effects of this molecule. The biochemical composition of the cell wall of *E. faecalis* strain has shown that the cell was endowed with a wall resistant to mechanical disruption, peptidoglycan, penicillin binding proteins (PBPs), muramidase enzyme and accessory wall polymers such as teichoic acid and lipoteichoic acid (Signoretto et al., 2000). The biochemical composition of cell-wall of Gram-positive bacteria has shown a very high proportion of the amino-acids such as: alanine, glutamic acid, lysine, diaminopimelic acid, aspartic acid and glycine. These

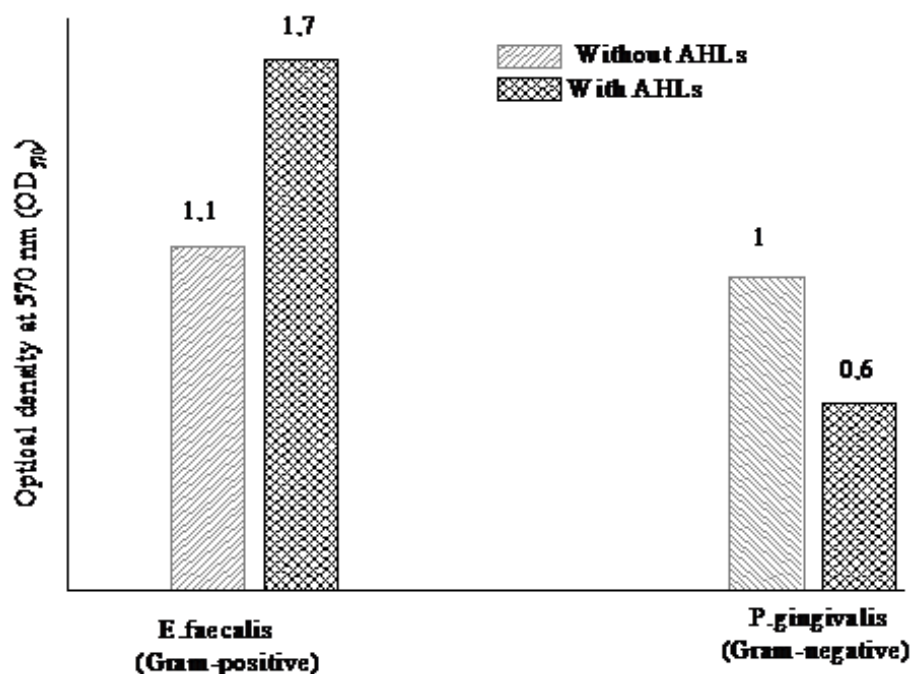


Figure 4. The different effects of AHLs molecules on bacterial biofilm formation of the Gram-positive bacterium *E. faecalis* and the Gram-negative bacterium *P. gingivalis*.

were associated with varying combinations of sugars and amino sugars (Cummins and Harris, 1956). The Gram-negative bacteria possess a lipid-rich outer membrane (as well as a plasma membrane) and a thin peptidoglycan layer. Gram-positive bacteria are enshrouded in thicker, more resilient cell walls (Beveridge, 1999).

Conclusion

The present study has shown that quorum sensing signal molecules named “AHLs” can have a significant effect on oral Gram-positive bacteria by promoting the biofilm formation of *E. faecalis* strain isolated from teeth of patients with endodontic infections in Algeria. Furthermore, it was evident that these AHLs molecules act differently on biofilm formation of Gram-positive or Gram-negative oral bacteria. AHLs promoted the biofilm formation of the Gram-positive oral bacterium (*E. faecalis*) but inhibited the biofilm of the Gram-negative oral bacterium (*P. gingivalis*). Finally, we have to take advantage of these results to well understand the biological activity of these signal molecules in oral biofilms in order to minimize human oral infections.

Conflict of interest

The authors declare that they have no conflict of interest

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Full Length Research Paper

The inoculation of cowpea culture with rhizobial lineage in Brazilian Cerrado Region

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Cowpea culture is spreading in the Brazilian Cerrado Region and specifically in Mato Grosso State. To evaluate the productive characteristics of cowpea inoculated with rhizobia lineages and grown in the Cerrado of Mato Grosso State, we conducted an experiment in a randomized block design with seven treatments, which consisted of five rhizobia lineages. Four lineages were previously isolated from cowpea (MT08, MT15, MT16 and MT23); one was a recommended lineage for cowpea in Brazil (BR3267). Two controls were included: one was equivalent to 75 kg N ha⁻¹ nitrogen and another was without nitrogen fertilization and without inoculation of rhizobia lineage. They were arranged in six blocks totaling 42 parts of 12.5 m² each. At 40 days after sowing (d.a.s.), six plants of each part of floor area were collected to determine the variables plant height, dry mass of shoots and roots, total dry mass, number of nodules, dry mass of nodules, SPAD reading and relative efficiency of each lineage. Data were subjected to analysis of variance using the statistical analysis program SISVAR. To compare the means, it was evaluated with Tukey test at 5% probability and contrast analysis. Regarding the lineage used, the most satisfactory result for plant height was BR3267 lineage; however, there were statistical differences between treatments with nitrogen fertilization. There were significant differences between dry weight of shoots, total dry weight and dry weight of nodules. The BR3267 lineage presented better results in the dry mass of roots and for relative efficiency. The most satisfactory results for SPAD readings and number of nodules were observed in MT15 lineage. The BR3267 and MT15 lineages tested and analyzed had high symbiotic effectiveness for inoculation in cowpea.

Key words: *Vigna unguiculata*, rhizobium, nitrogen fixation.

INTRODUCTION

The cowpea (*Vigna unguiculata* (L.) Walp.) is a native legume of Africa and largely grown in the tropical regions of Africa, Asia and America, being the main source of protein, especially for low-income populations (Silva et

al., 2006). This culture is highlighted in the Mid-North Brazilian as being of great socioeconomic importance as the source of vegetable protein for the population, especially the rural areas (Almeida et al., 2010).

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According to Zilli et al. (2009), Brazil is the third largest producer of cowpea; however, there is still a deficit in product offering as has been observed. The Brazilian Northeast is known for being the largest domestic producer of this grain, which is a major component of population diet, besides being an important generator of employment and income for families who cultivate the practice of family farming (Santos et al., 2008).

Despite this, its productivity is considered low due to few technological resources used in farming; there are regions in the States of Amazonas, Goiás, Mato Grosso do Sul and Mato Grosso where productivity has achieved good results using nitrogen fertilizer (Silva, 2009). According to Barbosa et al. (2010), the culture of cowpea is not only a subsistence activity and is becoming culture of high economic impact.

It is a very demanding culture in relation to nitrogen supply because most of this nutrient is exported to the grains during the plant reproductive period (Santos et al., 2003).

Furthermore, most part of this nutrient can be obtained directly from the atmosphere through association with rhizobial in a symbiotic relationship where the plant provides the metabolites to microorganisms while the bacterium in the root nodules performs the conversion of atmospheric nitrogen into ammonia (Figueiredo et al., 2008; Taiz and Zeiger, 2004).

In order to raise the productivity of cowpea, reduce production costs and increase the income of farmers, the biological nitrogen fixation (BNF) by adopting the practice of seed inoculation with efficient rhizobial lineages has been extensively explored (Zilli et al., 2009).

Thus, the aim of this study was to evaluate the productive characteristics of cowpea inoculated with rhizobia lineages and grown in the Cerrado of Mato Grosso, Brazil.

MATERIALS AND METHODS

Tillage and fertilization

The experiment was conducted from January to April of 2013 in the Federal University of Mato Grosso, Rondonópolis-MT campus, geographically located in the Southern region of the State which is situated at latitude 1° 28'15" south and longitude 54° 38'08" west. A heavy disk was used for soil leveling and to remove invasive plants. The soil was classified as Oxisol Red (EMBRAPA, 2009).

Soil samples were collected for chemical analysis and the results were: pH (CaCl₂) = 5.0, P = 3.7 and K = 60 mg dm⁻³ respectively, Ca = 1.2, Mg = 9.0, Al = 0.7, H = 0.0 and CTC = 6.0 cmol_c dm⁻³ respectively. Organic material = 24.8 gdm⁻³, base saturation = 44.8% and aluminum saturation = 55.2%.

Using the results of the soil chemical analysis, liming was performed in order to raise the base saturation of 60%, according to the culture needs.

The fertilization was performed according to Junior et al. (2002), applying 444 kg ha⁻¹ of superphosphate, 86 kg ha⁻¹ of potassium chloride and addition of micronutrients. 75 kg ha⁻¹ of urea for nitrogen fertilizer was applied. This application was in two stages and next to the furrow. In the first stage, at 20 days after sowing (d.a.s.), 50% of the dose was applied. At 30 d.a.s, the second nitrogen fertilization was performed by applying the remaining dose.

Cultivar

The cultivar of cowpea used was BRS New Era, which corresponds to the lineage MNC00-553D-8-1-2-2, obtained from crosses between lineages TE97-404-1F and TE97-404-3F. This cultivar presents semi erect branches, short lateral branches and insertion of pods above the foliage. The color of the pods during physiological maturity and harvest is light yellow and may have purple pigmentation on the sides of the pods (Freire Filho et al., 2008).

Experimental design

The experimental design was a randomized complete block design with seven treatments, which consisted of five rhizobia lineages, including four previously isolated from cowpea (MT08, MT15, MT16, MT23), a rhizobia lineage recommended for cowpea in Brazil (BR3267) and two controls (one fertilized with 75 kg N ha⁻¹ nitrogen and other without nitrogen fertilization and without inoculation with rhizobia lineages).

Each part consisted of an area of 12.5 m². The three central lines were used for floor area, excluding 0.5 m of edging. Each treatment consisted of six replicates, totaling 42 parts.

Inoculation and sowing seed

Seed inoculation was made by means of peat inoculant. The lineages were grown in liquid medium LMA (Vincent, 1970). After the growth of rhizobia lineages, 10 ml of bacterial broth was used for each 35 g of peat. Later, homogenization was done, and the inoculum was incubated at 30°C for 24 h (Guimarães et al., 2007).

Subsequently in this period, the seeds were pelleted with inoculant and made dry in the shade. And then seeding was performed with density of eight seeds per meter.

Collection of samples for analysis

At 40 d.a.s, the samples were collected, using six plants from each part of the floor area. In the samples, the followings were evaluated: SPAD reading, plant height (PH), dry mass of shoot (DMS), dry mass of root (DMR), total dry mass (TDM), number of nodules (NN), dry mass of nodules (DMN) and relative efficiency of the lineages (RE).

SPAD reading was realized using ClorofilLOG® CFL 1030 from five samples randomly chosen from each part of the floor area. Height of plants was determined using a measuring tape. The dry mass of shoot and root was determined in semi-analytical balance, after being kept in an incubator at 65°C until constant weight. The dry mass of nodules was determined through the same process however using an analytical balance.

The number of nodules was determined in the laboratory by tweeze extraction. Relative efficiency of each lineage was determined by dividing the dry mass of shoot from inoculated treatments by the nitrogen fertilizer treatment (Bergersen et al., 1971).

Data were subjected to analysis of variance using the statistical program SISVAR (Ferreira, 2008) and means were compared by Tukey test at 5% probability and contrast analysis.

RESULTS

The results concerning SPAD readings, plant height, root dry mass and number of nodules is shown in Table 1. From SPAD readings, it was observed that plants fertilized by nitrogen showed the highest values with an

Table 1. Plant height, SPAD reading, number of nodules (NN), dry mass of nodules (DMN), 40 d.a.s. in cowpea plants grown in the Cerrado Oxisol.

Treatment	Height (cm)	SPAD	NN*	DMN (g)*
MT 08	48.21 ^b	60.55 ^{ab}	6.54 ^{ab}	1.07 ^{ns}
MT 15	48.26 ^b	59.18 ^{ab}	9.22 ^a	1.043
MT 16	48.60 ^b	58.15 ^{ab}	7.43 ^{ab}	1.062
MT 23	48.60 ^b	59.28 ^{ab}	6.41 ^{ab}	1.084
BR3267	49.80 ^{ab}	57.85 ^b	7.18 ^{ab}	1.027
Nitrogen fertilization	54.58 ^a	63.21 ^a	4.26 ^b	1.043
Control	49.30 ^b	61.46 ^{ab}	3.58 ^b	1.032
CV (%)	5.54	4.44	39.05	4.6

Means followed by the same letter in the column do not differ by Tukey test at 5% probability. *Data transformed for Y +1; ns, not significant.

Table 2. Dry mass of shoot (DMS), total dry mass (TDM), dry mass of root (DMR) and relative efficiency (RE) of the lineages held at 40 d.a.s. in cowpea plants grown in the Cerrado Oxisol.

Treatment	DMS (g)	TDM (g)	DMR (g)	RE (%)
MT 08	45.76 ^{ns}	49.62 ^{ns}	3.85 ^{ab}	71.70 ^{bc}
MT 15	48.26	52.53	4.27 ^{ab}	81.40 ^{ab}
MT 16	53.10	57.27	4.16 ^{ab}	89.73 ^{ab}
MT 23	52.85	56.82	3.97 ^{ab}	89.03 ^{ab}
BR3267	57.47	62.64	5.17 ^a	94.97 ^{ab}
Nitrogen fertilization	63.82	68.50	5.02 ^{ab}	100.00 ^a
Control	47.74	51.24	3.50 ^b	47.74 ^c
CV (%)	19.21	18.92	20.45	16.96

Means followed by the same letter in the column do not differ by Tukey test at 5% probability; ns, not significant.

average of 63.2, with no statistical difference compared to MT08, MT15, MT16, MT23 lineages and control treatment; statistical difference was only observed in BR3267 lineage.

Among the inoculated treatments, the MT08 lineage is spotlighted with SPAD reading of 60.55 which is equivalent to 95.80% of SPAD reading of nitrogen fertilizer treatment. That result indicates an effective nitrogen fixation for MT08 lineage. However, the control treatment showed SPAD reading of 61.46 which may be related to the presence of native rhizobia in the tested soil.

The SPAD reading is a very important feature because it determines the plant efficiency in solar radiation absorption. The higher the solar radiation absorption efficiency is the higher the photosynthetic rates, which result in higher grain yield (Nascimento et al., 2011).

The highest average of plant height was obtained from nitrogen fertilization treatment with 54.58 cm per sample and had no statistical difference from BR 3267 lineages treatment. This result demonstrates the high efficiency of rhizobia symbiosis with cowpea. The other inoculated treatments MT08, MT15, MT16 and MT23 presented

values below those observed with nitrogen fertilizer and commercial inoculant of BR3267 lineage.

The highest number of nodules was observed in MT15 treatment with average of 9.22 nodules per sample. There was no significant statistical difference compared to the other inoculated treatments. Concerning dry mass of nodules, there was no statistical significant difference between treatments and the highest quantity was observed in MT23 treatment with 1.084 g per sample; while BR3267 treatment showed less satisfactory results for this variable with 1.027 g.

It was observed that there was no statistical difference between treatments for dry mass of the shoot and the highest quantity was found for the nitrogen fertilizer treatment with an average of 63.82 g and lower result for the control treatment with 47.74 g (Table 2). The BR 3267 treatments presented 90% of result from dry mass of shoot in comparison to nitrogen fertilizer treatment, followed by MT16 (83.20%), MT23 (82.81%) and MT15 (75, 62%).

Regarding the production of total dry mass (TDM), there was no statistical difference between the treatments; however, the plants with better results were those

Table 3. Contrast results for height of the lineages held at 40 d.a.s. in cowpea plants grown in the Cerrado Oxisol.

Treatment	Treatment (average)							Contrast estimative
	Control	Fertilized	BR3267	MT08	MT15	MT16	MT23	
	49.30	54.58	49.80	48.21	48.26	48.60	48.60	
Contrast coefficient								
C1	6	-1	-1	-1	-1	-1	-1	-0.376
C2		5	-1	-1	-1	-1	-1	5.878
C3			4	-1	-1	-1	-1	1.379
C4				3	-1	-1	-1	-0.273
C5					2	-1	-1	-0.348
C6						1	-1	0.000

Table 4. Contrast results for SPAD reading of the lineages held at 40 d.a.s. in cowpea plants grown in the Cerrado Oxisol.

Treatment	Treatment (average)							Contrast estimative
	Control	Fertilized	BR3267	MT08	MT15	MT16	MT23	
	61.46	63.21	57.85	60.55	59.18	58.15	59.28	
Contrast coefficient								
C1	6	-1	-1	-1	-1	-1	-1	1.761
C2		5	-1	-1	-1	-1	-1	4.214
C3			4	-1	-1	-1	-1	-1.440
C4				3	-1	-1	-1	1.681
C5					2	-1	-1	0.469
C6						1	-1	-1.128

who received nitrogen fertilization treatment (Table 2). Among the inoculation treatments, the highest value for this variable was observed in BR3267 with 91% of the total dry mass compared to nitrogen fertilizer treatment; it is followed by MT16 (83%); and the MT08 lineage was the treatment that provided lowest value of total dry mass in this experiment.

For root dry mass variable, the treatment that stood out was the BR3267 commercial lineage but did not differ significantly from the other treatments except the control. The production of the treatment inoculated with BR3267 lineages was superior to treatment with nitrogen fertilizer in approximately 3%. Regarding other inoculation treatments the MT15 lineage showed higher result with 83% of production observed in the commercial inoculant BR3267, followed by the lineages MT16, MT23 and MT08. All inoculation treatments had higher dry mass production of roots compared to control.

For the relative efficiency of lineages, the treatment showing the best result was BR3267 with 94.97% efficiency compared to nitrogen fertilizer. The MT16 and MT23 lineages were almost the most efficient of the BR3267 lineage (Table 2).

For contrast analysis, they were divided into six studies

as follows: C1: control vs all treatment; C2: fertilized vs all treatment; C3: Br3267 vs (MT 08 + MT15 +MT 16 +MT 23); C4: MT 08 vs (MT15 +MT 16 +MT 23); C5: MT15vs (MT 16 +MT 23); C6: MT 16vs MT 23.

The cowpea height presented statistical difference for C2 ($P < 0.01$); it shows that N was more efficient in the development of the cultivar at 40 days compared to treatments using commercial inoculant as treatments inoculated with native lineage of MT 08, MT 14, MT 16 e MT 23 (Table 3). The SPAD reading presented significant difference for contrast 2 (C2), with $P < 0.01$ (Table 4). This demonstrates that treatment that uses chemical fertilizers based on nitrogen promoted more satisfactory results offering better conditions and nutritional support for the development of cowpea plants. Regarding the contrasts between inoculated treatments, there was no statistically significant difference.

The number of nodules variable presented statistically significant difference for the contrasts C1 and C5, both with $P < 0.05$. The results obtained for C1 showed the effectiveness of soil native rhizobia and its potential in relation to the BNF. Results of C5 demonstrate the effectiveness of rhizobia isolated from samples of bait plants grown in cerrado soil (MT 08, MT 14, MT 16 and

Table 5. Contrast results for number of nodules of the lineages held at 40 d.a.s. in cowpea plants grown in the Cerrado Oxisol.

Treatment	Treatment (average)							Contrast estimative
	Control	Fertilized	BR3267	MT08	MT15	MT16	MT23	
	3.58	4.26	7.18	6.54	9.22	7.43	6.41	
Contrast coefficient								
C1	6	-1	-1	-1	-1	-1	-1	-41.250
C2		5	-1	-1	-1	-1	-1	-34.900
C3			4	-1	-1	-1	-1	-10.500
C4				3	-1	-1	-1	-10.444
C5					2	-1	-1	44.833
C6						1	-1	18.666

Table 6. Contrast results for dry mass of shoot of the lineages held at 40 d.a.s. in cowpea plants grown in the Cerrado Oxisol.

Treatment	Treatment (average)							Contrast estimative
	Control	Fertilized	BR3267	MT08	MT15	MT16	MT23	
	3.4	5.02	5.17	3.85	4.27	4.16	3.97	
Contrast Coefficient								
C1	6	-1	-1	-1	-1	-1	-1	-0.908
C2		5	-1	-1	-1	-1	-1	0.733
C3			4	-1	-1	-1	-1	1.110
C4				3	-1	-1	-1	-0.277
C5					2	-1	-1	0.204
C6						1	-1	0.198

Table 7. Contrast results for relative efficiency of the lineages held at 40 d.a.s. in cowpea plants grown in the Cerrado Oxisol.

Treatment	Treatment (average)							Contrast estimative
	Control	Fertilized	BR3267	MT08	MT15	MT16	MT23	
	47.74	100.00	94.97	71.70	81.40	89.73	89.03	
Contrast coefficient								
C1	6	-1	-1	-1	-1	-1	-1	-34.034
C2		5	-1	-1	-1	-1	-1	-21.549
C3			4	-1	-1	-1	-1	12.007
C4				3	-1	-1	-1	-15.021
C5					2	-1	-1	-7.989
C6						1	-1	0.695

MT 23), which for this variable presented better values than the commercial inoculant BR3267 (Table 5).

The dry mass of the roots of cowpea presented difference for C3 ($P < 0.01$) and contrast C1 ($P < 0.05$) (Table 6). The results obtained in C3 prove the effectiveness of the biological potential commercial inoculant BR3267; it has highest average when analyzing this variable and establishing the most effective treat-

ment. The contrast C1 showed satisfactory results but below those verified in C3.

For relative efficiency variable, the results were significant for C2 ($P < 0.01$) and C4 ($P < 0.05$) (Table 7). This result demonstrates the effectiveness of native cerrado lineages (MT 08, MT 14, MT 16 and MT 23) and proves that they are efficient in biological fixation in symbiosis with cowpea under the evaluated conditions.

DISCUSSION

The cowpea culture can get N through symbiosis with bacteria of the genus *Rhizobium* in FBN process. This process is one way to increase the productivity of this legume (Franco et al., 2002). According to Rumjanek et al. (2005), FBN is efficient for this culture that can achieve high levels of productivity when the crop produces good nodulation. In this experiment, five rhizobia lineages were tested to evaluate the productive characteristics of the culture. However, the supply of nitrogen by biological fixation was not satisfactory for all inoculate lineage tested.

Study performed by Santana et al. (2010) to cultivate beans in the same soil type in Cerrado of Goiás State found the highest values of SPAD (47.0) for the treatment that received high doses of mineral nitrogen (ranging from 60 to 120 kg ha⁻¹). These values are below those observed in this study where the fertilized soil showed a value of 63.21 for the same variable. These results demonstrate that the rate of mineral nitrogen applied on the soil in the experimented region is sufficient for obtaining acceptable results for SPAD index.

Chagas Junior et al. (2010) working with cowpea inoculated in the Cerrado of Tocantins State found greater crop production in the treatment with BR 3262 lineage.

In the actual experiment, the SPAD reading was a variable that achieved statistical significant difference when compared with the treatment fertilized with mineral nitrogen, inoculated treatments and the control treatment; higher values were obtained for the treatment fertilized with mineral nitrogen. This result may be related to the fact that mineral nitrogen is more readily absorbed by plants. In general, in the early development of the plant there is a preference for the absorption of nitrogen in the ammonium form. With the development of the vegetative phase, absorption of nitrogen as nitrate increases (Brown et al. 1983a, b; Blackmer, 2000).

Almeida et al. (2010) observed cowpea grown in Piauí State. This increased the number of nodules in treatments with lineages BR3267, which was also used as inoculum in this experiment. For this study, the highest number of nodules was obtained in treatment with the MT15 lineages; however, it did not differ statistically from the BR3267 and other inoculated treatments.

The results of this study are similar to results obtained by Chagas Junior et al. (2010) who planted the same variety of cowpea and obtained inoculant BR3267 as better treatment. In this study, the MT15 inoculant produced the highest number of nodules but there was no statistically significant difference relative to BR3267 inoculant.

The distribution of dry mass in the plant is a variable that allows one to discuss a process poorly studied which is the translocation of assimilates and that in many cases facilitates the understanding of plant response in terms of

productivity (Benincasa, 2003). In addition, Gualter et al. (2011) affirm that nitrogen-fixing bacteria may contribute significantly to higher N to the plant and consequently lead to an increase of plant dry mass.

The results obtained by the dry mass of shoot in this experiment are similar to that of Almeida et al. (2010) who also found no differences in the dry mass of shoots in cowpea when comparing the fertilized ground, inoculants and control treatment.

The results for total dry mass of cowpea showed no statistically significant difference between treatments with the most satisfactory production for the treatment fertilized with N. This result was expected due to the availability of this nutrient confirming the results reported by Chagas Junior et al. (2010).

The relative efficiency of the lineages revealed that inoculation is an efficient practice for the process of biological nitrogen fixation and may even totally or partially replace nitrogen fertilization on cowpea. Nascimento et al. (2010) found positive results for the inoculum BR3267 while planting cowpea in Pernambuco State. The average percentage of the inoculum did not show statistical significant difference compared to the fertilized treatment, demonstrating efficiency in agricultural production.

Gualter et al. (2011) and Chagas Junior et al. (2010) obtained better results as to the relative efficiency of inoculated treatment. In general, the treatments that received inoculation of rhizobia lineage were promising for the supply of nitrogen through BNF in cowpea plants. This confirms the importance of this association and is also less aggressive and harmful to the agriculture environment.

The productivity of cowpea in this study was not presented because the analyses were performed 40 d.a.s. at the end of the experiment.

Conclusion

The rhizobia lineages that best express the potential of BNF in association with cowpea grown in the Cerrado Oxisol are MT15 and BR3267.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

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Full Length Research Paper

Serotyping and antimicrobial susceptibility of *Salmonella* isolated from children under five years of age with diarrhea in rural Burkina Faso

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Diarrheal infections are an important public health problem worldwide but there is limited information on the molecular characteristics of responsible agents in Burkina Faso, especially in the countryside. The objective of this study was to investigate the prevalence, serotypes and antibiotic susceptibility patterns of *Salmonella enterica* in diarrheal children in rural areas. Stool specimens were collected from 400 diarrheal children under five years of age between July 2009 and June 2010. *Salmonella* spp. was investigated by using conventional culture techniques and susceptibility to 12 antimicrobial agents was determined by the disc diffusion method. Twenty four (6%) *Salmonella enterica* ssp. *enterica* strains (14 different serotypes) were isolated: *S. Typhimurium* (in 9 patients), *S. Poona* (2), *S. Virchow* (2), and *S. Kiambou*, *S. Rubislaw*, *S. Muenster*, *S. Gaminara*, *S. Tounouma*, *S. Cubana*, *S. Fresno*, *S. Montevideo*, *S. Duisburg*, *S. Hvitvingfoss* and *S. Ouakam* (in 1 patient each). Eight (8) *S. Typhimurium* were multiresistant to ampicillin, chloramphenicol, streptomycin, sulfonamides and trimethoprim. All the other *Salmonella* serotypes were sensitive to all antimicrobials tested. This study highlights the serotypes of *Salmonella* circulating in rural Burkina Faso. Therefore, constant monitoring for *Salmonella* infection and antibiotic resistance is needed in the control of this pathogen.

Key words: *Salmonella* serotypes, antimicrobial susceptibility, children, Burkina Faso.

INTRODUCTION

Diarrheal disease is a public health problem worldwide, mostly affecting children in developing countries (Bryce et

al., 2005; Vilchez et al., 2009). Diarrhea is one of the leading causes of morbidity and mortality among children

under five years in the developing world (Moyo et al., 2011). The aetiological agents include a wide range of viruses, bacteria and parasites (Giordano et al., 2001; Bonkougou et al., 2011; Nitiema et al., 2011). *Salmonella* is one of the zoonotic pathogens, which is of great importance in public health science because it is often associated with gastroenteritis (Fashae et al., 2010; Shi et al., 2012). Rural areas are characterized by inadequate hygienic measures, self-medication and poor quality of health care system. Gastroenteritis is associated with about 2500 different serotypes of *Salmonella* (Matheson et al., 2010) and serotyping is an epidemiological marker for the study of *Salmonella* spp. infections in a given population and location at a given time (Schutze et al., 1995). Treatment with antimicrobials is crucial for proper management of severe or invasive human salmonellosis (Fashae et al., 2010). Until recently, *Salmonella* spp. were highly susceptible to the most commonly used antibiotics. However, in the last decade, the emergence of multidrug-resistant strains, including isolates resistant to quinolones, have been reported from some African countries (Gordon et al., 2008; Sire et al., 2008; Hendriksen et al., 2009; Fashae et al., 2010) leading to a major problem associated with the control of diarrhea (Urio et al., 2001). This situation is often associated with bad use of antibiotics in human and animal health. Moreover current information regarding antimicrobial susceptibility pattern of bacteria causing diarrhea in children is limited and thus it is uncertain whether the recommended antibiotics are still effective (Moyo et al., 2011). In Burkina Faso, few data on animal and human *Salmonella* serotyping and its susceptibility to antibiotics have been reported in urban areas (Bonkougou et al., 2013; Kagambèga et al., 2013) while there is no information as far as rural areas are concerned. The aim of this study was to investigate the prevalence, serotypes and antibiotic susceptibility patterns of *S. enterica* in diarrheal children in rural Burkina Faso.

MATERIALS AND METHODS

Study design, population and settings

A prospective cross sectional study was conducted to determine the serotypes and antimicrobial susceptibility of *Salmonella* species among diarrheic children visiting hospitals in rural settings of Burkina Faso. This study was conducted between July 2009 and June 2010 (during one year) in two remote rural areas, in north (Gourcy, distance 140 km) and western (Boromo, distance 185 km) of the capital Ouagadougou, Burkina Faso (Figure 1). The main

sources of income in these rural settings are subsistence farming, animal husbandry and small scale trade.

Stool samples were collected from 400 children (183 from Gourcy and 217 from Boromo) under five years of age who had acute diarrhea (as described by WHO, 2000) and/or were admitted to medical district hospital of Gourcy and Boromo.

Specimen collection

Stool samples were taken by trained healthcare personnel using a swab transport system (M40 transystemAmies agar gel without charcoal; Copan Italia Spa, Brescia, Italy) and transported to laboratory within 24 h of their collection for analysis. Information regarding the age and sex were recorded for each child using a questionnaire.

Salmonella isolation and identification

Selenite broth (Emapol, Pologne) was used for enrichment of specimens followed by incubation at 37°C for 18 h. Subsequently, samples were cultured on Hecktoen Enteric agar (Liofilchem, Italy) and incubated at 37°C for 24 h. The identity of typical-looking *Salmonella* colonies on Hecktoen was examined by using orthonitrophenyl-β-D-galactopyranoside (ONPG), citrate, mannitol, lysine decarboxylase tests and the Kligler Hajna medium (Liofilchem, Italy). Finally the isolates were confirmed by API 20E (BioMérieux, Marcy l'Etoile, France) (Kagambèga et al., 2013).

Serotyping

All *Salmonella* isolates were serotyped by the *Salmonella* Reference Laboratory. The isolates were serotyped with the somatic O and flagellar H anti-sera according to the Kauffman-White scheme (Popoff et al., 2004).

Antimicrobial susceptibility testing

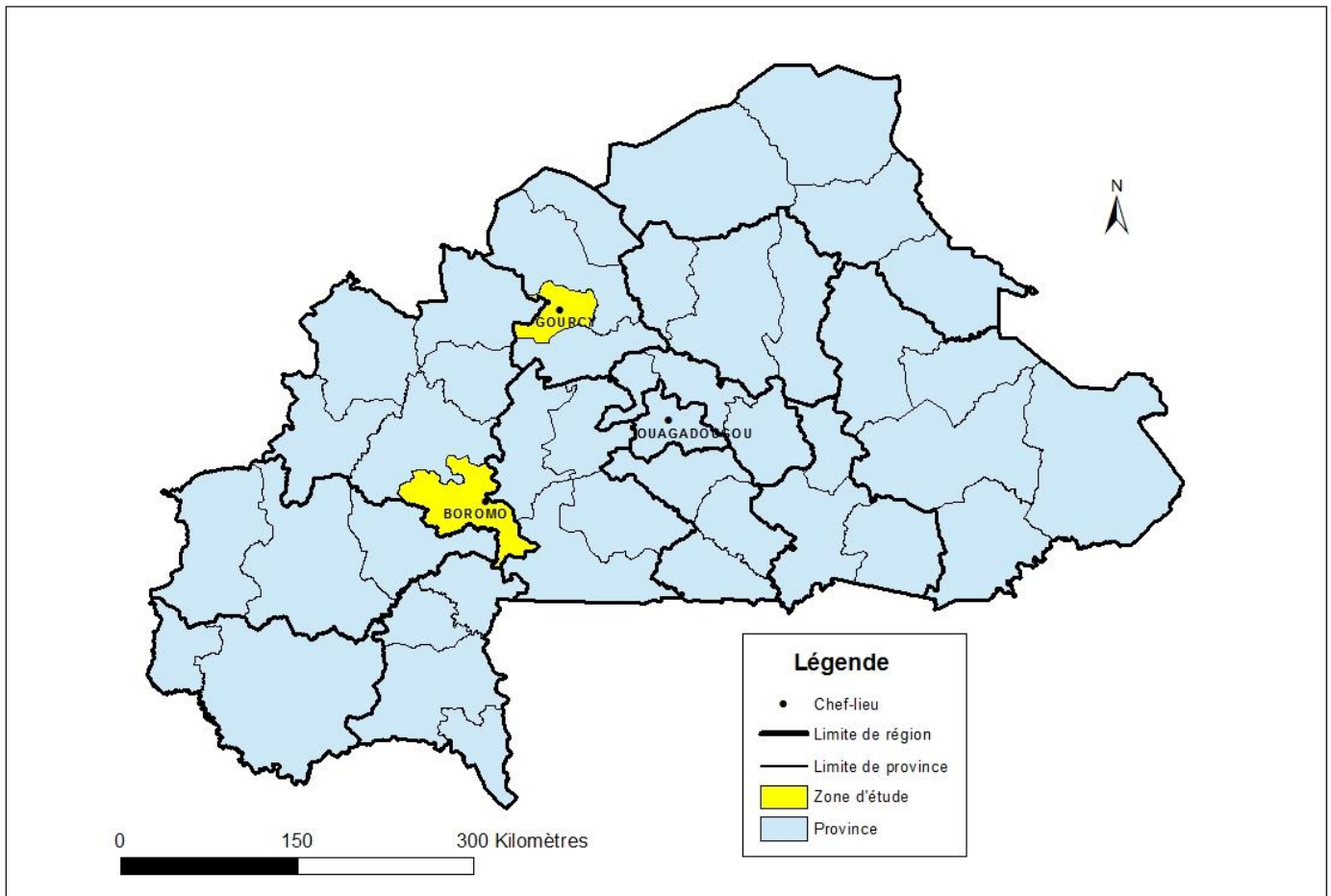
The susceptibility of strains to 12 antimicrobials was evaluated by the disc diffusion method on Mueller-Hinton agar (Oxoid, England) at 37°C for 24 h and *Escherichia coli* RHE 6715 (ATCC 25922) was used for validating the antimicrobial test results (CLSI, 2009). The antimicrobials evaluated were ampicillin (10 µg), chloramphenicol (30 µg), streptomycin (10 µg), sulfonamide (3 µg), tetracycline (30 µg), trimethoprim (5 µg), ciprofloxacin (5 µg), nalidixic acid (30 µg), mecillinam (10 µg), imipenem (10 µg), gentamycin (10 µg), and cefotaxime (30 µg). The inhibition zone were measured and tested isolates were classified as "resistant", "intermediate sensitive" or "sensitive" according to CLSI, 2009.

Ethical considerations

The study protocol was approved by the Ethical Committee of Burkina Faso. An informed verbal consent was obtained from the parents/guardians of every child before taking the stool samples.

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SOURCE : BNDT

DATE : 06 / 2014

Figure 1. Map of Burkina Faso. In yellow = Gourcy and Boromo where the study was conducted.

Table 1. Age distribution of patients with *Salmonella* infection.

Age (months)	Number of patients (%)		Total
	Boromo	Gourcy	
0-12	10 (62.5)	2 (25)	12 (50)
13-24	2 (12.5)	5 (62.5)	7 (29)
25-59	4 (25)	1 (12.5)	5 (21)
Total	16 (100)	8 (100)	24 (100)

RESULTS

Prevalence of *Salmonella*

Of the 400 stool specimens analyzed, 24 (6%) *Salmonella enterica* ssp. *enterica* were isolated.

Age and sex distribution of patients with *Salmonella* infection

Our results show that 50% (38% of males and 12% of

females) of the *Salmonella* serotypes were isolated from patients under 12 months of age. Twenty-nine (17% of males and 12% of females) of patients aged 13-24 months of age had *Salmonella* and 21% (17% males and 4% females) of patients aged 25-59 months reported *Salmonella* (Table 1). The sex distribution showed 75% of male versus 25% of females for the patients less than 12 months of age, 57% of males versus 43% of females for patients aged 13-24 months and 80% of males versus 20% of females for patients aged 25-59 months.

Table 2. The different serotypes of *Salmonella* isolated with their percentages.

<i>Salmonella</i> serotype	Source		Total N (%)
	Boromo N (%)	Gourcy N (%)	
<i>S. Typhimurium</i> 4,5,12:i:1,2	7 (78)	2 (22)	9 (38.1)
<i>S. Kiambou</i> 4,12:Z:1.5	1 (100)	0 (0)	1 (4.1)
<i>S. Rubislaw</i> 11:r:enx	1 (100)	0 (0)	1 (4.1)
<i>S. Muenster</i> 3,10:ch:1,5	1 (100)	0 (0)	1 (4.1)
<i>S. Gaminara</i> 16:d:1,7	0 (0)	1 (100)	1 (4.1)
<i>S. Tounouma</i> 8,20:b:Z6	0 (0)	1 (100)	1 (4.1)
<i>S. Cubana</i> 13,23:Z29:-	1 (100)	0 (0)	1 (4.1)
<i>S. Fresno</i> 9,46:Z38:-	1 (100)	0 (0)	1 (4.1)
<i>S. Montevideo</i> 6,7:gms:-	1 (100)	0 (0)	1 (4.1)
<i>S. Duisburg</i> 4,12:d:e,n,z15	1 (100)	0 (0)	1 (4.1)
<i>S. Hvitingsfoss</i> 16:b:e,n,x	0 (0)	1 (100)	1 (4.1)
<i>S. Ouakam</i> 9,46:z29:-	1 (100)	0 (0)	1 (4.1)
<i>S. Poona</i> 13,22:z:1,6	1 (50)	1 (50)	2 (8.4)
<i>S. Virchow</i> 6,7:r:1,2	0 (0)	2 (100)	2 (8.4)
Total (%)	16 (67)	8 (33)	24 (100)

Diversity of *Salmonella* serotypes

The 24 isolates belonged to 14 different *Salmonella* serotypes (Table 2). The most commonly isolated serotype were *S. Typhimurium* (n = 9; 37.6%), *S. Poona* (n = 2; 8.1%) and *S. Virchow* (n = 2; 8.1%). Several uncommon serotypes were among the isolated *Salmonella* such as *S. Kiambou* (n = 1; 4.2%), *S. Rubislaw* (n = 1; 4.2%), *S. Muenster* (n = 1; 4.2%), *S. Gaminara* (n = 1; 4.2%), *S. Tounouma* (n = 1; 4.2%), *S. Cubana* (n = 1; 4.2%), *S. Fresno* (n = 1; 4.2%), *S. Montevideo* (n = 1; 4.2%), *S. Duisburg* (n = 1; 4.2%), *S. Hvitingsfoss* (n = 1; 4.2%), and *S. Ouakam* (n = 1; 4.2%) (Table 2). Sixteen (66.7%) of the *Salmonella* isolated were from Boromo while eight (33.3%) were from Gourcy.

Salmonella antimicrobials susceptibility

The *Salmonella* serotypes were all susceptible to ciprofloxacin, gentamicin, nalidixic acid, cefotaxime, mecillinam and imipenem (Table 3). Low frequency of resistance to tetracycline was observed with *S. Typhimurium* (4%). Considering all the 24 *Salmonella* isolated, a higher frequency of antimicrobial resistance was observed in 33% of each following antibiotic: ampicillin, chloramphenicol, sulfonamide, trimethoprim and streptomycin. Eight of the nine *S. Typhimurium* isolates were multiresistant to the same antimicrobials (ampicillin, chloramphenicol, streptomycin, sulfonamides and trimethoprim). *S. Poona*, *S. Virchow*, *S. Tounouma*, *S. Duisburg*, *S. Hvitingsfoss*, *S. Ouakam* were all intermediate to streptomycin.

DISCUSSION

Salmonella spp. remains an important cause of diarrheal illness in developing countries. This was the first study in rural Burkina Faso to investigate the prevalence, serotypes and antibiotic susceptibility patterns of *S. enterica* among diarrheal children. The frequency of isolation of *Salmonella* comprising several serotypes was 6%, which is lower than in our previous report in urban Burkina Faso (Bonkougou et al., 2013). This was unexpected since hygiene and sanitation are better in urban than rural areas and thus may reduce *Salmonella* incidence. However, some practices like street food system which is more developed in Ouagadougou may justify the high prevalence in urban area. Similar data were reported in Malawi, Tanzania, Congo and Ethiopia (Sigauque et al., 2009; Gordon et al., 2010; Moyo et al., 2011; Beyene and Tasew, 2014).

Serotyping is an important data for understanding the epidemiology of salmonellosis in a geographical area and measuring trends in serovars over time can provide information about emerging serotypes (Fernandes et al., 2006). The relative high proportion of *S. Typhimurium* isolates in this study is similar to results of previous studies from Burkina Faso (Bonkougou et al., 2013), other African countries such as Tanzania (Moyo et al., 2011), Botswana (Urio et al., 2001) from Australia (Doyle et al., 2009), Colorado (Berg, 2008) and Denmark (Ethelberg et al., 2008). In contrast, *S. Enteritidis*, which is the most common serotype reported from human isolates globally (Galanis et al., 2006), was not found in this study. This result may be due to increased awareness of the community about food and

Table 3. Resistance to individual antimicrobial among *Salmonella* serotypes

Serotype	Antimicrobials Resistance n (%)											
	Ampicillin	Chloramphenicol	Streptomycin	Sulfonamide	Trimethoprim	Tetracycline	Ciprofloxacin	Gentamycin	Nalidixic acid	Cefotaxime	Mecillinam	Imipenem
S. Typhimurium (n = 9)	8(89)	8(89)	8(89)	8(89)	8(89)	1(11)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
S. Kiambou (n = 1)	0 (0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
S. Rubislaw (n = 1)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0 (0)	0(0)	0(0)	0(0)	0(0)	0(0)
S. Muenster (n = 1)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
S. Gaminara (n = 1)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
S. Tounouma (n = 1)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
S. Cubana (n = 1)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
S. Fresno (n = 1)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
S. Montevideo (n = 1)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
S. Duisburg(n = 1)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
S. Hvitingsfoss (n = 1)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
S. Ouakam (n = 1)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
S. Poona (n = 2)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
S. Virchow (n = 2)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
Total (n = 24)	8(33)	8(33)	8(33)	8(33)	8(33)	1(4)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)

environmental hygiene from the continuous interventions made by the health care agents recently because the main reservoir of *S. enteritidis* is food. In addition, some of *Salmonella* serotypes (*S. Kiambou*, *S. Rubislaw*, *S. Gaminara*, *S. Tounouma*, *S. Cubana*, *S. Montevideo*, *S. Duisburg*, *S. Hvitingsfoss*) were never detected in human stool in Burkina Faso up to this study. Some of those serotypes have been isolated in food and animal products and thus it could be suggested that humans have been contaminated from food or animal reservoirs. In fact, animals husbandry (chickens particularly) is

well developed both in Boromo and Gourcy. Sure enough, besides through food, direct transmission from chicken to humans (children) could easily happen in Burkina Faso, since chickens roam free scattering their feces anywhere in the house yards (Kagambèga et al., 2013). However, the following serotypes (*S. Typhimurium*, *S. Muenster*, *S. Fresno*, *S. Ouakam*, *S. Poona* and *S. Virchow*) were isolated in Burkina Faso from poultry (Kagambèga et al., 2013) and the close association between humans and domestic animals could play an important role in human infection. In general, the primary sources of salmonellosis are considered

to be food-producing animals such as cattle, poultry and swine (Thorns, 2000). It is interesting to note that the majority of the *S. Typhimurium* was isolated from Boromo whereas very few found from Gourcy. This result might be due to location of Boromo as crossroads between Ouagadougou, the capital city, and Bobo-Dioulasso, the second largest city in Burkina Faso. Street-foods are produced and sold there which may explain this higher rate of *Salmonella* in comparison to Gourcy. Children and young adults are the most affected *Salmonella* infection. The children under 12 months group was the most infected followed

by 13-24 months of age group and 25-59 age group. Our results are consistent with those reported in southwest Ethiopia (3.9% for children under 12 months and 5.4% for 13-60 months) (Beyene and Tasew, 2014). The antimicrobial susceptibility tests showed that all *Salmonella* strains except *S. Typhimurium* were susceptible to all the tested antibiotics, suggesting the efficacy of old antimicrobials for treatment of these strains of *Salmonella*. Most of *S. Typhimurium* were resistant to ampicillin, chloramphenicol, streptomycin, sulfamethoxazol, tetracycline and trimethoprim. It has been reported that resistance to antimicrobials may increase the difficulty and cost of treating seriously ill patients (Doyle et al., 2009). This is likely due to the uncontrolled overuse of these antimicrobials for various sicknesses leading to the emergence of resistant strains. In many ways, the *S. Typhimurium* isolates in this study match the resistance profile of the multidrug-resistant invasive *S. Typhimurium* ST313 clone found in sub-Saharan Africa (Kingsley et al., 2009) but further investigation by MLST typing is needed before concluding. No resistance was detected to the newer generation antimicrobials (ciprofloxacin, gentamycin, nalidixic acid, cefotaxime, mecillinam, Imipenem) in our study these could thus be used in the future for empiric treatment.

Diarrhea caused by bacterial pathogens, especially *Salmonella*, remains one of the major causes of morbidity and mortality among infants and young children in Burkina Faso. Our results show that part of diarrhea in rural Burkina Faso is caused by antimicrobial-resistant *Salmonella*. Resistance of *Salmonella* spp. has shown a progressive increase over the course of time in many areas of the world.

Approaches to minimize the development of antimicrobial resistance and optimize therapy include educational interventions for physicians and parents, appropriate use of antimicrobial agents, reduction in the use of growth promoters in animal feed.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Conidial production of *Beauveria bassiana* on agricultural products and effect of storage temperature on its formulations

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Rice, wheat, maize, sorghum, mini potato tubers, rice bran and rice straw were evaluated for mass production of three *Beauveria bassiana* (Balsamo) Vuillemin strains. Results show rice as the most suitable substrate for examined fungi as it yielded highest conidial count (31.8×10^5 conidia/g) and colony forming unit (30.5×10^5 cfu/g) whereas rice straw recorded minimum conidial count (12.2×10^5 cfu/g) and colony forming unit (9.5×10^5 conidia/g). This study also shows the preparation of formulations of *B. bassiana* on best substrate and its viability at different storage temperature. Results show Talc based formulation at refrigeration temperature as the best and the cells were viable upto three months of storage.

Key words: *Beauveria bassiana*, formulations, temperature.

INTRODUCTION

Beauveria bassiana Bals. Vuill. (Ascomycota: Sordariomycetes) is one of the important entomopathogenic fungus used as biocontrol agent. Among fungal biopesticides, it is the potential microbial alternative to chemical insecticides as this strain grows on variety of substrates, has high virulence, transcuticular penetration, broad host range and is safe to human beings, animals and the environment (McCoy, 1990). *B. Bassiana* is an entomopathogenic fungus that attacks a wide range of agricultural pests by contact and penetration (Feng et al., 1994; Nadeau et al., 1996). The success of microbial control of insect pests depends not only on the isolation, characterization and pathogenicity, but also on

the successful mass production (Bhadauria et al., 2012). Species of genus *Beauveria* (*B. bassiana* and *Beauveria brongniartii*) are produced by different companies to control various insect pests including termites, whiteflies, aphids, maize borer and other insects (Strasser et al., 2000; Wraight et al., 2001). Both solid and liquid state fermentation are used for the mass production of *B. bassiana* (Pham et al., 2010). The liquid phase provides active growing mycelia and blastospores, while the solid phase provides support for development of the dry aerial conidia (Lomer et al., 1997). The conidia produced by *Beauveria* can be used directly as natural granules or extracted through sieving and formulated as powder,

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granules or oil concentrate or any other suitable formulation depending on the target insect pest (Yadav et al., 2013).

The mycoinsecticides used as biological control agents for insect species has increased the global attention during last few decades. The development of a suitable formulation is essential for successful utilization of mycoinsecticides. Biological and physical properties of the formulation must remain viable for at least one year for commercialization to take place (Couch and Ignoffo, 1981). According to Moore et al. (2000), fungal spores are living organisms and their viability diminishes with time depending on environmental conditions. For the commercial production of fungal spores, there is need to obtain an ideal, cheap and highly productive culture medium. The shelf life of microbial pesticide is an important factor for effective insect control (Consolo et al., 2003). So, the present study was conducted to identify best agricultural substrate and carrier for production of *B. bassiana* formulations and evaluation of the storage temperature on its viability.

MATERIALS AND METHODS

The present research was conducted in the Biocontrol Laboratory of Department of Entomology, College of Agriculture, Punjab Agricultural University, Ludhiana.

Microorganisms

Three isolates of *B. bassiana* were used in this study. These isolates, *B. bassiana* (Accession No 6291), *B. bassiana* (Accession No 2028), *B. bassiana* (Accession No 4495) were obtained from Institute of Microbial Technology (IMTECH), Chandigarh. These cultures were maintained at 4°C in potato dextrose agar (PDA) slants and subcultured after every three months.

Substrates

Seven solid substrates such as rice, sorghum, wheat, maize, mini potato tubers, rice straw and rice bran were selected to study the suitability of different substrates for mass production of *B. bassiana*. One hundred gram of each substrate was washed, drained and soaked in water overnight except rice which was soaked for 3 h before starting the experiment. The excess water was drained by decanting and shade drying for half an hour to remove the excess moisture. The substrates were packed separately in individual autoclavable polythene bags which were plugged with cotton wool and autoclaved at 15 psi for 50 min. After autoclaving, the substrates were cooled at room temperature and preserved in the refrigerator till further use. These substrates were inoculated individually with all the three isolates of *B. bassiana*.

Inoculum

B. bassiana isolates were produced in Erlenmeyer flasks (250 ml) containing 100 ml of PDA, incubated at 25°C for 15 days under static conditions. The conidia suspension was prepared by adding 40 ml of sterilized distilled water containing Tween 80 (0.1%) and stirred for 30 min on a magnetic stirrer. The spores were counted in Neubauer chamber.

Growth of *B. bassiana* on different solid substrates

The solid substrate mass production provides a physical support for the fungus to produce aerial conidia the infective propagules which were best suited for storage and formulations. Different solid substrates like rice, sorghum, wheat, maize, mini potato tubers, rice straw and rice bran were used for estimating the sporulation of different isolates of *B. bassiana* and its viability at 25±2°C. 1 ml of the spore suspension of fungi was aseptically inoculated into each polythene bag which was incubated at 25±2°C at relative humidity ≥50% for 15 days (Sahayaraj and Namasivayam, 2008; Rajanikanth et al., 2010). To avoid clumping, after a week of inoculation, the bags were shaken vigorously to separate the grains and to break the mycelial mat. After incubation of 20 days, 1 g of homogenous grain samples were taken from each polythene bag and transferred to 9 ml of sterilized distilled water containing Tween 80 (0.01%) solution. The suspension was filtered through sterilized double layered muslin cloth. Counting of spores were made after the serial dilution of the suspension using double ruled Neubauer haemocytometer for determining the number of conidia in 1 g of the sample by using the following formula:

$$\text{Conidia/g} = \text{Average number of cells per square} \times \text{dilution factor} \times 10^4$$

To determine colony forming unit per gram (cfu/g), prepared serial dilutions were plated on PDA medium (1 ml/plate). The plates were gently rotated for uniform spreading of spore suspension and incubated at 25±2°C. Each treatment had three replications. The cfu counts were recorded on 4th day after plating and calculated by using the following formula:

$$\text{cfu/g} = \frac{\text{Average number of colonies} \times \text{Dilution factor}}{\text{Volume plated}}$$

Formulation of *B. bassiana* conidia with talc and charcoal

To prepare dry formulations, talc powder and charcoal were used as carriers. Polyethylene bags showing full sporulation of *Beauveria* growth were cut open and grains were crushed to fine powder which were passed through sieve of 80 µm mesh size. This powder was dried at room temperature (28±2°C) under aseptic conditions. 100 g of dried conidial powder of *B. bassiana* isolates were mixed with carrier material. (talc and charcoal) in proportion of 1:2 and dried under aseptic conditions for 24 h. Carboxy methyl cellulose (1%) was added. After thorough mixing these formulations were packed into sterilized polyethylene bags and stored at 4 and 25°C for three months. The viability of *B. bassiana* was calculated by determining the number of colony forming units by standard pour plate method, 1 g portions was homogenized in 9 ml of sterilized water, serial dilutions were prepared and aliquots of the dilutions were placed on PDA after every fortnight (Prasad and Rangeshwarran, 1999).

Formulation of *B. bassiana* conidia with oil

Conidia of *B. bassiana* isolates were also formulated in sunflower oil. Oil was initially blended with Tween 80 in 9:1 ratio and 45 ml of mixture was blended with 15 g of conidia to get the Suspension concentrates (SC) formulation and stored (Devi and Hari, 2009). They were stored at 4 and 25°C for three months To determine the number of colony forming units of the formulation, 1 ml portions were homogenized in 9 ml sterile water, serial dilutions were prepared and aliquots of the dilutions were placed on PDA within 15 days intervals.

The statistical analysis was done through analysis of variance (two-way ANOVA) at 5% level of significance.

Table 1a. Conidial count of *B. bassiana* isolates cultivated on different substrates.

Substrate	Conidial count (1×10^5 conidia/g) of different <i>Beauveria bassiana</i> isolates cultivated on different substrates (Mean \pm S.D*)			Mean
	<i>Beauveria bassiana</i> MTCC 2028	<i>Beauveria bassiana</i> MTCC 6291	<i>Beauveria bassiana</i> MTCC 4495	
Rice	40.5 \pm 1.94	36.4 \pm 0.87	25.1 \pm 1.10	34.0
Wheat	17.7 \pm 1.20	25.8 \pm 2.35	14.4 \pm 2.20	19.3
Sorghum	32.1 \pm 1.90	32.6 \pm 1.77	21.3 \pm 0.75	28.6
Maize	30.5 \pm 0.70	27.4 \pm 1.50	10.2 \pm 1.11	22.7
Mini potato tubers	16.1 \pm 1.81	21.4 \pm 1.70	9.5 \pm 1.10	15.6
Rice bran	14.8 \pm 1.44	20.3 \pm 1.61	8.8 \pm 1.40	14.6
Rice straw	11.0 \pm 1.58	17.3 \pm 20.1	6.3 \pm 1.21	11.5
Mean	23.42	25.88	13.65	
CD (0.05)		CD (5%) substrates = 1.92 CD (5%) isolates = 0.97 CD (5%) substrates x isolates = 2.58		

*Mean = Mean of three replications ** S.D = standard deviation.

Table 1b. Colony forming unit (cfu) of fungal isolates cultivated on different substrates.

Substrate	Colony forming units (1×10^5 cfu/g) of <i>Beauveria bassiana</i> isolates cultivated on different substrates (Mean \pm S.D*)			Mean
	<i>Beauveria bassiana</i> MTCC 2028	<i>Beauveria bassiana</i> MTCC 6291	<i>Beauveria bassiana</i> MTCC 4495	
Rice	36.6 \pm 6.11	32.3 \pm 2.62	22.6 \pm 4.50	30.5
Wheat	16.6 \pm 4.16	23.6 \pm 5.79	12.6 \pm 4.04	17.6
Sorghum	30.6 \pm 2.08	28.6 \pm 3.30	18.6 \pm 6.11	25.9
Maize	26.3 \pm 6.50	25.0 \pm 4.08	12.3 \pm 5.85	21.2
Mini potato tubers	14.3 \pm 3.05	21.6 \pm 3.05	10.0 \pm 3.60	15.3
Rice Bran	11.3 \pm 1.52	15.3 \pm 3.29	6.6 \pm 2.51	11.0
Rice Straw	9.6 \pm 2.08	13.3 \pm 2.62	5.6 \pm 3.05	9.5
Mean	20.7	22.8	12.1	
CD (0.05)		CD (5%) substrates = 4.1 CD (5%) isolates = N.S (non-significant) CD (5%) substrates x isolates = 7.18		

*Mean = Mean of three replications ** S.D = standard deviation.

RESULTS AND DISCUSSION

Effect of substrates

Solid substrates rice, sorghum, wheat, maize, mini potato tubers, rice bran and rice straw exhibited significant differences in their utility to yield conidia and colony forming unit. Among these substrates, maximum mean conidial count (34.0×10^5 conidia/g) (Table 1a) and colony forming units (30.5×10^5 cfu/g) (Table 1b) was recorded in rice medium. This was followed by sorghum and maize which were at par with each other. Rice straw gave minimum conidial count (11.5×10^5 conidia/g) and cfu

count (9.5×10^5 cfu/g) (Figure 1). Among *B. bassiana* strains, the mean conidial count varied. The maximum mean conidial count (25.8×10^5 conidia/g) and cfu count (22.8×10^5 cfu/g) was recorded for *B. bassiana* MTCC 6291 whereas the minimum mean spore count (13.6×10^5 conidia/g) and cfu count (12.1×10^5 cfu/g) was recorded for *B. bassiana* MTCC 4495. The substrates which were rich in nutritional composition produced not only high spore count but also higher colony count (Rajnikanth et al., 2010). Hence, rice was the best substrate followed by sorghum for mass multiplication of *B. bassiana* which may be due to the presence of rich source of carbon and adequate source of nitrogen.

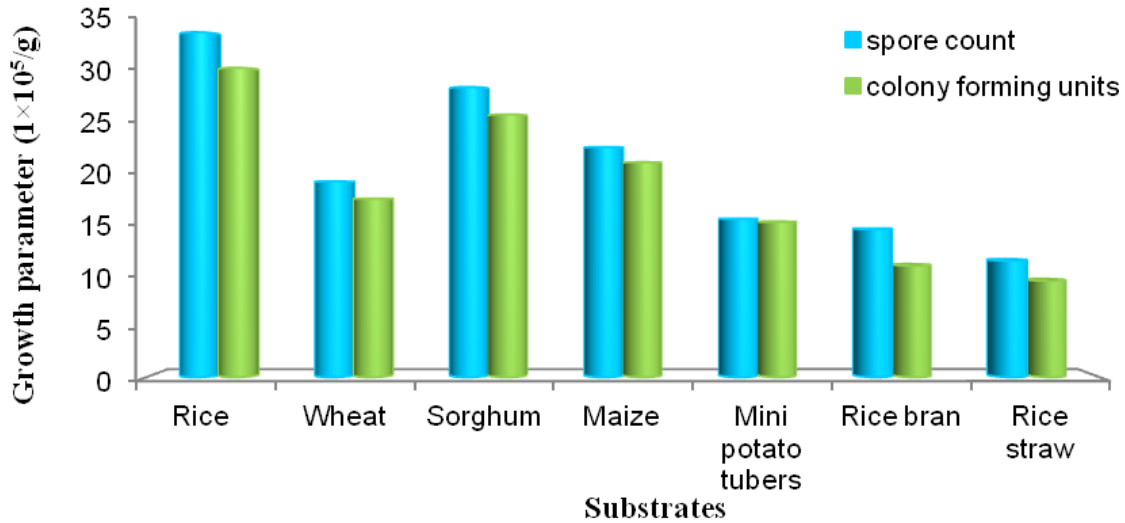


Figure 1. Conidial count and colony forming unit of *Beauveria bassiana* cultivated on different solid substrates.

It has been reported that the rice grain consists of 75-80% starch, 7% protein and sorghum contains 75% starch, 25% amylase, which are rich sources of carbon and nitrogen that enhanced the growth and sporulation (Oko et al., 2012). Mar and Lumyong (2012) also reported that *B. bassiana* showed maximum growth on rice. Sahayaraj and Namasivayam (2008) found sorghum to be the suitable substrate for large multiplication of *B. bassiana*. The rice straw and rice bran were the weakest supportive for *B. bassiana* growth. The growth in rice bran was less as compared to rice and sorghum. The reason may be that medium becomes compact mass after sterilization which does not allow *B. bassiana* to ramify with the medium (Puzari et al., 1997). The reason why rice straw did not support the growth of the fungus may be due to the presence of lignin (20-47%) and cellulose (30-45%) which act as binding material and results in hardness. Secondly, less nitrogen content (3.27%) and high silica content (94%) inhibits the fungal growth (Siwach and Jaipal, 2004).

All cereals used in this study, were coarsely broken to provide enough surface area to volume ratio for aeration and formation of conidia. Similarly, Karanja et al. (2010) also investigated the effect of broken rice, maize, machicha and maize husk for mass production of *B. bassiana* isolate (Bb575) and two *Metarhizium anisopliae* isolates (M58 and M60) and reported that enough surface provides better conditions for fungal growth, sporulation and such form of the substrate is a source of assimilable nutrients.

Effect of storage temperature on formulations

B. bassiana MTCC 6291 and *B. bassiana* MTCC 2028 biomass produced on rice were formulated with talc,

charcoal and oil. These formulations were stored at 4 and 25°C upto 90 days and their viability was evaluated fortnightly. In *B. bassiana* MTCC 6291 formulation, maximum cfu count (44.4×10^5 cfu/g) was recorded at 4°C on 15th day of storage in talc based formulation, which was at par with count recorded initially (control) (44.6×10^5 cfu/g). This was comparably at par with the count recorded upto 90th day at the same temperature. However, significantly lower count (12.3×10^5 cfu/g) was recorded at 25°C after 90 days of storage (Table 2a).

Hence, it can be concluded that in *B. bassiana* MTCC 6291 formulation, viable count of a *Beauveria* was at par with initial count upto three months at refrigeration temperature. Charcoal based formulation stored at refrigeration temperature recorded maximum mean colony forming units upto 30th day of storage and were at par with the control which recorded 39.3×10^5 cfu/g. However, significantly least count (6.6×10^5 cfu/g) was recorded at 25°C after 90 days of storage. In oil based formulation, colony forming unit ranged from 7.6 to 35.6×10^5 cfu/g at different temperatures. Colony forming unit (cfu) recorded upto 75th day of storage at 4°C were at par with control (36.0×10^5 cfu/g). However, significantly least cfu count (7.6×10^5 cfu/g) was recorded at 25°C on 90th day of storage. Similar results were recorded in *B. bassiana* MTCC 2028 formulation (Table 2b).

Thus, from the above results discussed, we conclude that storage of these formulations at refrigeration temperature recorded minimum decrease of viable count in both talc based *Beauveria* formulations (Figure 2). As at refrigeration temperature, the fungus undergoes the stationary phase of growth and the growth rate is decreased therefore, the nutrients requirement is low, so culture remain viable for more time as compared to 25°C. This supports reported findings, that decrease in temperature increases the longevity of *B. bassiana*

Table 2a. Effect of storage temperature on *B. bassiana* MTCC 6291 formulations.

Day	Colony forming units (1×10^5 cfu/g) of <i>B. bassiana</i> MTCC 6291 ((Mean \pm S.D*) (% decrease))					
	Talc		Charcoal		Oil	
	4°C	25°C	4°C	25°C	4°C	25°C
0 (Control)	44.6 \pm 8.02		39.3 \pm 11.50		36.0 \pm 8.18	
15	44.4 \pm 8.14 (0.8)	40.0 \pm 7.54 (10.3)	39.0 \pm 12.00 (0.7)	36.0 \pm 7.93 (8.3)	35.6 \pm 6.50 (1.1)	31.6 \pm 13.20 (12.2)
30	43.3 \pm 10.4 (2.9)	34.6 \pm 7.50 (22.2)	38.6 \pm 7.02 (1.7)	33.6 \pm 7.63 (14.5)	34.3 \pm 5.68 (4.7)	27.3 \pm 8.02 (24.1)
45	42.6 \pm 7.02 (4.4)	28.6 \pm 4.50 (35.8)	35.6 \pm 5.85 (8.6)	28.0 \pm 7.54 (28.7)	34.0 \pm 8.50 (5.5)	25.6 \pm 10.69 (28.8)
60	43.0 \pm 9.00 (3.5)	20.6 \pm 3.05 (47.5)	34.3 \pm 7.50 (12.7)	20.6 \pm 2.08 (47.5)	33.6 \pm 6.42 (6.6)	23.3 \pm 4.16 (35.2)
75	42.3 \pm 6.11 (5.1)	14.6 \pm 5.03 (67.2)	33.6 \pm 4.04 (14.5)	12.3 \pm 1.52 (68.7)	33.3 \pm 5.50 (7.5)	13.6 \pm 2.51 (62.2)
90	41.3 \pm 9.07 (7.3)	12.3 \pm 2.51 (72.4)	33.3 \pm 4.04 (15.2)	6.6 \pm 3.21 (83.2)	32.3 \pm 7.50 (10.2)	7.6 \pm 1.52 (78.8)
CD (5%)	CD (5%) Temperature = 3.45					

*Mean = Mean of three replications *S.D = standard deviation. Figure in parentheses are percentage decrease. (Decrease (%) = Initial count - final count/Initial count x 100)

Table 2b. Effect of storage temperature of *B. bassiana* MTCC 2028 formulations.

Days	Colony forming units (1×10^5 cfu/g) <i>B. bassiana</i> MTCC 2028 (Mean \pm S.D*) (% decrease)					
	Talc		Charcoal		Oil	
	4°C	25°C	4°C	25°C	4°C	25°C
0 (Control)	35.3 \pm 9.29		34.6 \pm 5.68		35.6 \pm 14.04	
15	35.0 \pm 4.04 (0.8)	32.0 \pm 7.21 (9.3)	34.0 \pm 3.60 (1.7)	31.0 \pm 2.64 (10.4)	35.3 \pm 5.68 (0.8)	33.6 \pm 8.02 (5.6)
30	35.0 \pm 3.60 (0.8)	29.0 \pm 7.54 (17.8)	33.6 \pm 6.80 (2.8)	28.6 \pm 6.11 (17.3)	35.0 \pm 5.00 (2.7)	31.3 \pm 9.07 (12.0)
45	34.6 \pm 6.02 (1.9)	26.0 \pm 9.64 (26.3)	33.6 \pm 6.50 (2.8)	22.6 \pm 4.04 (34.6)	34.3 \pm 6.42 (3.6)	28.3 \pm 6.02 (20.5)
60	34.0 \pm 5.56 (3.6)	22.3 \pm 8.73 (36.8)	33.0 \pm 4.58 (4.6)	18.6 \pm 5.03 (46.2)	33.6 \pm 8.32 (6.6)	22.6 \pm 3.05 (36.5)
75	34.3 \pm 4.16 (2.8)	16.6 \pm 3.21 (52.9)	32.3 \pm 6.50 (6.6)	14.6 \pm 4.04 (57.8)	32.6 \pm 5.68 (8.4)	15.3 \pm 3.21 (57.0)
90	32.6 \pm 4.12 (7.6)	10.6 \pm 5.03 (69.9)	31.6 \pm 9.16 (8.6)	8.6 \pm 4.16 (75.1)	32.3 \pm 4.72 (10.2)	10.3 \pm 2.51 (71.0)
CD (5%)	CD (5%) Temperature = 3.51					

*Mean = Mean of three replications *S.D = standard deviation. Figure in parentheses are percentage decrease (Decrease (%) = Initial count - final count/Initial count x 100).

conidia (Hong et al., 1997). Morley-Davies et al. (1995) reported that too low temperature can harm the conidia of *M. anisopliae* and *B. bassiana* fungi and concluded that temperature of long-term storage should be maintained preferably between 0 and 20°C. Shimizu and Mitani

(2000) reported that high-temperature treatment killed *B. bassiana* conidia in oil formulations. Results of the present investigations are also similar to findings of Ramegowda (2005) who reported 82.47% conidial viability of *Normuraea rileyi* formulations stored for 180

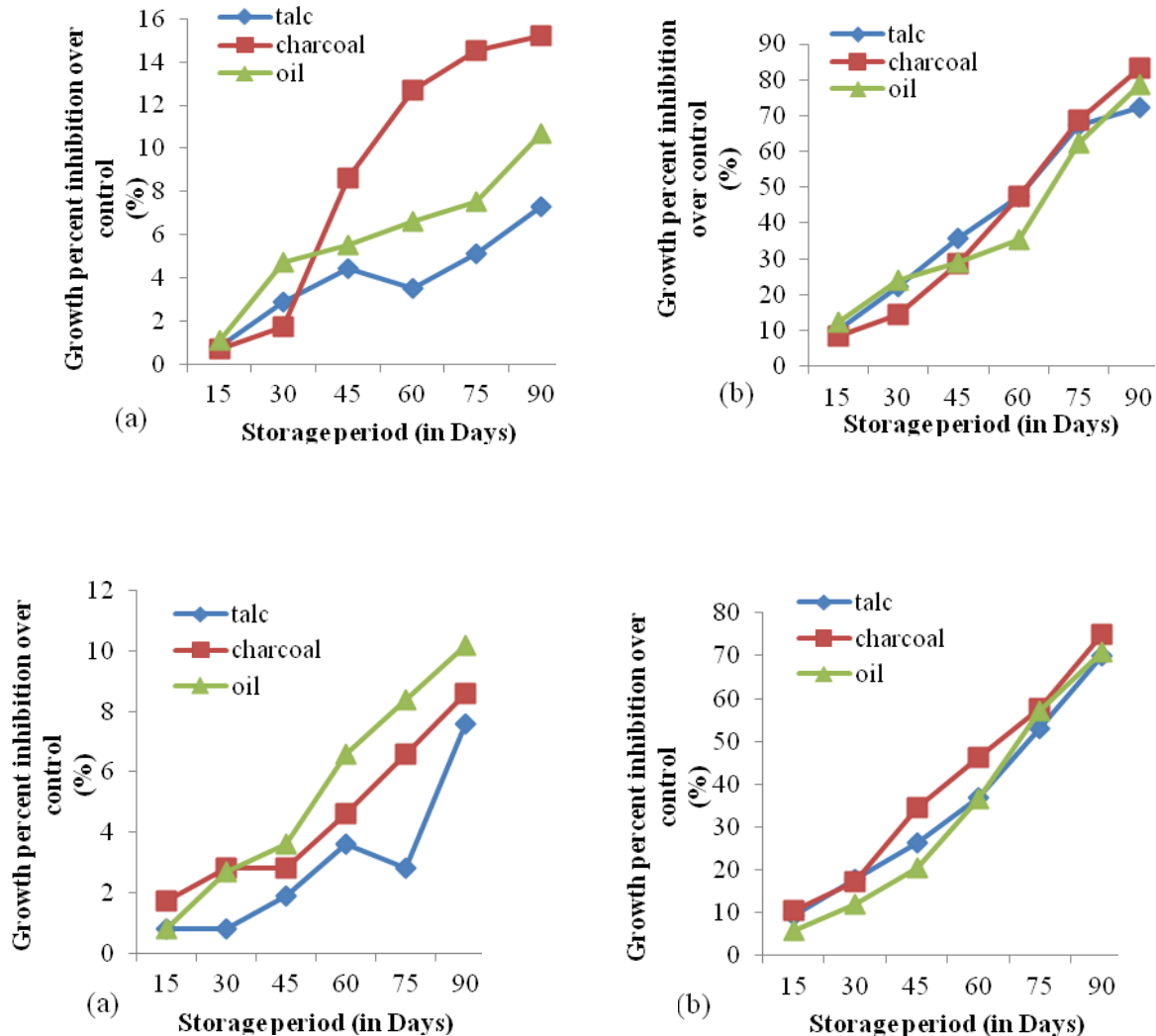


Figure 2. Growth percent reduction over control of (I) *B. bassiana* MTCC 6291 (a) At 4°C (b) At 25°C (II) *B. bassiana* MTCC 2028 (a) At 4°C (b) At 25°C in different formulation (talc, charcoal and oil).

days in refrigerated condition, as compared to 63.23% under room temperature formulation with talc at 4°C.

Described experimental work showed that for mass production of *B. bassiana* formulation on solid substrate, rice is the best substrate. Studies of effect of carriers on the formulations have shown that viability of formulations on storage was optimum in talc based formulation as compared to charcoal and oil at refrigeration temperature over the period of 90 days of storage.

Conflict of Interests

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

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Full Length Research Paper

Isolation and molecular Identification of fungi associated with the spoilage of farms fruit in Southern Côte d'Ivoire

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The spoilage of pawpaw (*Carica papaya* L.), pepper (*Capsicum* sp.) and tomato (*Lycopersicon esculentum*) from three selected farms in Agboville area, south of Côte d'Ivoire were investigated. 50 samples of each type of fruit (pawpaw, pepper and tomato) showing spoilage signs were examined for the presence of fungal pathogens inducing spoilage. Eight fungal species, *Mucor velutinosus*, *Meyerozyma guilliermondii*, *Colletotrichum higginsianum*, *Rhizopus oryzae*, *Mucor circinelloides* f. *circinelloides*, *Fusarium oxysporum*, *Rhizopus stolonifer* and *Geotrichum* sp. identified by polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE), were found associated with the deterioration of the fruit. All fungi found in the fruit were common to pawpaw (*C. papaya*) and tomato (*L. esculentum*) except *M. guilliermondii* which was only found in pawpaw (*C. papaya*). *F. oxysporum*, *R. stolonifer* and *R. oryzae* were associated with the spoilage of pepper (*Capsicum* sp.). *F. oxysporum* had the highest rate of occurrence among the isolated fungi (30%) followed by *R. oryzae* with 15% occurrence. *M. velutinosus*, *Geotrichum* sp., *M. circinelloides* f. *circinelloides*, *R. stolonifer* and *C. higginsianum* had all 10% occurrence. *M. guilliermondii* had the lowest rate at 5%. The result reveals that the presence of fungi before harvesting may be damageable to the fruit, so the use of natural control agents to ensure an effectiveness of the production, the marketing, product quality food safety appears to be very important.

Key words: Tomato (*L. esculentum*), pawpaw (*Carica papaya*), pepper (*Capsicum* sp.), polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE).

INTRODUCTION

Fruit represent an important part of food produce in African tropical countries and they play a significant role

in human diet through the supply of vitamins and minerals to the organism. Tropical fruit in Côte d'Ivoire

include crops such as pawpaw, tomato and pepper, which are consumed fresh, cooked or processed to products such as juice and jam.

In 2010, world production of fruit and vegetables amounted to 1,639 million tons, 67, 13, 10, 9 and 1% respectively for Asia, America, Europe, Africa and Oceania. World trade in fresh fruit and vegetables displayed, meanwhile, 97 million tons (FAOSTAT, 2010).

Among the various fruit grown in Côte d'Ivoire, pawpaw (*C. papaya*, var. Solo8) is one of the most important tropical fruit exported every year toward the European Union. The pawpaw fruit contain minerals such as Na, K, Ca, Mg, P, Fe, Cu, Zn and Mn. It is also a source of carotenoids, vitamins C, thiamin, riboflavin, vitamin B6 and vitamin K (Adetuyi et al., 2008). The hot pepper (*Capsicum annuum*) is also considered to be the most known and nutritive legume. It is rich in nutrients and is used for its flavor, as a spice, and as colorant to food due to its color properties (Mueller et al., 2010; Kouassi and Koffi-Nevry, 2012). The hot peppers are abundant and savory during the months of August and September and are consumed during all year around. The tomato (*Lycopersicon esculentum*) is a climacteric fruit, with very high nutritional qualities due to its composition which includes dry matter, soluble solids, amino acids, pigments, simple sugar, organic acids, citric acid and others volatile compounds and more than 400 aromatic compounds contributing to its taste, flavor, and savor (Thybo et al., 2006). However, the quality of tropical fruit is commonly affected by postharvest diseases such as fruit rot, which are mostly caused by improper handling and storage during transportation and marketing. The fruit are affected by a large range of micro-organisms such as pathogenic fungi, which cause their degradation as indicated by the changing in the taste, the odor, the appearance or the texture, resulting in less appealing and toxic fruit. These microorganisms can contaminate the seed prior to sowing, during plant growth in the farms, harvesting, post-harvest handling, or while these fruit are stored and during distribution (Barth et al., 2009). About 20 to 25% of the harvested fruit are rotten by these agents during the post-harvest handling even in the developed countries, negatively influencing the economic value of the fruit (Droby, 2006). The amount of global food losses and waste annually represents about 40-50% of roots, fruit and vegetables (FAO, 2014). Fungal activities can also lead to contamination with mycotoxins, and could represent a health risk to the consumers. Such risks could be reviewed in studies on papaya fungal and viral postharvest diseases accomplished in Côte d'Ivoire (Diallo et al., 2007; Koffi-Nevry et al., 2011).

However, little has been done to prevent fungi spoilage of the fruit in farms of Côte d'Ivoire. These recent years, the frequency of the fungal attack on the fruit has requires a special attention of current researches in order to isolate and identify fungi associated to these various deteriorations on farm fruit. The purpose of the present study was to identify the fungal strains profiles associated to the rotting of pawpaw, tomato and hot pepper in different farms of Côte d'Ivoire.

MATERIALS AND METHODS

Sampling

Three types of fruit (hot pepper, pawpaw, tomato) found with symptoms of fungal infection were sampled in three different farms in the area of Azaguié and Tomassé in the morning (8.00 AM to 12.00PM). The fruit were harvested at random in 2011 in the region of Agneby-Tiassa. It is located between 5° 38'00" North and 4° 5'00" west on the road to Agboville about 35 kilometers from Abidjan (Côte d'Ivoire). The sequences obtained were deposited in GenBank. For this study, 50 samples of pawpaw were sampled in a farm at Tomassé, 50 samples of tomatoes and 50 samples of hot pepper were sampled in two different farms at Azaguié. The farm fruit presenting symptoms were harvested on the plants, placed in sterilized stomacher bag and stored in a cooler at 8°C.

Isolation of fungi

After receiving the samples, physical observation of the diseases were described. Each of the fruit was washed with (1/10) sodium hypochlorite solution. A little infected portion of each fruit was cut and inoculated. The various portions cut describing rots were placed directly onto sterile Sabouraud chloramphenicol medium plates (Figure 1). All plates were incubated at 25 °C for 72 h. Each fungal strain is inoculated on three different Sabouraud chloramphenicol medium plates for isolation and purification. The isolated strains were macroscopically characterized by a visual observation, based on the description of the morphology and color of the colonies.

Molecular identification of fungi

The strains isolated were identified by a Denaturing Gradient Gel Electrophoresis -PCR (DGGE-PCR) method characterized by the extraction of the total fungal DNA, the amplification of the DNA by the PCR, and the DGGE separation (Li et al., 2008; El Sheikha et al., 2011). For the PCR amplification, the D1/D2 region of the 28S rDNA from the fungi was amplified by two GC-rich primer set U1f (5'- CGC CCG CCG CGC GCG GCG GCGGG GCG GGG GTG AAA TTG TTG AAAGGG AA - 3') and U2r (5'- GAC TCC TTG GTC CGT GTT - 3'). For the DGGE method, a GC-clamp of 30 nucleotides was added to the U1f primer at the 5'-end in order to ensure that the resulting DNA fragments will partially remain (Sheffield et al., 1989). Then the denaturing gradient gel

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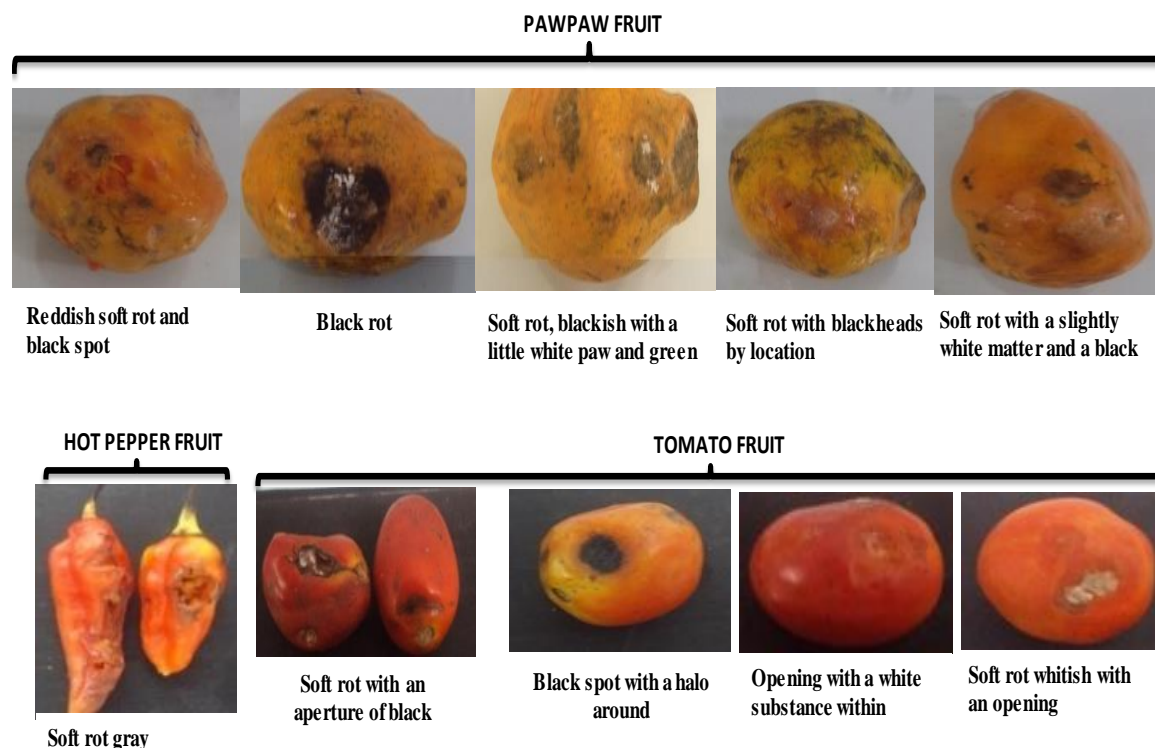


Figure 1. Description of fungi disease symptoms on fruits.

Table 1. Macroscopic description of the strains

N° strains	Macroscopic description of the strains
1	Whitish mycelium with invasive character as duvet.
2	Colony with cottony globular aspect that becomes brown-black when it is matured. There are also creeping branches, markedly elevated and away from the substrate.
3	Colony cottony, white-yellow color with invasiveness. Colonies are fast growing, flat, dusty the waxy, white cream on the surface.
4	A strain having an aspect in form cottony white, creamy white then turning purple with a purple background color.
5	The strain gave invasive gray colonies, presenting also a globular aspect at the beginning and becomes dark at maturity.
6	A cottony colony mycelium sparse becoming gray with maturity with a yellow-whitish center.
7	A cottony strain, whitish at the beginning and becoming brownish gray to blackish gray after the third day.
8	A colony of cottony aspect and an orange color with a background colorless after 3 days of growth.

electrophoresis (DGGE) was done after ensuring that the PCR product from acrylamide gel (8%) (37, 5/1, v/v/v, Promega, France) contains a chemical denaturing gradient (40 to 70 % urea) and formamide. The DGGE was performed according to the method used by Kouakou et al. (2012). For the sequencing of the DNA fragment from DGGE bands, the bands were cut from the DGGE gel with a sterilized scalpel and eluted in 100 ml of TE buffer at 4°C for 24 h. The DNA is then re-amplified in the same condition using a primer without GC-clamp before the products were sent for sequencing at GATC Biotech (Germany). The 28S rDNA sequences obtained were compared with those available in GenBank database, at the National Center for Biotechnology Information (NCBI) website using the program BLAST in order to determine the nearest known sequences (Table 2).

RESULTS AND DISCUSSION

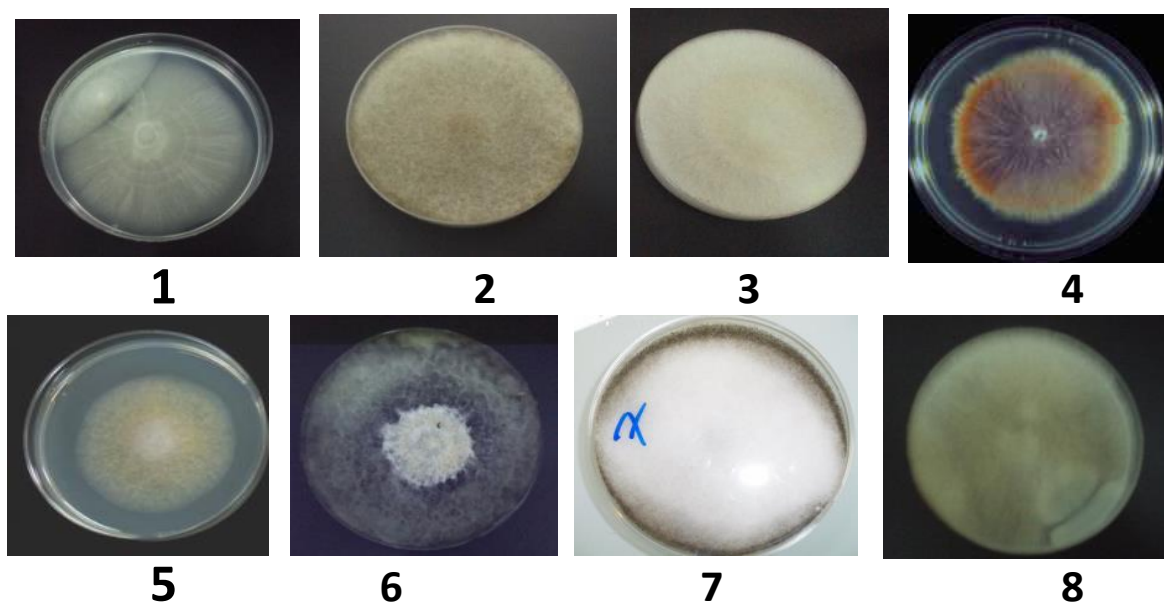
Descriptions of the fungal symptoms on fruits

Symptoms of microbial contamination occurred in the form of necrosis of soft rots apparently reddish, blackish, whitish, greenish or grayish color with or without openings and also the presence of round spots as shown in Figure 1 and Table 1. These symptoms are characteristics of fungal diseases similar to those described by Cannon et al. (2012) on exported tropical fruit.

Table 2. Summary of strains identified for each fruit.

Strains	Identity (%)	Genbank accession number	Fruits		
			Pawpaw	Tomato	Pepper
<i>Mucor velutinosus</i>	98	JN874486	+	+	-
<i>Meyerozyma guilliermondii</i>	98	JX423568	+	-	-
<i>Colletotrichum higginsianum</i>	86	CACQ02008496	+	+	-
<i>Geotrichum sp</i>	97	AB741076	+	+	-
<i>Mucor circinelloides f. circinelloides</i>	97	JN939203	+	+	-
<i>Fusarium oxysporum</i>	95	JX081386	+	+	+
<i>Rhizopus stolonifer</i>	100	JN938904	+	+	+
<i>Rhizopus oryzae</i>	99	JN938902	+	+	+

+, Strain present; -, strain absent.

**Figure 2.** Macroscopic description of the strains

Isolation and macroscopic identification of fungal strains

From the infected fruit, 24 strains of fungi were isolated. The analysis of the morphology, after isolation and purification, permitted obtaining eight (08) morphological groups strains different from each other by their appearance, density, color, size, and the mycelium as described in Figure 2.

Molecular identification of the fungal strains

Strains were identified using the molecular method of PCR-DGGE. The quality of the DNA was confirmed by

PCR amplification of the fungal conserved 28S rDNA region using GC U1F⁻ - CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GTG AAA TTG TTG AAA GGG AA - 3[']), U2r (5['] - GAC TCC TTG GTC CGT GTT - 3[']) primers with control DNA from pure strains as are presented (Figure 3).

PCR products provided unique, unambiguous and intense bands between 298 and 220 bp, which corresponded to the expected size of 260 bp for fungi. The negative control was performed with the reaction mixture without the addition of DNA extract. The absence of a band for the negative control showed that there was no contamination of the PCR reaction mixture. The bands of the DGGE profile obtained were finally sequenced for strains identification. Identification of strains revealed (08)

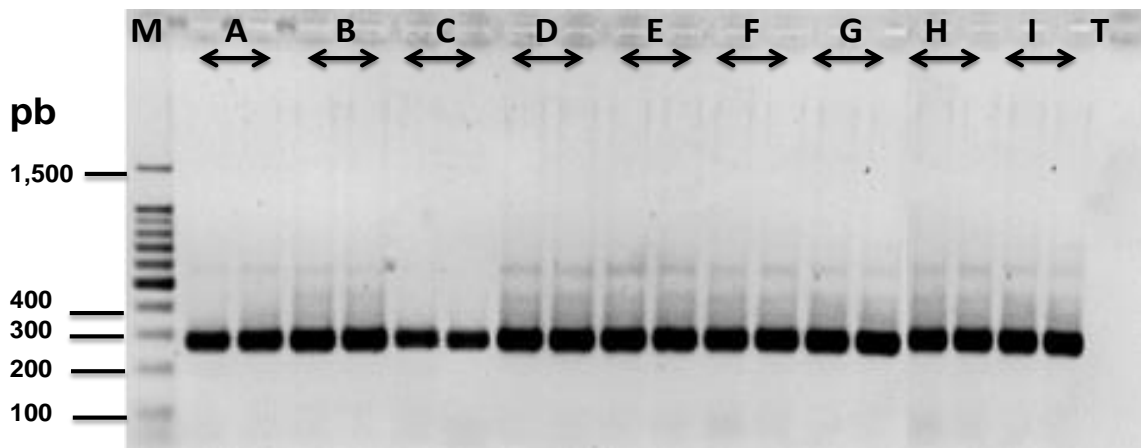
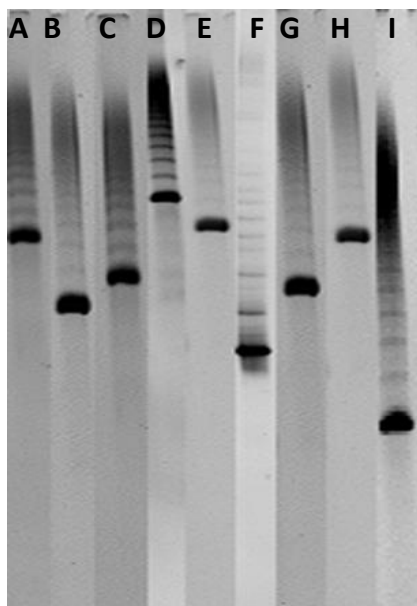


Figure 3. Gel of PCR of fungi DNA verification. T, negative control; M, Marker; bp, base pair; ABCDEFGHI, different fungi



- A:** *Mucor velutinosus*
B: *Meyerozyma guilliermondii*
C: *Rhizopus oryzae*
D: *Geotrichum sp.*
E: *Mucor circinelloides f. circinelloides*
F: *Fusarium oxysporum*
G: *Rhizopus stolonifer*
H: *Mucor circinelloides f. circinelloides*
I: *Colletotrichum higginsianum*

Figure 4. DGGE Gel and identified fungi strains.

distinct species such as: *M. velutinosus*, *M. guilliermondii*, *C. higginsianum*, *R. oryzae*, *M. circinelloides f. circinelloides*, *F. oxysporum*, *R. stolonifer*, and *Geotrichum sp.* (Figure 4).

These results show that these fruit and vegetables (pawpaw, tomato and chili) collected in different farms are true hosts of these fungal agents. So they are responsible for diseases affecting pawpaw fruit on the farms. This result is in conformity with those of Chukwuka et al. (2010) who recently reported that *Fusarium sp.*, *Mucor sp.* and other germs were responsible for the rot of

pawpaw in the Nigeria farms. *M. guilliermondii* was only isolated from pawpaw sample from Tomassé farms indicating that the strains vary according to the farm. Fungi isolated from tomato (*L. esculentus*) included *F. oxysporum*, *M. velutinosus*, *C. higginsianum*, *R. oryzae*, *M. circinelloides f. circinelloides*, *R. stolonifer* and *Geotrichum sp.* Almost all of the species found on papaya were also present on tomatoes except for *M. guilliermondii* (Table 2).

These results show that pawpaw and tomato might be infected by the same germs, causing diseases in the

Table 3. Frequency of occurrence of strains on fruits

Strain	Number	Frequency of occurrence (%)
<i>Mucorvelutinosus</i>	2	10
<i>Meyerozyma guilliermondii</i>	1	5
<i>Colletotrichum higginsianum</i>	2	10
<i>Geotrichum sp</i>	2	10
<i>Mucor circinelloides f. circinelloides</i>	2	10
<i>Fusarium oxysporum</i>	6	30
<i>Rhizopus stolonifer</i>	2	10
<i>Rhizopus oryzae</i>	3	15
Total	20	100

farm. The presence of these germs on these studied fruit and vegetables would be favored by the climatic conditions of the country because these organisms have the ability to grow at 37°C as highlighted by Sugui et al. (2011). Among the isolated germs, *F. oxysporum*, *R. stolonifer* and *Mucor* sp. were pathogenic to tomato as confirmed by studies of Chuku et al. (2008) and Akinmusire (2011), who have taxed these germs to be responsible for soft rotting of tomato. *F. oxysporum* was also held responsible for rotting pawpaw, tomato, banana, and guava (Latiffah et al., 2012). Furthermore works of Kleemann et al. (2008) associated *C. higginsianum* with anthracnose disease affecting these fruit. Other studies on fungi have shown that *F. oxysporum*, *R. stolonifer*, *Mucor* sp., and *Geotrichum* sp. were associated with soft rot and sour of tomato and pawpaw (Chuku et al., 2008; Nnebue et al., 2013). *R. oryzae* would be responsible for the disease causing rotting of these fruit as in the case of sunflower due to favorable climatic condition such as the temperature (Yildiz and Baysal, 2006).

Concerning the pepper, it revealed the presence of *F. oxysporum*, *R. stolonifer* and *R. oryzae* pathogens of this fruit. However, Hammami et al. (2014) found species of *Aspergillus tamarii*, and *Aspergillus Flavus* in Egyptian chili powder.

The germs isolated from the disease-affected zones of the fruit (pawpaw, pepper and tomato) are pathogenesis of these fruit at different levels as reported by Akintobi et al. (2011). According to the works of these authors, *R. stolonifer* has a high degree of pathogenicity compared to *Fusarium* sp. Moreover *M. guilliermondii* was isolated only on the pawpaw in that study. Although it was isolated on pawpaw rots, it is not necessary a pathogen of the fruit. Recent studies showed that *M. guilliermondii* inhibits the growth of fungi during the storage of maize in Cameroon, according to Su-lin et al. (2012). However, *M. velutinosus* isolated on the pawpaw and the tomato was also pathogens agent for human because they were responsible for skin infection as reported by Sugui et al.

(2011) suggesting that they represent a threat to the health of farmers and consumers. *F. oxysporum*, *R. stolonifer* and *R. oryzae* were common pathogens strains of the three types of fruit in this study. But for the frequency of occurrence of the isolated fungi associated to the fruit deterioration, *F. oxysporum* was the most frequently isolated (30%) on the three types of fruit. It was followed by *R. oryzae* with an infection rate of 15% while *M. guilliermondii* was the less observed (5%) (Table 3).

Conclusion

Eight (8) spoilage fungi such as *M. velutinosus*, *M. guilliermondii*, *C. higginsianum*, *R. oryzae*, *M. circinelloides f. circinelloides*, *F. oxysporum*, *R. stolonifer*, and *Geotrichum* sp. were found responsible for the rotting and the diseases of local pawpaw, tomato, and chili fruit in the farms of south of Côte d'Ivoire with *F. oxysporum* being the major contaminant. This result implies tough realities for the farmers in guaranteeing safe products for consumption and for trading. The high rate of some fungi contamination requires the adoption of appropriate control measures against farms infection. Therefore, the deterioration of fruit can be controlled in the farms by using natural antifungal extracts from plants and by the correct handling during the harvesting in order to avoid cuts and reduce losses.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

New records and new distribution of known species in the family Orbiliaceae from China

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The family Orbiliaceae belongs to Orbiliales, Orbiliomycetes, Pezizomycotina and Ascomycota. It presently includes *Orbilia*, *Hyalorbilia*, and *Pseudorbilia*, which have caused more attention in due that some members of their 10 anamorphic genera are the nematode-trapping fungi. During the survey of the distribution of Orbiliaceae since the summer of 2005, three new records including *Orbilia xanthostigma*, *Orbilia tenebricosa*, *Hyalorbilia fusispora* and new distribution of five known *Hyalorbilia* species are firstly reported from Mainland China and provided clearer illustrations.

Key words: Orbiliaceae, *Orbilia xanthostigma*, *Orbilia tenebricosa*, taxonomy.

INTRODUCTION

Orbilia Fr., *Hyalorbilia* Baral et al. and *Pseudorbilia* Zhang et al. are the only genera presently accepted in the family Orbiliaceae Nannf (Eriksson et al., 2003; Zhang et al., 2007; Kirk et al., 2008). The model genera *Orbilia* possessed easily separated asci and paraphyses, the excipulum composed of globose cells, the asci arising from H- or L-shaped bases, and on the contrary the main characters of *Hyalorbilia* are that the asci arising from crosiers and conglutinated with the paraphyses by a gel, the excipulum cells composed of rectangular cells; the key features of *Pseudorbilia* are between *Hyalorbilia* and

Orbilia (Zhang et al., 2007). The shape and size of spore bodies (SBs) and their distribution have been recognized as the key characteristic in specific classification within Orbiliaceae.

Before 2005, Orbiliaceae is rarely studied in China, only 6 species were recorded (Teng, 1939; Zhuang and Korf, 1989; Zhuang, 1997, 1999; Zhuang and Wang, 1998a, b; Zhuang and Hyde, 2001; Liu et al., 2006). During latest eight years, Orbiliaceae and its anamorphs have been studied more in China. Besides a new genus *Pseudorbilia* (Zhang et al., 2007), eight new *Orbilia*

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species, eight new *Orbilbia* records and three new *Hyalorbilia* species were reported from China (Wu et al., 2007; Zhang et al., 2009; Su et al., 2010). Together with anamorphs or single, other scattered new species were reported (Zhang et al., 2006; Liu et al., 2005a, b; Mo et al., 2005a,b; Yu et al., 2006, 2007a, b; Yu et al., 2009a,b; Li et al., 2009; Qiao et al., 2011). In Chinese publication, Liu et al. (2007a, b) and Guo et al. (2007) reported seven new records of *Orbilbia* and of *Hyalorbilia*.

During the survey of the distribution of Orbiliaceae since the summer of 2005, three new records including *O. xanthostigma*, *O. tenebricosa*, *H. fusispora* and new distribution of five known *Hyalorbilia* species are firstly reported from Mainland China and provided clearer illustrations.

MATERIALS AND METHODS

The specimens were collected by J.W. Guo and S.F. Li from the south to Qinling-Huai Line including Henan Province, Anhui Province, Jiangsu Province, Jiangxi Province, Hubei Province, Hunan Province, Sichuan Province, Zhejiang Province, Fujian Province, Guangdong Province, Guangxi Province, Guizhou Province and Yunnan Province since September 2005 to July 2010, and deposited in Laboratory for Conservation and Utilization of Bio-Resources of Yunnan University. In addition, the specimens were collected by J.W. Guo and T.Z. Ye in Daweishan Mountain Reserve, Honghe City, Yunnan since October 2011, and deposited in Key Laboratory of Higher Quality and Efficient Cultivation and Security Control of Crops for Yunnan Province, Honghe University.

The living ascospores were observed according to the methods presented by Baral (1992). Specimens were sectioned longitudinally using a freezing microtome at a thickness of 5-10 µm to observe the vertical structure of apothecia. Observations, measurements, and photographs were carried out with an Olympus BX51 microscope of differential interference contrast.

RESULTS

During the samplings Orbiliaceous fungi were gathered on rot branches, more than 1500 specimens were collected from the south to Qinling-Huai Line. From these collections eight species of the genera *Orbilbia* and *Hyalorbilia* were identified; three of them are first recorded from China and other five known *Hyalorbilia* species are stated new distribution in China.

New records of Orbiliaceae in Mainland, China

Orbilbia xanthostigma (Fr.) Fries, Summa Vegetabilium Scandibaviae 2: 357, 1849 (Figure 1)

Basionym: *Pezzia xanthostigma* Fries, Observations Mycologicae 1: 166, 1815.

Calloria xanthostigma (Fr.) W. Phillips, A Manual of the British Discomycetes: 329, 1887

Specimens examined: PR China, Sichuan Province, Jiuzhaigou Nature Reserves, on rotten branch of uniden-

tified broadleaf tree, lying on moist ground under subtropical broadleaf forest, J.W. Guo and S.F. Li, 7 August 2007, lwh-1.

Apothecia massed or scattered on the periderm of a partly decayed branch of unknown deciduous tree, disc smooth at the margin, flat, 0.3-1.2 mm diam., subsessile with a very short and broad obconical base, reddish to wate-r-whitish, semitranslucent. Ectal excipulum composed of polygonal to subglobose cells, hyaline, thin-walled, most 5.2-15.2 µm diam. subglobose and larger cells at the frank. Asci cylindrical to clavate, 16.0-22.8 × (2.8-) 3.4-5.3 µm, 8-spored, rounded to truncate at the apex, eventually shrunken down the base and forked. Ascospores hyaline, without septa, symmetrically and crossed arranging in each ascus, slightly helical, strongly curved with sickled-shaped, both ends obtuse, 6.0-8.5(-10.0) × 1.0-1.4 µm, a refractive tear-shaped spore body (SB) at the upper, 1.6-2.5 × 0.5-0.9 µm. Paraphyses hyaline, vacuolar bodies (VBs) globose to cylindrical, slightly inflated up to 2.3-3.5 µm diam. at the apex, exudates 1.0-1.3 µm thick over paraphyses and asci.

Known distribution: Russia, Georgia, Sloviakia, British, Venezuela and so on.

New distribution: Sichuan Province, China.

Orbilbia xanthostigma resembles *O. vermiformis*, whereas the spores of the latter are more curving and slender; in addition, it distinguishes that *O. vermiformis* has anamorph *dactylellia*-type but *O. xanthostigma* has anamorph *dicranidion*-type.

Orbilbia tenebricosa (Svrček) Baral, Mycotaxon 96: 167, 2006 (Figure 2)

Basionym: *Patinella tenebricosa* Svrček, Česká Mykol. 31(3): 135, 1977

Specimens examined: PR China, Yunnan Province, Kunming City, Xishan Forest Park, on rotten branch in moist evergreen broadleaf forest, J.W. Guo and S.F. Li, 16 May 2007, xs -6.

Apothecia scattered on the tip of decayed branch of unknown tree, 0.7-1.4 mm diam., nonsessile, fresh disc yellowish, flat and semitranslucent, yellow when dried. Asci cylindrical, 22.0-28.0 × 4.3-5.0 µm (living state), 8-spored including 2-3 inverted spores in each ascus; rounded to truncate at the apex, eventually shrunken down the base which forked with "h-" or "k-shaped". Ascospores hyaline, nonseptate, spindle to rod shaped, slightly curved or straight with both shrunken ends, 5.0-8.0 × 1.1-1.4 µm (living state), a refractive globose- or ellipse-shaped SB at the upper, 0.7-1.5 × 0.5-1.0 µm. Paraphyses 2.0 µm width at the base, slightly inflated up to 2.5 µm width at the apex, exudates over paraphyses and asci.

Known distribution: Czech, Australasia

New distribution: Yunnan Province, China.

Orbilbia tenebricosa similar to *Orbilbia rectispora* but having shorter and wider spores (5.0-8.0 × 1.1-1.4 µm vs. 7.0-9.9 × 0.9-1.2 µm).

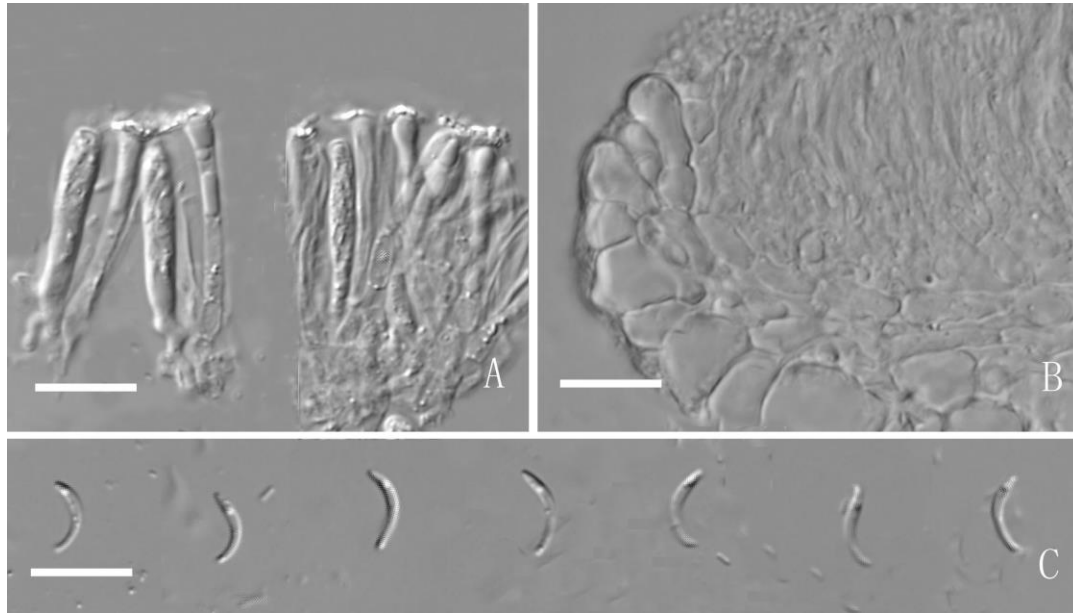


Figure 1. *O. xanthostigma* A. Asci and paraphyses; B. Vertical section of part apothecium; C. Ascospres. Bars: A-C=10 μ m.

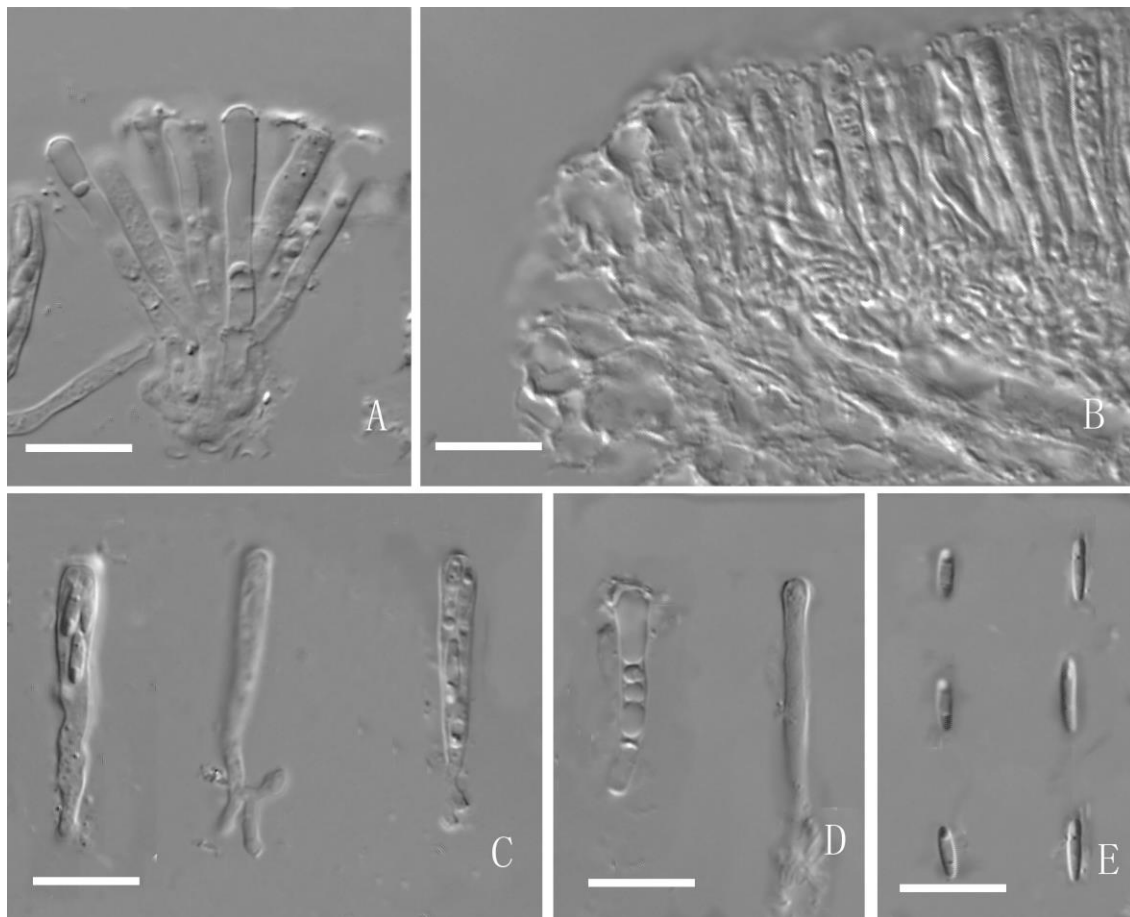


Figure 2. *O. tenebricosa* A. Asci and paraphyses; B. Vertical section of part apothecium; C. Asci; D. Paraphyses; E. Ascospores with spore body. Bar: A-E=10 μ m.

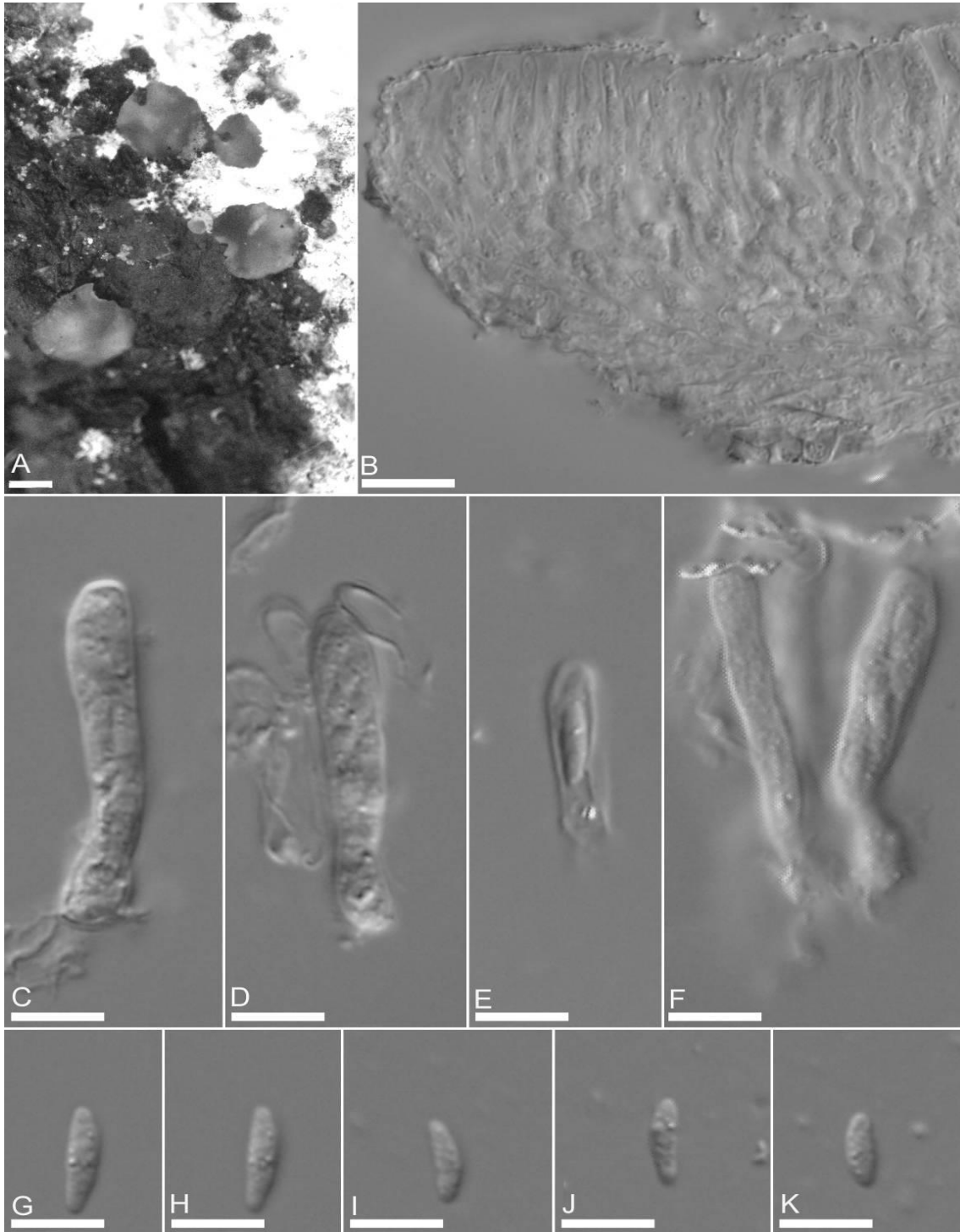


Figure 3. *H. fusispora* A. Rehydrated apothecia; B. Vertical section of part apothecium; C-E. Asci; F. Asci and paraphyses; G-K. Ascospores. Bar: A= 0.5 mm, B= 10 μ m, C-K = 5 μ m.

Hyalorbilia fusispora (Velen.) Baral & G. Marson, *Micologia* 2000: 44, 2001 (Figure 3)

Basionym: *Orbilia fusispora* Velen., *Monogr. Discom. Bohemiae*: 96, 1934.

Specimens examined: China, Anhui Province,

Jiuhuashan, on rotten branch of broadleaf tree, lying on moist ground, J.W. Guo, 28 September 2009, jhs-31; China, Anhui Province, Huangshan, on rotten branch of unidentified tree, lying on moist ground, J.W. Guo, 13 September 2009, hs-7, hs-8 and hs-21; China, Jiangsu

Province, Nanjing City, Zijinshan, on rotten branch of unidentified tree, lying on moist ground, J.W. Guo, 10 September 2009, zjs-34; China, Jiangxi Province, Wuyuan County, Wengong Mountain, on rotten branch of broadleaf tree, lying on moist ground, J.W. Guo, 7 September 2009, wgs-6; China, Zhejiang Province, Linan City, Tianmushan Mountain, on rotten branch of unidentified broadleaf tree, lying on moist ground, J.W. Guo, 3 September 2009, tms-5; China, Yunnan Province, Kunming City, Shuanglong Town, on rotten branch of broadleaf tree, lying on moist ground, J.W. Guo, 19 May 2006, sl-j-6; China, Yunnan Province, Kunming City, Xishan Forest Park, on rotten branch of unidentified tree, lying on moist ground, J.W. Guo, 16 June 2006, xs-j-12; China, Yunnan Province, Yuxi City, Yimen County, Longquanshan Forest Park, on rotten branch of unidentified tree, lying on moist ground, J.W. Guo, 23 August 2006, ym-j-179.

Apothecia scattered, superficial, sparse, slightly concave to cup-shaped and whitish when fresh, flat and straw yellowish when dried, 0.3-1.5 mm diam., sessile or short-stipitate, margin even, waxy, translucent. Ectal excipulum of texture rectangular to texture angular, 12.3-60.0 μm thick, cells hyaline, thin-walled, 5.0-14.5 \times 3.2-9.5 μm . Hymenium strongly coherent, 15.4-33.9 μm thick, asci and paraphyses immersed in a gelatinous matrix. Asci cylindrical, apex thin-walled and rounded, unforked at the base, shrunken and curving near the base, 16.6(19.5-)-22.5(-26.2) \times 2.8(3.5-)-4.3(-5.9) μm , 8-spored. Ascospores fusoid, straight or some slightly curved, most strongly inflated and slightly curved near one third of one end, hyaline, nonseptate, 4.2(4.5-)-6.1(-6.3) \times 1.3(1.6-)-1.6(-1.8) μm , one elongate SB and three to five further peripheral globose SBs (living state) of 0.4-0.5 diam in each end, most of ascospores biserial or only exceptionally irregularly uniseriate. Paraphyses apex slightly inflated, cylindrical, hyaline, nonseptate, 21.1-28.6 \times 1.8-2.6 μm .

Known distribution: Czech.

New distribution: Anhui Province, Jiangsu Province, Jiangxi Province, Zhejiang Province, and Yunnan Province in China.

Orbilbia fusispora was firstly described by Velenovský (1934). The key character is the approximately fusoid spores. According to the new taxonomic treatments (Baral and Marson, 2001), it was transferred to *Hyalorbilia*. Liu et al. (2007b) reported *H. fusispora* collected in China distributing in Beijing, Liaoning Province, Henan Province, Hubei Province and Jiangsu Province, but it should be *H. ulicicola* (a new species in the unpublished monograph of H.O. Baral) according to the shape of ascospores.

The similar species is *H. juliae* (Velen.) Baral, Priou & G. Marson. They are similar in the shape of ascospores and the number of SBs, but obviously differ in the distribution of SBs.

New distribution of Orbiliaceae in Mainland, China

Hyalorbilia juliae (Velen.) Baral, Priou & G. Marson, Bull. Mens. Soc. Linn. Lyon, S74: 55, 2005 (Figure 4)

Basionym: *Orbilbia juliae* Velen., Monogr. Discom. Bohemiae: 95, 1934.

Specimens examined: China, Anhui Province, Huangshan, on rotten branch of broadleaf tree, lying on moist ground, J.W. Guo, 13 September 2009, hs-11; China, Yunnan Province, Kunming City, Songhuaba Forest, on rotten branch of unidentified tree, lying on moist ground, J.W. Guo, 16 May 2006, shb-j-2; Baoshan City, Shidian County, on rotten branch of unidentified tree, lying on moist ground, J.W. Guo, 12 July 2006, sd-j-5; China, Yunnan Province, Yuxi City, Yimen County, Longquanshan Forest Park on rotten branch of unidentified tree, lying on moist ground, J.W. Guo, 1 August 2006, ym-j-4; China, Sichuan Province, Aba County, Jiuzhaigou Nature Reserves, on rotten branch of unidentified tree, lying on cool and moist ground, J.W. Guo, 8 October 2006, ys-j-8.

Apothecia scattered, superficial, slightly concave to cup-shaped and yellowish when fresh, 0.6-1.0 mm diam., sessile or short-stipitate, margin even, waxy, translucent; marginal hairs, hyaline, septate. Ectal excipulum of rectangular cells, 15.9-47.8 μm thick, cells hyaline, thin-walled, 9.6-17.8 \times 3.1-7.6 μm . Hymenium strongly coherent, 26.8-33.9 μm thick, asci and paraphyses immersed in a gelatinous matrix. Asci cylindrical, apex thin-walled and rounded, unforked at the base, shrunken and curving near the base, 19.5-28.1 \times 4.3-5.7 μm , 8-spored. Ascospores cylindrical to subfusoid, strongly inflated near one third of one end, straight or slightly curved, hyaline, nonseptate, 7.4-12.8 \times 1.2-1.7 μm , four to seven globose SBs of 0.6-0.9 μm diam. in each half, most of ascospores overlapping fascicles and few of them irregularly triseriate. Paraphyses apex slightly inflated, cylindrical, hyaline, nonseptate, 18.9-23.4 \times 1.5-2.1 μm .

Known distribution: Europe; Beijing City and Zhejiang Province in China.

New distribution: Anhui Province, Yunnan Province and Sichuan Province in China.

This is a new record of *Hyalorbilia* from China. *Orbilbia juliae* was firstly described by Velenovský (1934), the description as follows: asci 25 \times 6-8 μm , obtuse cylindrical, ascospores 6-10 μm . Svrček (1954) and Spooner (1987) considered it as a synonym of *H. inflatula*. Actually, the latter spores with less SBs in each half are shorter and more thinner than those of *O. juliae*. Finally, Priou (2005) transferred *O. juliae* to *Hyalorbilia*.

Hyalorbilia arcuata H.O. Baral, M.L. Wu & Y.C. Su, Fungal Diversity 25: 235, 2007 (Figure 5)

Specimens examined: China, Yunnan Province, Honghe City, Pingbian County, Daweishan Nature Reserves, on rotten branch of broadleaf tree, lying on moist ground,

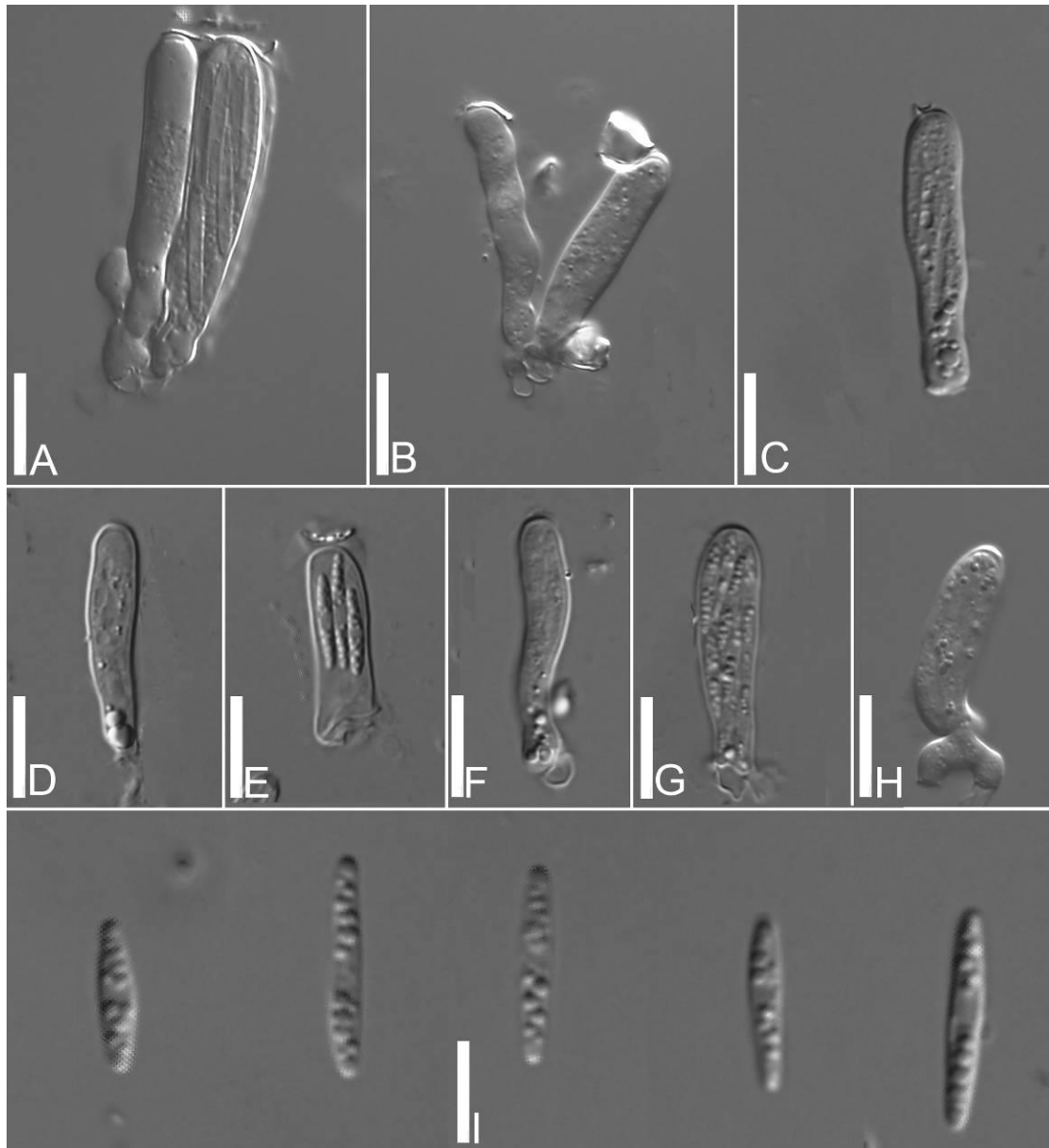


Figure 4. *H. juliae* A-B. Asci and paraphyses; C-H. Asci; I. Ascospores. Bar: A-H = 10 μ m, I = 5 μ m.

J.W. Guo, 30 October 2013, DWS1-13 and DWS1-25; China, Jiangxi Province, Wuyuan County, Wolonggu Valley, on rotten branch of broadleaf tree, lying on moist ground, J.W. Guo, 7 September 2009, wyg-6, wyg-7 and wyg-29; China, Zhejiang Province, Linan City, Tianmushan Mountain, on rotten branch of unidentified broad-leaved tree, lying on moist ground, J.W. Guo, 3 September 2009, tms-7, tms-28, tms-34 and tms-36; China, Fujian Province, Shanghang City, Meihuashan Mountain, on rotten branch of unidentified broad-leaved tree, lying on moist ground, J.W. Guo, 1 September 2009, MQS-17; China, Hunan Province, Yizhang County, Mangshan Nature Reserves, on rotten branch of

unidentified broad-leaved tree, lying on moist ground, J.W. Guo, 29 August 2009, CZMS-6 and CZMS-25; China, Guangdong Province, Zhaoqing City, Dinghushan Mountain, on rotten branch of unidentified broad-leaved tree, lying on moist ground, J.W. Guo, 26 August 2009, DHS-14; China, Guangxi Province, Longsheng County, Huaping Nature Reserves, on rotten branch of unidentified broadleaf tree, lying on moist ground, J.W. Guo, 24 August 2009, HPH-2; China, Hubei Province, Yizhang County, Shennongjia Nature Reserves, on rotten branch of unidentified broadleaf tree, lying on moist ground, J.W. Guo, 20 August 2009, sn1-19; China, Guizhou Province, Yuping County, Fanjingshan Mountain,

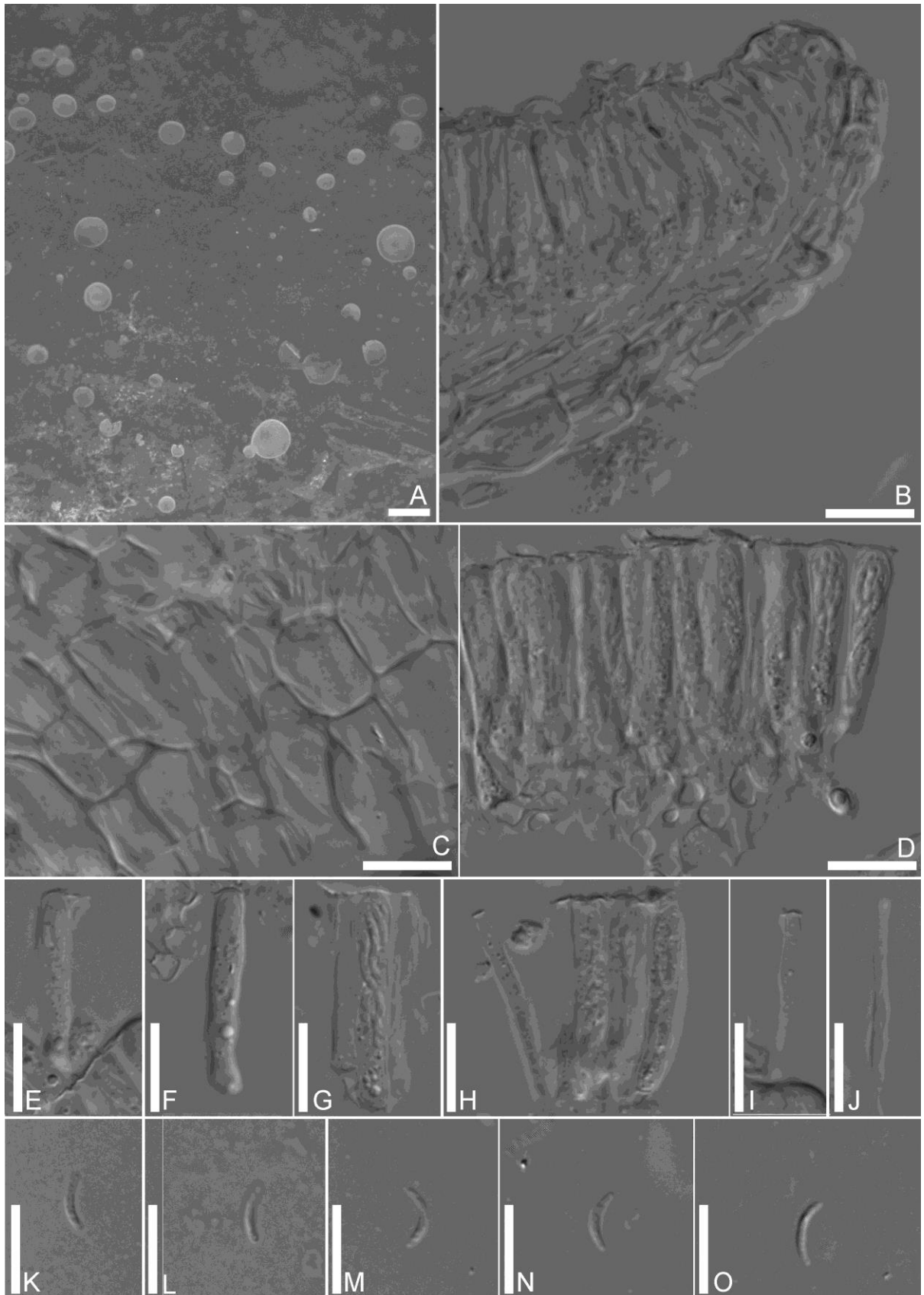


Figure 5. *H. arcuata*. A. Rehydrated apothecia; B. Vertical sections of part apothecium; C. Excipulum cells; D,H. Asci and paraphyses; E-G. Asci; I-J. Paraphyses; K-O. Ascospores. Bars: A = 0.5 mm, B-O = 10 μ m.

on rotten branch of unidentified broadleaf tree, lying on moist ground, J.W. Guo, 20 July 2009, FJH-3, FJH-8 and FJH-12; China, Yunnan Province, Kunming City, Shuanglong Town, on rotten branch of unidentified tree, lying on moist ground, J.W. Guo, 19 May 2006, sl-j-9; China, Yunnan Province, Kunming City, Jindian Forest Park, on rotten branch of unidentified tree, lying on moist ground, J.W. Guo, 13 June 2006, jd-j-16.

Known distribution: Taiwan; Kunming City, Yunnan Province, China.

New distribution: Guizhou Province, Guangxi Province, Guangdong Province, Hunan Province, Hubei Province, Fujian Province, Jiangxi Province mentioned above; in addition, some specimens of this species were also collected from Wenshan, Dali, Yuxi and Honghe in Yunnan Province, as well as Sichuan Province, Jiangsu Province, Henan Province, Anhui Province.

Hyalorbilia sp. that was the teleomorph of *Dactylella lignatilis* M.H. Mo & K.Q. Zhang, was firstly reported and widely distributed in Yunnan, China (Mo et al. 2005b). It was given specific name by Wu et al. (2007). The medium to strongly sickle-shaped ascospores is its key character. The shape of the ascospores and the distribution of its SBs of the present specimens are similar to the model species, but there is a slight difference in the size of ascospores [(4.6-)5.8-(-7.4)9.0 × 0.8-1.3 μm vs 5.5-7.5(-8) × 0.9-1.2 μm]. An illustration of the species is also provided here.

Hyalorbilia erythrostroma (Berk. & Broome) Baral & G. Marson, *Micologia* 2000: 44, 2001 (Figure 6)

Basionym: *Peziza erythrostroma* Berk. & Broome, *Ann. Mag. Nat. Hist.*, ser. 3, 18: 126, 1866

Dasyscyphus erythrostroma (Berk. & Broome) Sacc. *Syll. Fung.* 8: 453, 1889

Specimens examined: China, Yunnan Province, Pingbian County, Daweishan Nature Reserves, on rotten branch of broadleaf tree, lying on moist ground, J.W. Guo, 7 June 2013, pb-j-1; China, Zhejiang Province, Kaihua County, Gutianshan Nature Reserves, on rotten branch of unidentified broadleaf tree, lying on moist ground, J.W. Guo, 5 September 2009, GTS-38; PR China, Yunnan Province, Yuxi City, Tonghai County, Xiushan Forest Park, on rotten branch in moist evergreen broadleaf forest, J.W. Guo and S.F. Li, 16 July 2007, Th2-1.

Apothecia moist 0.1-0.3 mm diam., saprotrophic, scattered on inner bark surface of *Dalbergia* sp. under subtropical broad-leaved forest, sometimes parasitic on the black fruiting bodies of unknown fungi or together with *O. sarraziniana* Boud, fresh water-whitish, cupulate, short-stipitate or sessile, smooth at the margin, translucent. Ectal excipulum composed of subglobose to globose cells, 5.0-6.3 × 6.4-9.0 μm, thin-walled, hyaline. Hymenium 21.7-31.6 μm thick. Asci in the living state 20.2-31.2 × 2.5-4.5 μm, 8-spored, cylindrical, truncate rounded at the apex, thin-walled, unforked below, arising

from crosiers. Ascospores 2.5-3.3 × 2.0-2.3 μm in living state, ellipsoid, hyaline, mono-celled, uniseriate; living ascospores with 1 obvious globose SB neighbor to one end. Paraphyses embedded in a gel and not higher than asci, nonseptate, hyaline, 22.0-27.4 × 2.1-3.6 μm.

Known distribution: Europe; Fujian Province and Jiangxi Province in China.

New distribution: Zhejiang Province and Yunnan Province in China.

This is a rarely species in China, and usually parasitic on the ascomata of other fungi [such as *Chaetosphaerella* (Durieu & Mont.) E. Müll. & C. Booth and *Nitschkia acanthostroma* (Mont.) Nannf.]. It is similar to *H. brevistipitata* but its spores have less SBs and larger size.

Hyalorbilia* aff. *inflata (P. Karst.) Baral & G. Marson, *Micologia* 2000: 44, 2001 (Figure 7)

Basionym: *Peziza inflata* P. Karst., *Not. Soc. Sällsk. Fauna Fl. Fenn.* 10: 175, 1869

Orbilia inflata (P. Karst.) P. Karst., *Not. Sällsk. Fauna Fl. Fenn.* 11: 248, 1870

Specimens examined: China, Yunnan Province, Honghe City, Pingbian County, Daweishan Nature Reserves, on rotten branch of broadleaf tree, lying on moist ground, J.W. Guo, 30 October 2013, DWS1-22, DWS1-26 and DWS1-31; China, Jiangxi Province, Wuyuan County, Wolonggu Valley, on rotten branch of broadleaf tree, lying on moist ground, J.W. Guo, 7 September 2009, wyg-2; China, Zhejiang Province, Kaihua County, Gutianshan Nature Reserves, on rotten branch of unidentified broadleaf tree, lying on moist ground, J.W. Guo, 5 September 2009, GTS-7; China, Fujian Province, Shanghang City, Meihuashan Mountain, on rotten branch of unidentified broadleaf tree, lying on moist ground, J.W. Guo, 1 September 2009, MGS-33; China, Guangdong Province, Zhaoqing City, Dinghushan Mountain, on rotten branch of unidentified broadleaf tree, lying on moist ground, J.W. Guo, 26 August 2009, DHS-6 and DHS-19; China, Guizhou Province, Yuping County, Fanjingshan Mountain, on rotten branch of unidentified broadleaf tree, lying on moist ground, J.W. Guo, 20 July 2009, FJH-17; China, Sichuan Province, Jiuzhaigou Nature Reserves, on rotten branch of unidentified broadleaf tree, lying on moist ground under broadleaf forest, J.W. Guo and S.F. Li, 8 October 2006, ys-j-4.

Apothecia moist 0.5-1.0 mm diam., fresh water-whitish, cupulate, short-stipitate or sessile, smooth at the margin, translucent; marginal hairs 1.0-1.3 diam., hyaline, septate. Ectal excipulum 21.6-51.2 μm thick, composed of rectangular to angular cells, 7.5-18.4 × 3.6-6.8 μm, hyaline, thin-walled. Hymenium 26.7-32.7 μm thick. Asci in the living state 19.1-19.7 × 1.7-2.8 μm, 8-spored, cylindrical, truncate rounded at the apex, thin-walled, unforked below, arising from crosiers. Ascospores 5.2-6.3 × 0.8-1.0 μm in living state, calvate, hyaline, mono-celled, symmetrically biseriolate; living ascospores

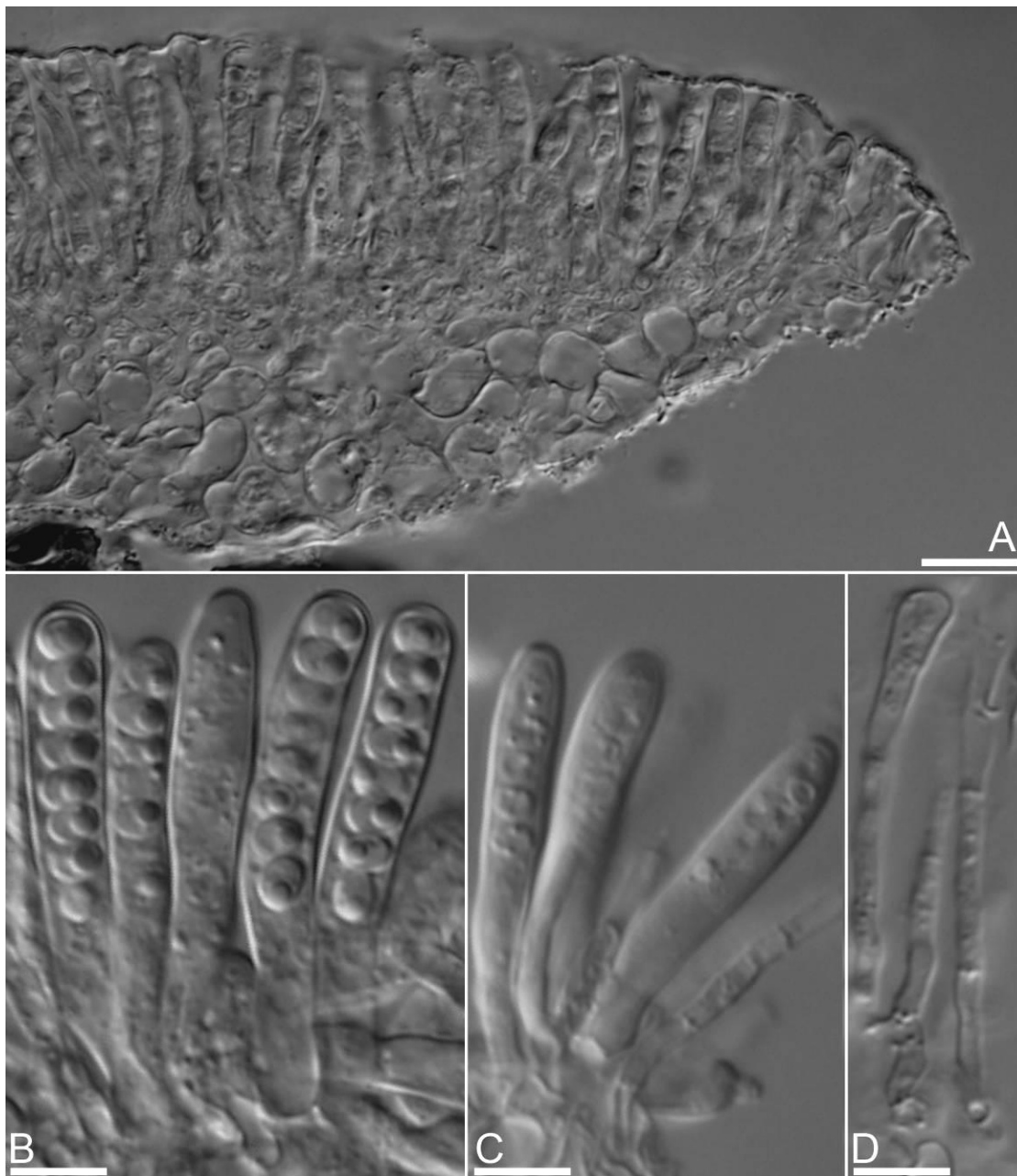


Figure 6. *Hyalorbilia erythrostroma* A. Vertical sections of part apothecium; B-C. Asci and paraphyses; D. Paraphyses. Bars: A = 10 µm, B-D = 5 µm.

with 2-4 obvious globose SB of 0.3-0.5 µm diam. neighbor to one end. Paraphyses embedded in a gel, nonseptate, hyaline, 27.2-30.6 × 1.7-2.4 µm.

H. inflatula is a common species in Europe. Our specimens have smaller size of its spores but resembled the shape with *H. inflatula*. Here these specimens are proposed as *H. aff. inflatula*.

Distribution: Henan, Anhui, Jiangsu, Zhejiang, Hunan, Guangdong, Guangxi, Guizhou, Sichuan and Yunnan, China.

Hyalorbilia inflatula (P. Karst.) Baral & G. Marson, *Micologia* 2000: 44, 2001 (Figure 8)

Basionym: *Peziza inflatula* P. Karst., *Not. Soc. Sällsk. Fauna Fl. Fenn.* 10: 175, 1869

Orbilia inflatula (P. Karst.) P. Karst., *Not. Sällsk. Fauna Fl. Fenn.* 11: 248, 1870

Specimens examined: China, Yunnan Province, Honghe City, Pingbian County, Daweishan Nature Reserves, on rotten branch of broadleaf tree, lying on moist ground, J.W. Guo, 30 October 2013, DWS1-21, DWS1-23 and

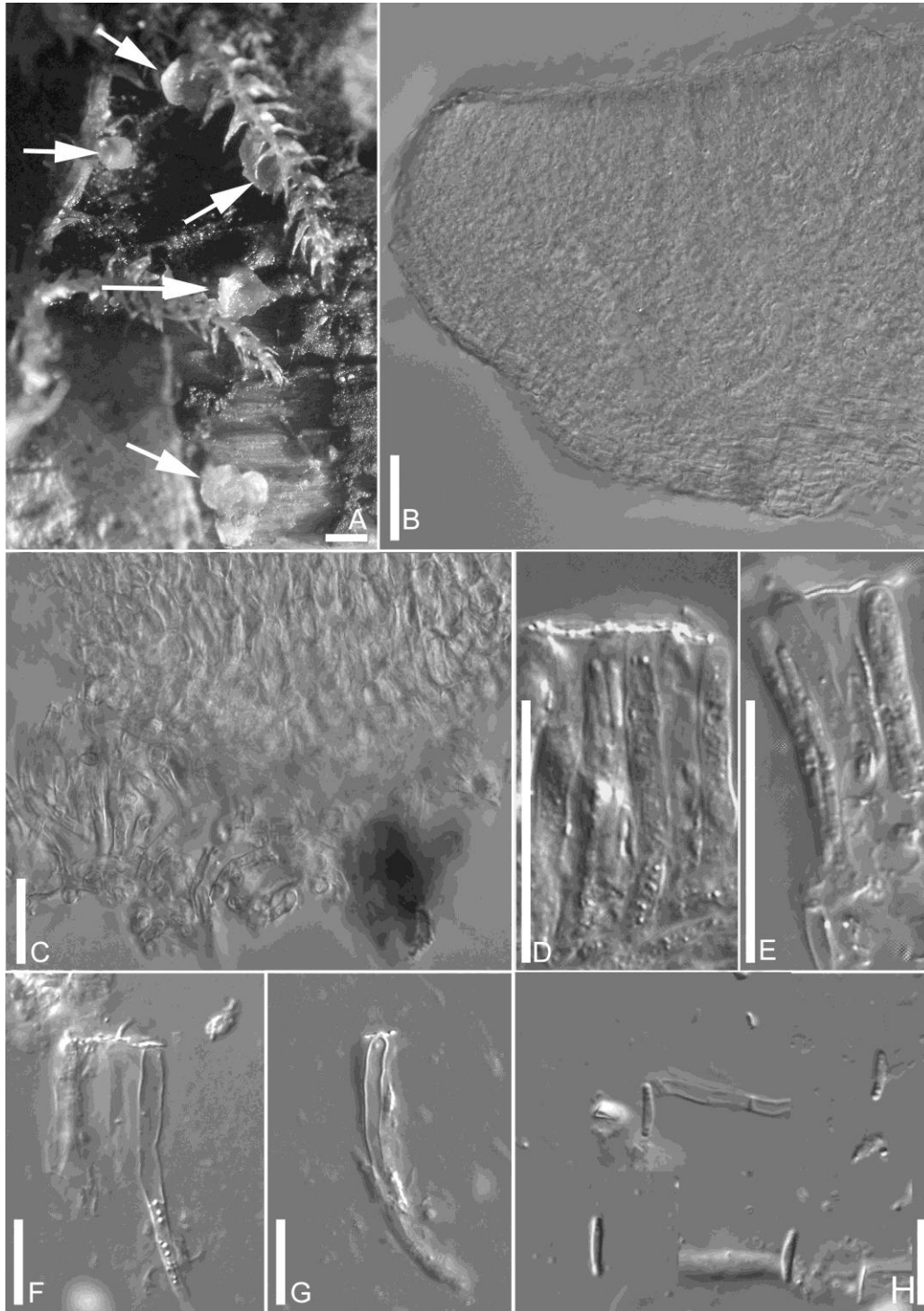


Figure 7. *H. aff. inflatula*. A. Rehydrated apothecia (arrows); B. Vertical sections of part apothecium; C. Medullary excipulum cells; D-E. Asci and paraphyses; F-G. Paraphyses; H. Ascospores. Bars: A = 0.5 mm, B-C = F-K = 10 μ m, D-E = 20 μ m.

DWS1-38; China, Jiangxi Province, Ningdu County, Cuiweifeng Forest Park, on rotten branch of broadleaf tree, lying on moist ground, J.W. Guo, 5 September 2009, cwf-8; China, Yunnan Province, Yuxi City, Yimen County,

Longquanshan Forest Park, on rotten branch of unidentified tree, lying on moist ground, J.W. Guo, 13 July 2009, ym092-8; China, Sichuan Province, Jiuzhaigou Nature Reserves, on rotten branch of

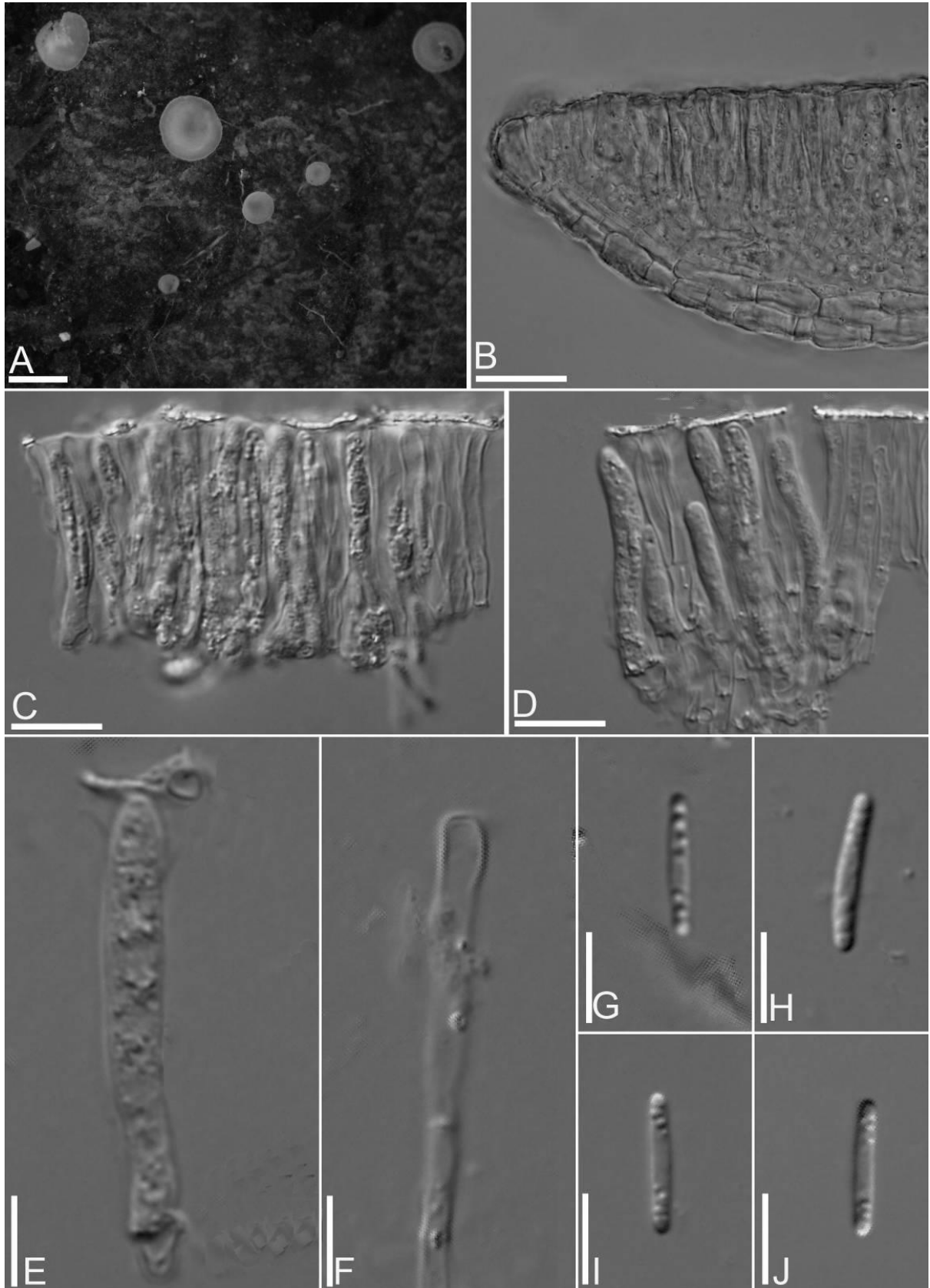


Figure 8. *H. inflatula*. A. Fresh apothecia on bark; B. Vertical sections of part apothecium; C-D. Asci and paraphyses; E. Asci; F. Paraphyses; G-J. Ascospores. Bars: A = 0.5 mm, B = 20 μ m, C-D = 10 μ m, E-J = 5 μ m.

unidentified broadleaf tree, lying on moist ground under broadleaf forest, J.W. Guo and S.F. Li, 7 August 2007, bji-5.

Known distribution: Europe; Tibet and Hubei, China.

New distribution: Jiangxi, Guangdong, Guangxi, Sichuan and Yunnan, China.

Hyalorbilia inflatula was a widely distributed species in Europe and China. The ascospores of Chinese specimens are 5.9-10.3 × 0.9-1.2 μm and have 3-4 SBs diam. 0.4-0.7 μm at each half.

Hyalorbilia berberidis (Velen.) Baral, *Micologia* 2000: 44, 2001

Basionym: *Orbilia berberidis* Velen., *Monogr. Discom. Bohemiae*: 99-100, 1934

Specimens examined: China, Yunnan Province, Yuxi City, Yimen County, Longquanshan Forest Park, on rotten branch of unidentified tree, lying on moist ground, J.W. Guo, 13 June 2009, ym091-16; China, Yunnan Province, Yuxi City, Tonghai County, Xiushan Forest Park, on rotten branch of unidentified tree, lying on moist ground, J.W. Guo, 7 June 2007, Th2-8.

Known distribution: Europe; Kunming City, Yunnan Province in China.

New distribution: Yuxi City, Yunnan Province in China.

Ten samples of this fungus were collected in Kunming City, Yimen County and Tonghai County during May and June of 2005-2010. The sickle spores are similar to *H. arcuata*, but it has more longer spores up to 10-12 μm.

Key to Orbiliaceae from Mainland China

1. Asci arising from crosiers, hymenial elements strongly coherent(*Hyalorbilia*) 2
1. Asci mostly branched below, asci with paraphyses easily separated.....(*Orbilia*) 6
2. Ascospores ellipsoid, 2.5-3.3 × 2.0-2.3 μm in living state.....*H. erythrostigma*
2. Ascospores slightly inflated and clavate.....3
2. Ascospores strongly inflated and slightly curved near one third of one end.....4
2. Ascospores sickle-shaped.....5
3. Ascospores with 2-4 SBs at each ends, 5.2-6.3 × 0.8-1.0 μm.....*H.aff. inflatula*
3. Ascospores with 3-4 SBs at each ends, 5.9-10.3 × 0.9-1.2 μm.....*H. inflatula*
4. Ascospores fusoid, 4.2(4.5)-6.1(-6.3) × 1.3(1.6)-1.6(-1.8) μm..... *H. fusispora*
4. Ascospores obtuse cylindric, 7.4-12.8 × 1.2-1.7 μm..... *H. juliae*
5. Ascospores, 10.9-13.6 × 1.2-1.4 μm.....*H. berberidis*
5. Ascospores, (4.6-) 5.8-(-7.4)9.0 × 0.8-1.3 μm.....*H. arcuata*
6. Ascospores spindle to rod shaped, straight or slightly curved, 5.0-8.0 × 1.1-1.4 μm diam. 0.4-0.7 μm at each

half

6. Ascospores helical and curved with sickled-shaped, 6.0-8.5(-10.0) × 1.0-1.4 μm.....*O. xanthostigma*

Conflict of Interests

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

ACKNOWLEDGMENTS

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Full Length Research Paper

Isolation and characterization of Chilembwe and Sindarock phosphate solubilizing soil microorganisms

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This study was conducted to isolate and characterize soil microorganisms capable of solubilizing Chilembwe and Sinda rock phosphates readily available in Zambia. Single isolates were obtained by direct plating and enrichment cultures with succinate, cellulose and glucose as the carbon sources. Isolates were differentiated using colony morphology (form, margin, elevation and colour) and a subset of 20 was chosen for identification using the 16S rRNA gene sequences. After being cultured in nutrient broth and defined medium with tricalcium phosphate as the sole phosphate source, ten isolates were characterized for acid phosphatase enzyme activity. Over 80 isolates were obtained from different rock phosphate-sugar combinations. Isolates had varying morphological characteristics and the 20 strains identified showed a large diversity of organisms belonging to the genera *Enterobacter*, *Burkholderia*, *Arthrobacter*, *Bacillus*, *Beijerinckia* and two genera not previously reported to have P-solubilizing capabilities, *Dyella* and *Curtobacterium*. Both the observed growth and genera to which isolates belonged were dependent on the type of rock phosphate. Almost all isolates tested showed acid phosphatase activity that were similar to previously reported levels, ranging from 0.62 to 4.8 μg *p*-nitrophenol per 10^8 cells per hour. This work will be useful as the basis for the development of appropriate seed inocula that can enhance Sinda and Chilembwe rock phosphate dissolution and increased plant available P for crop production.

Key words: Chilembwe, Sinda, Rock Phosphate, *Burkholderia*, *Enterobacter*, *Rhizobia*, *Dyella*, *Arthrobacter*, *Curtobacterium*, *Dyella*.

INTRODUCTION

Phosphorus is second only to nitrogen as an inorganic nutrient required by both plants and microorganisms. Its

major physiological role is in the storage and transfer of biochemically useful energy through the phosphate

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anhydride bond (Plante, 2007). It is also found in different cellular components as a constituent of genetic material and phospholipids. Phosphorous is primarily rock derived and therefore, large amounts of P are found either in soil minerals or organic matter (Syer et al., 2008). There is very little P present in soil solution, with concentrations usually 0.1 to 1 $\mu\text{g/g}$ at any given time (Plante, 2007), accounting for about 0.1% of the total soil P (Sharma et al., 2013).

The availability of native P in most Zambian agricultural soils is relatively low, especially in highly weathered and high P-sorption soils such as Alfisols, Ultisols and Oxisols (Yerokun, 2008). The limiting levels of available P necessitates the use of commercially produced fertilizers to supplement P for crop production. Alternatives to commercially available fertilizers include crop residues and animal manure; these sources cannot, however, on their own mitigate soil fertility decline because of limited quantities available on farms (Ademiluyi and Omotoso, 2007) and alternate uses of the residues as forage, silage and fuel. For example, the green biomass of *Tithonia diversifolia* has been recognized to be high in nutrients and effective as a nutrient source for lowland rice and maize (Ademiluyi and Omotoso, 2007). Particularly, *T. diversifolia* has been shown to have relatively high concentrations of N, P and K in its green biomass (Jama et al., 2000) and tissue concentrations of P can be greater than the critical 2.5 g kg^{-1} for net mineralization (Palm et al., 1999). Guano excreted by seabirds, seals or bats is another source of organic P that can be used as fertilizer. In Zambia, Guano enriched with P can contain as high as 4.03% P and contains beneficial fungi and bacteria that act as a natural control of plant diseases and that participate in organic matter decomposition (Sikazwe and de Waele, 2004).

Phosphorus can also be obtained from minerals; this mineral form of P is known as rock phosphate (RP), which consists of different forms of mineral apatite. Most of the world's fertilizers are produced from RP resources (Van Straaten, 2007). There are several deposits of RP in sub-Saharan Africa, including Zambia which are found in two types of igneous rocks: carbonatites and syenites (Van Straaten, 2002).

Plant roots associated with certain soil microorganisms can enhance the dissolution of these mineral forms of P by the production of organic acids such as acetic, citric, lactic, malic, oxalic, succinic, tartaric, glycolic, formic, and ketogluconic acids (Whalem and Sampedro, 2010). Organic acids dissolve Al- and Fe-P complexes and hasten the weathering of apatite-rich rock fertilizers as well as other primary minerals. Carbonic acid produced from the respiration of plant roots and soil microorganisms promotes the dissolution of calcium and magnesium P compounds (Plante, 2007). The production of other organic acids, such as gluconic acid, seems to be the most frequent means of mineral phosphate solubilisation by bacteria such as *Erwinia herbicola*,

Burkholderiacepacia, *Rhizobium leguminosarium*, *Rhizobium meliloti*, and *Bacillus firmus* (Brempong, 2013). *In vitro* assays have shown reduction in the pH of cultures by at least one unit due to the production of acid by phosphate solubilizing microorganisms (Kang et al., 2002). Microorganisms are also able to release inorganic P from minerals through other reactions such as the formation of chelating agents which are able to sequester aluminium, iron, calcium and magnesium ions from the mineral (Altomare et al., 1999). Several works have demonstrated that phosphate solubilising strains of *Rhizobium*, *Bradyrhizobium* and *Azotobacter* increase the growth and phosphate content in both non-leguminous and leguminous plants (Khan et al., 2007).

Soil microorganisms also produce other compounds such as acid and alkaline phosphatase enzymes, which hydrolyse ester bonds of organic matter releasing P into solution for plant and microbial uptake (Nannipieri et al., 2011). While acid phosphatase is derived mainly from soil microbial communities, production by roots has also been observed (Dick et al., 1983; Juma and Tabatabai, 1988a, b, c). Recent studies have shown that some microorganisms that have the capability of producing organic acids involved in RP solubilisation also have enzyme activity through the production of acid phosphatase and pyrophosphatase enzymes (Traoré et al., 2013).

The activities of phosphatases, and organic acids and chelating compounds in making organic and mineral P sources available, respectively, depend on several factors such as soil properties and interactions among soil microorganisms. For example, the effect of soil type on the ability of the microorganisms to solubilise P has been shown in Ghana on rice fields (Brempong, 2013). This suggests the importance of selecting microorganisms appropriate for particular soil types when developing inoculants.

This study was conducted to isolate and characterize Chilembwe and Sinda RP solubilizing soil microorganisms and to determine if these organisms can make organic forms of P available through acid phosphatase activity. This work serves as the first step to the development of inoculants that would increase the agronomic effectiveness and value of Chilembwe and Sinda RP readily available in Zambia for crop production.

MATERIALS AND METHODS

Soil sampling, processing and characterization

Soils were collected from the Pinney Purdue Agricultural Center in Wanatah, in northwestern Indiana, USA (latitude 41.44278, longitude -86.92905). Six random soil samples (approximately 3 kilograms each; P1-P6) were collected from the periphery of a soybean field and immediately put on ice for transportation back to the laboratory. Soils were then processed by sieving and apportioned for different tests before storing at either 4°C or -10°C. About 250 g of soil previously stored at 4°C was prepared and sent



Figure 1.Crushed Sinda and Chilembwe rock phosphates.

for chemical analysis at the A & L Great Lakes Laboratories (Fort Wayne, IN). Soils were characterized for organic matter, phosphorous, potassium, magnesium, and calcium concentrations, pH, and cation exchange capacity using standard laboratory procedures.

Culture medium preparation

To isolate soil microorganisms that have the capability to solubilize P and that use different carbon sources, two rock phosphates readily available in Zambia were used: Sinda (S-RP) and Chilembwe (C-RP) (Figure 1). Minimal media (MM) were prepared according to the procedure of Traoré et al. (2013) with modifications to the carbon source and their amounts. Three carbon sources were used to enrich for growth of soil microorganisms: succinate (S), glucose (G) and cellulose (C). The media contained per litre of distilled water: carbon source (1.9 g glucose, 1.4 g succinate or 1.9 g cellulose), 5 g rock phosphate (Sinda or Chilembwe), 5 g $MgCl_2 \cdot 6H_2O$, 0.25 g $MgSO_4 \cdot 7H_2O$, 0.2 g KCl, and 0.1 g $(NH_4)_2SO_4$. Plates for isolating rock phosphate dissolving microorganisms were made with the same medium with 10 g agar added. All media preparations were adjusted to pH 7 before autoclaving at 121°C and 15 psi for 30 min. With the three carbon sources and two rock phosphates, a total of six treatments were used and each replicated six times (according to the soil samples collected). No-soil and no rock-phosphate controls were also included.

Soil enrichments and direct plating

Culturing of soil microorganisms was done either as soil enrichments or as direct plating of soil dilutions. Direct plating was achieved using 100 µl of a 100-fold dilution of soil solution (in deionized water) that was spread evenly on a plate; plates were then sealed with parafilm and incubated upside down at 30°C until colonies developed. The soil enrichments were prepared by diluting soil a 100-fold into a total volume of 120 ml of MM solution. Flasks were then sealed with cotton plugs and incubated at 30°C in an environmental shaker (120 rpm) for a week before sub-culturing into fresh medium. At the time of sub-culturing, sub samples were obtained from the week old cultures and diluted serially to a million fold and 100 µl was plated onto corresponding agar plates made

with corresponding carbon sources. Microorganism growth on agar plates and in enrichment cultures was then observed and recorded.

Isolation and characterization of single isolates

As previously mentioned, plating of sub samples was conducted at each subculture and growth was observed. Single colonies were obtained from the plates, streaked onto fresh individual plates and purified. Morphological characterization of isolates was done based on colony form, colour, elevation, and margin. Colonies that showed growth of hyphae were recorded as 'fungal' and the colour of the mycelia recorded.

For genetic characterization and identification of isolates, 60 isolates were selected initially. Selected isolates were cultured overnight in Difco nutrient broth (NB); cultures with OD_{600} readings of 1-1.5 were immediately used for DNA extraction. DNA was isolated from only 36 out of the 60 selected isolates due to poor growth of some of the isolates in the nutrient broth. DNA was extracted from the cells using the FastDNA SPIN Kit (Q-Biogene; Solon, OH). The isolated DNA was then assessed for quality using gel electrophoresis (0.8% agarose gel run for 10 minutes at 100 V) and the 260/280 nm OD and quantified using a NanoDrop 3300 fluorospectrophotometer (Thermo Scientific, Wilmington, DE).

A subset of (20) isolates was carried through for identification using the almost full length 16S rRNA gene (approximately 1500 bp) that was PCR amplified using bacteria specific primers (27F, 5'-AGA GTT TGA TCC TGG CTC AG--3' and 1452R, 5'-AAG GAG GTG ATC CAG CCG CA—3'). The gene was amplified in a 50 µl PCR reaction mix containing 1× PCR buffer, 75 µmol $MgCl_2$, 4 nmol deoxynucleoside triphosphates, 100 pmol of forward and reverse primers, and 50-100 ng of template DNA. The thermal cycle protocol began with an initial denaturation at 95°C for 5 min, then thirty cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, followed by 10 min at 72°C. The PCR products were directly sequenced using the Sanger Sequencing method at the Purdue University Genomics facilities. Best matches to nucleotide sequences in the GenBank database (NCBI) were determined using the BLASTn program (Altschul et al., 1997). The best matches to bacterial type strains were determined using SeqMatch and the RDP database (Cole et al., 2014).

To illustrate the relatedness of the identified isolates to type strains of officially classified taxa, a phylogenetic tree was

Table 1. Selected chemical characteristics of soils* used in this study.

Sample	pH	Organic matter (%)	P (mg kg ⁻¹)	Exchangeable bases (mg kg ⁻¹)			Cation exchange capacity (cmol+/ kg)
				K	Mg	Ca	
P1	7.0	3.3	28	200	650	1800	14.9
P2	6.4	3.3	11	113	470	1500	12.9
P3	6.6	3.6	9	123	490	1350	12.3
P4	6.4	3.7	8	108	465	1400	12.4
P5	6.7	4.3	8	103	575	1700	14.8
P6	6.4	4.1	11	118	565	1700	15.9
Mean	6.6	3.7	12.5	128.7	535.8	1575	13.9

*Soils were from the Pinney Purdue Agricultural Center in Wanatah, Indiana, USA

generated. Clustal Omega (Sievers et al., 2011) was used for multiple sequence alignment and the neighbour-joining method to build the tree using 1000 reiterations. Only isolates for which the full 16S rRNA gene sequence was obtained were used for the phylogenetic tree along with the best matching type strains.

Biochemical characterization using the acid phosphatase assay method

Selected isolates were grown in nutrient broth and also in MM with tricalcium phosphate (TCP) as the sole P source instead of rock phosphate for the acid phosphatase assay. The second medium was used to test enzyme activity in the absence of any organic phosphate source. Growth in both media was observed and recorded. Acid phosphatase activity for each isolate was determined by the method of Eivazi and Tabatabai (1977), however rather than using 1 g of soil, 1 mL of broth culture was used. In brief, one 1 mL of broth culture was mixed with 4 mL of pH 6.5 modified universal buffer (MUB) and 0.2 mL of toluene to inhibit cellular metabolism, then 1 μ L of 0.05 M disodium *p*-nitrophenyl phosphate (PNP) tetrahydrate substrate solution was added. The mixture was incubated for 1 hour at 37°C. The reaction was stopped by adding 1 mL of 0.5 M CaCl₂ and 4 mL of 0.5 M NaOH. The resulting solution was filtered using Whatman #2 filter paper, the solution's yellow colour intensity was measured at 410 nm on a spectrophotometer and compared to a standard curve to determine the concentration of released substrate. Acid phosphatase activity was then normalized to μ g of PNP released/10⁸ cells. Cell density was estimated by measuring the optical density at 600 nm. This value was then converted to cells/mL using the *E. coli* cell density conversion of an OD₆₀₀ of 1.0 being equivalent to 8 x 10⁸ cells/mL.

RESULTS

Soil characteristics

Selected soil chemical characteristics are shown in Table 1. In order to set up subsequent isolation experiments, it was important that the selected soil samples were low in available phosphorus. The results indicated that only Pinney 1 had medium levels (28 mg kg⁻¹) of P while the others had either low or very low P concentrations (Table 1). The pH ranged from 6.4 to 7.0 with an average of 6.6. The levels of potassium ranged from medium to high

(103 to 200 mg kg⁻¹) with an average of 128.7 mg kg⁻¹. Magnesium levels were all very high and ranged from 465 to 575 mg kg⁻¹. Calcium levels were low in one of the samples (Pinney 3) but the rest were in the medium range (1350 to 1800 mg kg⁻¹) with an average of 1575 mg kg⁻¹.

Effect of carbon source and rock phosphate on microbial growth

Growth in all carbon source- rock phosphate combinations was observed, except in the cellulose + Sinda (C+S-RP) combination. Growth of glucose cultures was faster than in both the succinate and cellulose cultures (Table 2).

Growth was also observed in some of the no-soil (control) cultures (Figure 2; Table 2). No- soil control cultures with the all carbon sources tested and Chilembwe rock phosphate combinations (G+C-RP, S+C-RP, and C+C-RP) as well as G+S-RP and S+S-RP, showed growth of microorganisms encapsulating the rock phosphate. The growth observed in these control enrichment cultures differed and depended on the carbon-rock phosphate combinations. The G+S-RP combination resulted in three different forms of growth around the rock phosphate (Figure 2D-F). The cultures with C-RP showed more growth when compared to the S-RP cultures when cellulose was used as the carbon source, which exhibited no growth at all (Figure 3). The lack of growth was confirmed by failure of colonies to develop on corresponding C+S- RP plates when 100 μ l of culture was plated.

Morphological characterization of isolates

Once individual colonies were isolated, they were assigned a unique identity and their morphological characteristics recorded. Over 80 colonies were isolated and characterized (Supplemental Table 1). In summary,

Table 2. Summary of growth in enrichment cultures after 1-week incubation at 30°C.

Soil*	G+C-RP	G+S-RP	S+C-RP	S+S-RP	C+C-RP	C+S-RP
P1	+++++++	+++++++	-	-	+	-
P2	+++++++	+++++++	-	-	+	-
P3	+++++++	++	-	+	+	-
P4	+++++++	++	-	-	+	-
P5	++	+++++++	-	-	+	-
P6	+++++++	+++++++	-	-	+	-
No soil 1	+	-	++	-	+	-
No soil 2	+	+	-	+	+	-
No soil 3	-	++	-	-	+	-

G, Glucose; S, succinate; C, cellulose; C-RP, Chilembwerock phosphate; S-RP, Sindarock phosphate. *Soils were from the Pinney Purdue Agricultural Center in Wanatah, Indiana, USA. + indicates extent of growth after 1 week of culture; - indicates lack of observable growth after one week of culture.

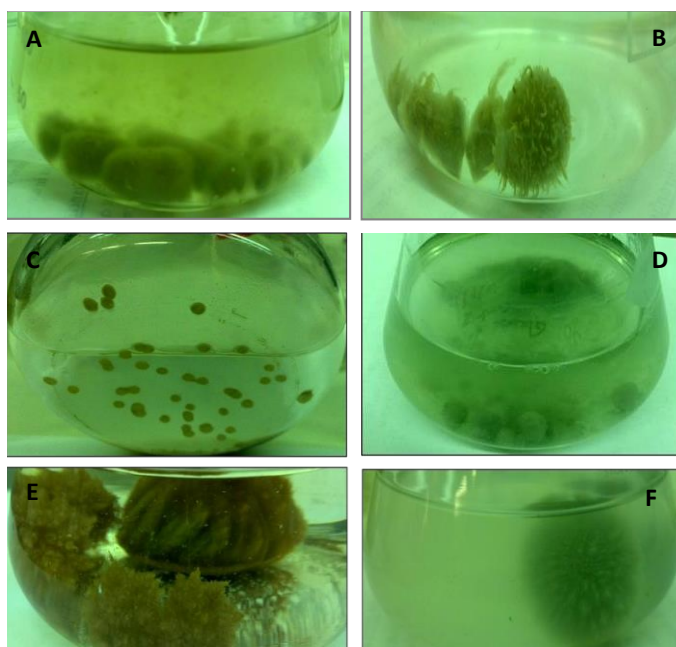


Figure 2. Growth of microorganisms encapsulating Chilembwe (CRP) and Sinda (SRP) rock phosphates in carbon enrichment cultures without soil addition. **A.** Glucose (G) + CRP; **B.** Succinate +CRP; **C.** Cellulose + CRP; **D-F.** G + SRP.

41, 28, 8, 4 and 3 isolates were obtained from G + C-RP, G+SRP, C+C-RP, S+CRP and S+S-RP carbon source - rock phosphate combinations, respectively, giving a total of 86 (Table 3). Based on their characteristics, isolates were assumed to be either bacterial or fungal (Figures 4 and 5). The bacterial isolates had circular, irregular, rhizoid and spindle forms, with white, cream, pink, yellow and translucent colouration. All the isolates from the S+C-RP combination were translucent (Figure 4; Supplemental Table 1). The types of bacterial colony elevations observed included flat, convex, pulvinate and umbonate.

Colony edges were filamentous, entire and undulate. The fungal isolates had mycelia with grey, green, white or yellow colouration. Of the 86 isolates, twenty representative bacterial isolates were chosen for further characterization.

Molecular characterization and tentative identification of isolates

Based on their 16S rRNA gene sequences, 20 isolates

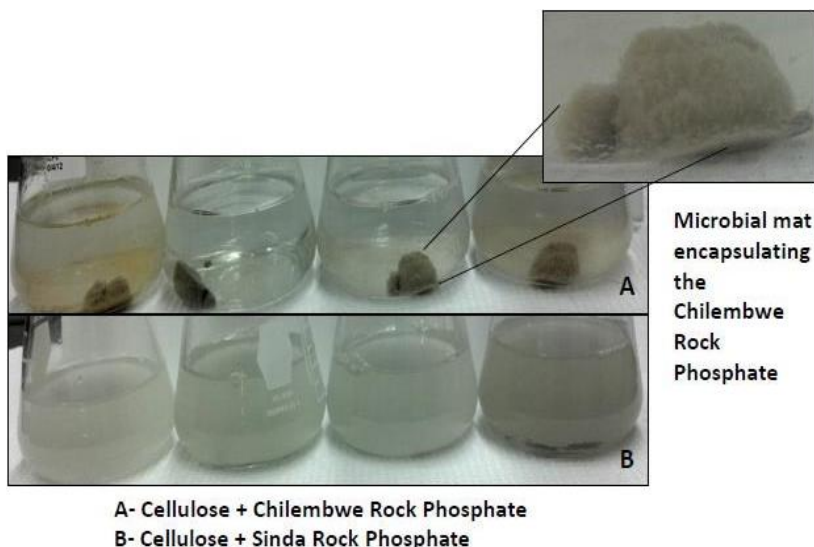


Figure 3. Growth of microorganisms in enrichment cultures with cellulose + Chilembwe rock phosphate cultures, 19 days sub-culture.

Table 3. Summary of 86 isolates purified from each carbon-rock phosphate-soil combination.

Soil*	G+C-RP	S+C-RP	C+C-RP	G+S-RP	S+S-RP	C+S-RP
P1	3	-	-	2	-	-
P2	13	-	-	1	-	-
P3	5	-	-	5	2	-
P4	7	-	-	4	-	-
P5	6	-	-	6	-	-
P6	3	-	-	5	-	-
No soil 1	1	4	1	-	-	-
No soil 2	2	-	7	1	1	-
No soil 3	1	-	-	4	-	-
Total	41	4	8	28	3	0

G, Glucose; S, succinate; C, cellulose; C-RP, Chilembwe Rock Phosphate; S-RP, Sinda rock phosphate. *Soils were from the Pinney Purdue Agricultural Center in Wanatah, Indiana, USA.

were identified as belonging to a total of seven different phyla. Five different bacterial genera were isolated from the C-RP enrichments, while four were isolated from the S- RP enrichments. Chilembwe and Sinda RP isolates had two genera in common, *Burkholderia* and *Enterobacter*. Isolates were most commonly identified as *Enterobacter*, representing 9 out of 20 sequenced isolates. The closest genetic matches for the remaining isolates were to type strains in the genera *Bacillus*, *Burkholderia*, *Arthrobacter*, *Dyella*, *Curtobacterium* and *Beijerinckia* (Table 4) and illustrated in a phylogenetic tree (Figure 6).

Acid phosphatase activity of selected isolates

Only a subset of six isolates identified by 16S rRNA gene

sequencing and two others were tested for growth in MM-TCP both and for phosphatase activity. All isolates grew in nutrient broth but three isolates could not use TCP as a phosphate source when grown in minimal medium (Table 5). The isolates that grew in both the nutrient and TCP broths were from the genus *Enterobacter* whereas *Bacillus*, *Curtobacterium* and two isolates without 16S rRNA gene sequences (Table 5, Figure 6) grew only in nutrient broth. Apart from AM16S-17, all isolates tested showed acid phosphatase activity ranging from 0.62 to 4.80, and 1.02 to 2.15 $\mu\text{g PNP } 10^8 \text{ cells}^{-1} \text{ hr}^{-1}$ in nutrient and MM-TCP broths, respectively (Figure 7). The highest enzyme activity ($4.80 \text{ PNP } \times 10^8 \text{ cells}^{-1} \text{ h}^{-1}$) was observed in an unidentified isolate (AM16S-43) from the control cultures of S+C-RP. Comparing isolates that grew in both media, the enzyme activity was higher in MM-TCP broth than NB for isolates from Chilembwe rock

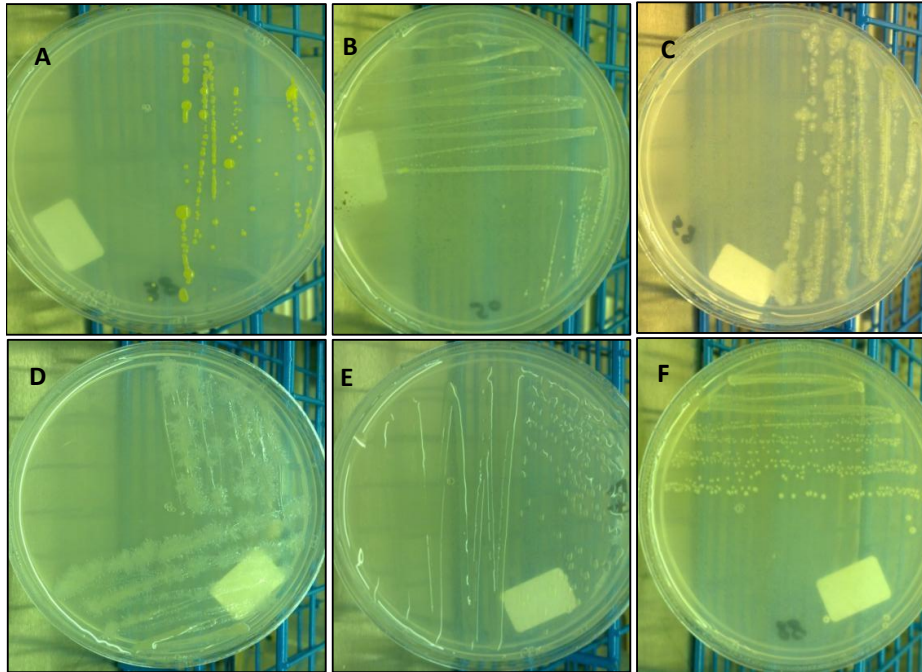


Figure 4. Selected streak plates showing bacterial isolates with varying colony forms, colours, edges, and elevation. **A.** Glucose (G) + Sinda rock phosphate (SRP) - P3-38; **B.** G+S-RP P4-50; **C.** G+ Chilembwe Rock Phosphate (CRP) P5-85; **D.** G+CRP P5-84; **E.** Succinate (S) +CRP Control 1-47; **F:** S+SRP Control-2-88.

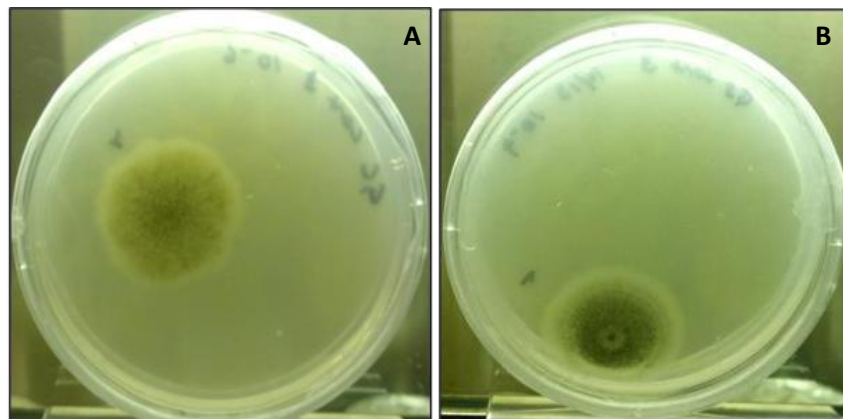


Figure 5. Selected streak plates showing fungal isolates. **A.** G + C-RP Control. **B.** G + S-RP Control.

phosphate (AM16S-19 and AM16S-21) whereas it was about the same for isolates from Sinda rock phosphate (AM16S-25 and AM16S-88).

DISCUSSION

A total of 86 bacterial and fungal isolates growing on

Chilembwe and Sinda rock phosphates were isolated from the soils and control cultures. Most (69) isolates were isolated from the cultures with glucose as the carbon source. This was attributed to more growth in glucose cultures resulting in more colonies on plates which were isolated compared to succinate and cellulose (Table 2). Glucose is more readily metabolised than cellulose and succinate by soil microorganisms. Out of

Table 4. Best matches of 20 microbial isolates to bacterial type strains based on 16SrRNA gene sequences.

Isolate ID	Carbon source	Rock phosphate source	Soil sample	Best type strain match (RDP)*	Genbank ID	Identity	%
AM16S-3	Glucose	Chilembwe	P6	<i>Arthrobacter nicotinovorans</i> DSM 420	X80743	1395/1398	99
AM16S-4	Glucose	Chilembwe	P4	<i>Arthrobacter defluvii</i> 4C1-a	AM409361	1391/1411	99
AM16S-6	Glucose	Sinda	P5	<i>Burkholderia stabilis</i> LMG14294	AF097533	1384/1391	99
AM16S-9	Glucose	Sinda	P3	<i>Curtobacterium pusillum</i> DSM 20527	AJ784400	1404/1412	99
AM16S-10	Glucose	Sinda	P4	<i>Enterobacterasburiae</i> JCM6051	AB004744	1349/1358	99
AM16S-11	Glucose	Sinda	P4	<i>Enterobacterasburiae</i> (T); JCM6051	AB004744	1310/1335	98
AM16S-17	Glucose	Chilembwe	P5	<i>Bacillus megaterium</i> IAM 13418	D16273	1441/1450	99
AM16S-19	Glucose	Chilembwe	P2	<i>Enterobacterasburiae</i> JCM6051	AB004744	840/843	99
AM16S-21	Glucose	Chilembwe	P2	<i>Enterobacterasburiae</i> JCM6051	AB004744	859/869	99
AM16S-25	Glucose	Sinda	P6	<i>Enterobacterasburiae</i> JCM6051	AB004744	737/742	99
AM16S-40	Glucose	Sinda	P5	<i>Beijerinckia fluminensis</i> UQM 1685	EU401907	1363/1374	99
AM16S-50	Glucose	Sinda	P4	<i>Enterobacterasburiae</i> JCM6051	AB004744	1345/1373	98
AM16S-51	Glucose	Sinda	P4	<i>Enterobacterasburiae</i> JCM6051	AB004744	1310/1346	97
AM16S-66	Glucose	Chilembwe	P2	<i>Burkholderia cepacia</i> ATCC 25416T	U96927	1400/1407	99
				<i>Burkholderia metallica</i> R-16017	AM747632	1403/1411	99
AM16S-67	Glucose	Chilembwe	P2	<i>Burkholderia cepacia</i> ATCC 25416T	U96927	1410/1414	99
				<i>Burkholderia metallica</i> R-16017	AM747632	1410/1414	99
AM16S-81	Glucose	Chilembwe	P3	<i>Dyellakoreensis</i> BB4	AY884571	1361/1388	98
AM16S-88	Succinate	Sinda	Control	<i>Enterobacterasburiae</i> JCM6051	AB004744	787/795	99
AM16S-93	Cellulose	Chilembwe	P3	<i>Enterobacter ludwigii</i> EN-119	AJ853891	1408/1414	99

*Best match to type strains determined using SeqMatch and the RDP database (Cole et al., 2014).

the total, 24 were isolated from control plates or enrichment cultures without soil (Table 3). The observed growth in the control cultures was attributed to the possibility that rock phosphate solubilizing fungi and bacteria had colonized both the Chilembwe and Sinda rock phosphates, and that these organisms must have been in a form that was able to withstand the moist heat and pressure of the autoclaving process. Some rod-shaped, endospore forming bacteria, such as those of the genus *Bacillus* have been shown to possess this characteristic (Wolska et al., 2007). The isolate AM16S-88, tentatively identified as

Enterobacterasburiae, from a control culture of succinate + Sinda-RP was among this group; species from this genus are known to be rod-shaped, non-spore forming Gram negative bacilli (Khunthongpan et al., 2013). This observation prompts further analysis of these isolates. The type of rock phosphate appeared to influence microbial growth. The cultures with Chilembwe-RP showed more growth of microorganisms when compared to the Sinda-RP cultures (Table 3 and Figure 3). The observed differences were attributed to the possible differences in the mineralogy and subsequent solubility between the

two rock phosphates. The Chilembwe-RP deposits has been characterized as being composed mainly of apatite, quartz, feldspar, biotite, hornblend, magnetite-haematite-goethite and ilmenite, with an average of 12 % P₂O₅ and a solubility of 1-1.7% P₂O₅ in neutral ammonium citrate (Appleton, 2002). In the absence of information on the mineralogical characteristics of the Sinda-RP, it can be inferred that Sinda-RP potentially has an even lower solubility than Chilembwe-RP, and therefore microorganisms metabolizing RP as a source of P would prefer the latter RP. Otherwise, other mineralogical attributes

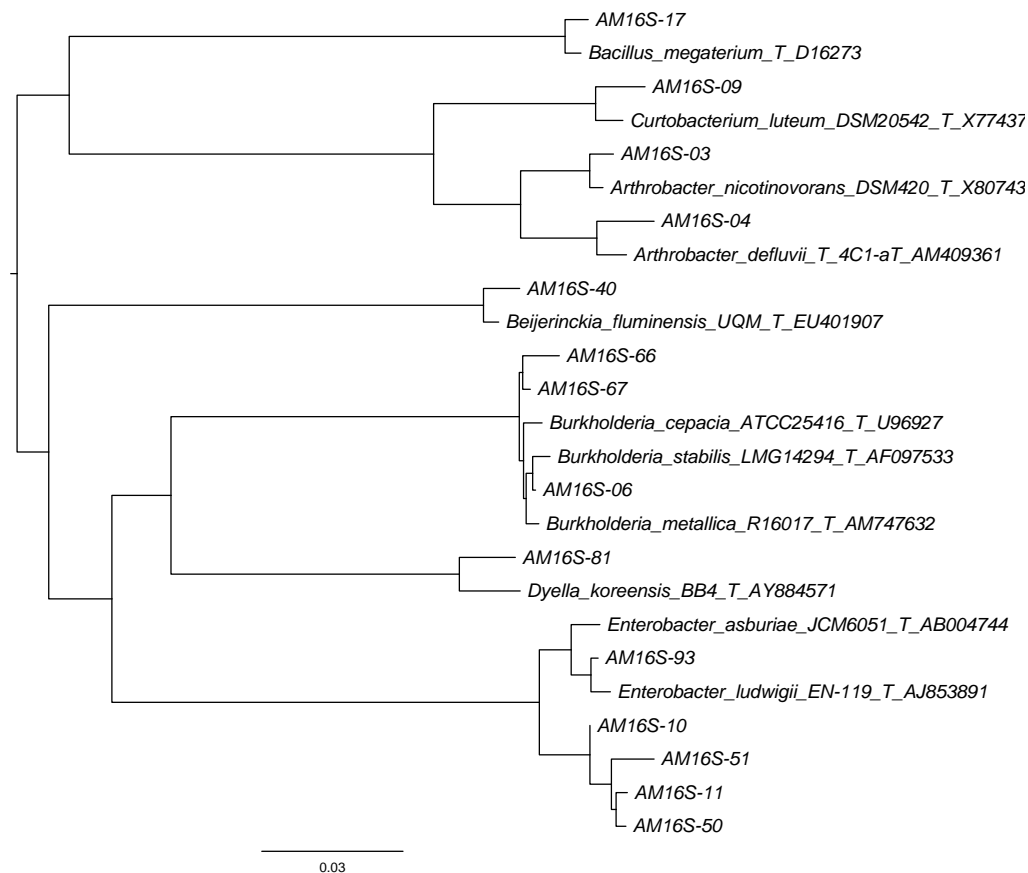


Figure 6. Phylogenetic tree of 16S rRNA gene sequences of representative isolates and most closely related type strains. Sequence alignment was made using CLUSTAL Omega and the phylogenetic tree generated using the neighbour-joining method with 1000 iterations. The type strain ID and GenBank accession numbers are included in the figure.

Table 5. Summary of growth in nutrient broth and minimal medium amended with tricalcium phosphate.

Strain ID	Carbon + rock phosphate combination [†]	Soil source*	Growth nutrient broth	Growth MM-TCP broth	Acid phosphatase activity
AM16S-9	G+S-RP	P3	+	-	Yes
AM16S-11	G+S-RP	P4	+	+	Yes
AM16S-17	G+C-RP	P5	+	-	No
AM16S-19	G+C-RP	P2	+	+	Yes
AM16S-21	G+C-RP	P2	+	+	Yes
AM16S-25	G+S-RP	P6	+	+	Yes
AM16S-43	S+C-RP	Control 1	+	-	Yes
AM16S-69	G+C-RP	P2	+	-	Yes
AM16S-88	S+S-RP	Control 2	+	+	Yes

[†]G, Glucose; S, succinate; C, cellulose; CRP, Chilembwerock phosphate; S-RP, Sindarock phosphate. *P, Pinney Purdue Agric. Center. Control 1 and 2 were enrichments with no soil added

may be considered to be responsible for the observed differences.

Based on the morphological and molecular characteri-

sation of the isolates, results show a wide range of characteristics and genera among the isolates so far identified. Previously, *Bacillus* spp., *Rhizobia* spp.,

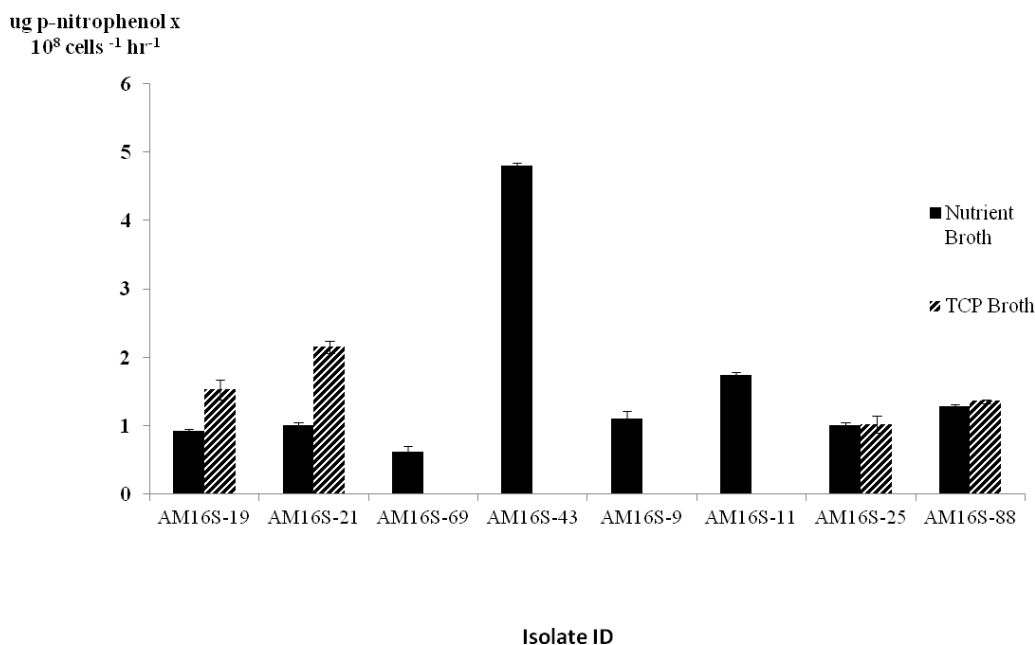


Figure 7. Acid phosphatase activity of microbial cells grown in nutrient and MM-tricalcium phosphate (TCP) broths.

Azotobacterspp., *Arthrobacter spp.* and *Burkholderia spp.* have been isolated and reported to have rock phosphate solubilizing capacities (Halder et al., 1991; Illmer and Schinner, 1992; Kumar et al., 2001). Fungal species such as *Trichodermaspp.*, *Aspergillus spp.*, *Rhizoctonia spp.* and *Penicillium spp.* have also been isolated and characterized for this capability (Fankem et al., 2014; Babana et al., 2013; Jacobs et al., 2002; Altomare et al., 1999; Motsara, et al., 1995). We report here the identification, based on the 16S rRNA gene sequences, of 20 isolates. The results indicate a large diversity of organisms capable of solubilizing rock phosphate across the two rock phosphates, including two genera that have not previously been associated with P-solubilization, *Dyella* and *Curtobacterium* (Table 4; Figure 6). However, because *Curtobacterium pusillum* (AM16S-9) could not grow in TCP broth, its capabilities to effectively solubilize RP remain questionable. The results show five different bacterial genera from the enrichments with Chilembwe-RP and four from the Sinda-RP.

The results of the phosphatase enzyme activity of the 10 isolates were comparable to previous research employing this method to measure acid phosphatase activity in bacterial isolates (Traoré, et al., 2013). Some of the microorganisms that had acid phosphatase activity failed to grow in TCP broth suggesting that they are incapable of utilizing both inorganic and organic forms of P. Previous studies have, however, shown that some microorganisms capable of solubilizing RP by producing low molecular weight organic acids also have acid phosphatase activity and that 70-80% of the microbial population in soils produce phosphatases (Plante, 2007).

In this study, that the tested organisms were incapable of solubilizing tricalcium phosphate after being isolated with RP as the only source of phosphorus may be explained by the composition of the RP. In addition, this observation does not agree with earlier findings that show that isolates preferred tricalcium phosphate to rock phosphate as a source of inorganic P in culture media (Asuming-Brempong and Keri, 2014).

In conclusion, over 80 isolates were isolated that were able to grow on Chilembwe and Sinda rock phosphates with varying morphological characteristics. Of these isolates, 20 were tentatively identified based on their 16S rRNA sequences, representing a large diversity of organisms capable of solubilizing rock phosphate. Biochemically, almost all the isolates tested had acid phosphatase enzyme activity. The ability to grow in culture, the genera represented as well as the enzyme activity of the isolates, all depended on the type of rock phosphate. The findings from this study provide a basis for developing appropriate seed inocula that can enhance availability of P from Chilembwe and Sinda RP resources for crop production.

Conflict of Interests

The authors have not declared any conflict of interests.

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Supplementary Table. Colony morphological characteristics.

Culture ID	Carbon- Rock Phosphate- Soil	Characteristic+S-RP				Fungal/ bacterial/ Dk
		Form	Colour	Elevation	Margin	
1	G+C-RP CONTROL 2	Circular	Cream	Flat	Entire	Bacterial
2	G+C-RP CONTROL 2	Fungal	Grey	-	-	Fungal
3	G+C-RP P6	Circular	Cream	Convex	Entire	Bacterial
4	G+C-RP P6	Circular	Cream	Convex	Entire	Bacterial
5	G+S-RP P5	Circular	Yellow	Flat	Entire	Bacterial
6	G+S-RP P5	Circular	Yellow	Flat	Entire	Bacterial
7	G+S-RP P5	Fungal	White	-	-	Fungal
8	G+S-RP P3	Circular	Cream	Pulvinate	Entire	Bacterial
9	G+S-RP P3	Circular	Cream	Pulvinate	Entire	Bacterial
10	G+S-RP P4	Irregular	Pink- white	Convex	Entire	Mixed
11	G+S-RP P4	Circular	Cream	Convex	Entire	Bacterial
12	G+S-RP P6	Fungal	Green	-	-	Fungal
13	G+S-RP P6	Fungal	Green	-	-	Fungal
14	G+C-RP P1	Fungal	Cream	-	-	Fungal
15	G+C-RP P1	Fungal	White	-	-	Fungal
16	G+C-RP P1	Fungal	Clear	-	-	Fungal
17	G+C-RP P5	Spindle	Cream	Convex	Entire	Bacterial
18	G+C-RP P5	Circular	Orange	Flat	Entire	Bacterial
19	G+C-RP P2	Circular	Cream	Convex	Entire	Bacterial
20	G+C-RP P2	Fungal	Brown			Fungal
21	G+C-RP P2	Circular	Cream	Convex	Entire	Bacterial
22	G+C-RP P3	Fungal	Green	-	-	Fungal
23	G+C-RP CONTROL 1	Fungal	Yellow&green	-	-	Fungal
24	G+C-RP P5	Circular	White	Flat	Entire	Bacterial
25	G+S-RP P6	Circular	White	Convex	Entire	Bacterial
26	G+S-RP P6	Circular	White	Convex	Entire	Bacterial
27	G+C-RP P4	Spindle	Pink	Convex	Entire	Bacterial
28	G+C-RP P4	Spindle	Pink	Convex	Entire	Bacterial
29	G+C-RP P4	Circular	Pink -white	Convex	Entire	Bacterial
30	S+S-RP P3	Circular	White	Raised	Entire	Bacterial
31	S+S-RP P3	Irregular	White	Raised	Entire	Bacterial
32	G+S-RP P5	Circular	White	Convex	Entire	Bacterial
33	G+C-RP P4	Rhizoid	Cream	Raised	Filamentous	Bacterial
34	G+C-RP P4	Filamentous	Translucent	Flat	Filamentous	Bacterial
35	G+S-RP P1	Irregular	White	Umbonate	Curled	Bacterial
36	G+S-RP P1	Circular	White	Convex	Entire	Bacterial
37	G+S-RP P3	Circular	Yellow	Pulvinate	Entire	Bacterial
38	G+S-RP P3	Circular	Yellow		Entire	Bacterial
39	G+S-RP P3	Circular	White	Umbonate	Entire	Bacterial
40	G+S-RP P5	Circular	Translucent	Pulvinate	Entire	Bacterial
41	G+S-RP P6	Circular	Yellow	Convex	Entire	Bacterial
42	G+C-RP P6	Fungal	White	-	-	Fungal
43	S+C-RP CONTROL 1	Irregular	Translucent	Flat	Undulated	Bacterial
45	S+C-RP CONTROL 1	Circular	Translucent	Convex	Entire	Bacterial
46	S+C-RP CONTROL 1	Circular	Translucent	Convex	Entire	Bacterial
47	S+C-RP CONTROL 1	Circular	Translucent	Convex	Entire	Bacterial
48	G+C-RP P3	Rhizoid	Cream white	Convex	Filamentous	Bacterial
49	G+C-RP P4	Circular	Cream white	Convex	Entire	Bacterial
50	G+S-RP P4	Circular	Cream white	Convex	Entire	Bacterial
51	G+C-RP P4	Circular	Cream white	Convex	Entire	Bacterial

Supplementary Table 1.Contd.

Culture ID	Carbon- Rock Phosphate- Soil	Characteristic+S-RP				Fungal/ bacterial/ Dk
		Form	Colour	Elevation	Margin	
52	G+S-RP P4	Circular	Cream white	Convex	Entire	Bacterial
53	G+S-RP P2	Rhizoid	White	Umbonate	Filamentous	Bacterial
54	G+S-RP CONTROL 2	Fungal	Green	-	-	Fungal
55	G+C-RP P2	Fungal	White	-	-	Fungal
56	G+C-RP P2	Fungal	Green	-	-	Fungal
57	G+S-RP CONTROL 3	Fungal	White	-	-	Fungal
58	G+S-RP CONTROL 3	Fungal	White	-	-	Fungal
59	G+S-RP CONTROL 3	Fungal	Green	-	-	Fungal
60	G+S-RP CONTROL 3 10-4	Fungal	Grey	-	-	Fungal
61	G+C-RP CONTROL 3 10-6	Fungal	Grey	-	-	Fungal
63	G+C-RP P2	Fungal	Green	-	-	Fungal
64	G+C-RP P2	Fungal	Green	-	-	Fungal
65	G+C-RP P2	Circular	Cream	Convex	Entire	Bacterial
66	G+C-RP P2	Irregular	White	Raised	Undulated	Bacterial
67	G+C-RP P2	Circular	Cream	Raised	Entire	Bacterial
68	G+C-RP P2	Circular	White	Raised	Entire	Bacterial
69	G+C-RP P2	Circular	White	Convex	Entire	Bacterial
70	G+C-RP P2	Circular	White	Raised	Entire	Bacterial
71	C+C-RP CONTROL 2 10 -5	Circular	White	Umbonate	Entire	Bacterial
72	C+C-RP CONTROL 1	Fungal	White	-	-	Fungal
73	C+C-RP CONTROL 2 10-4	Circular	White	Umbonate	Entire	Bacterial
74	C+C-RP CONTROL 2 10-4	Circular	White	Raised	Umbonate	Bacterial
75a	C+C-RP CONTROL 2 10-4	Fungal	White	-	-	Fungal
75b	C+C-RP CONTROL 2	Filamentous	Cream	Flat	Filamentous	Bacterial
76a	C+C-RP CONTROL 2 10-4	Fungal	White	-	-	Fungal
76b	C+C-RP CONTROL 2	Filamentous	Cream	Flat	Filamentous	Bacterial
77	C+C-RP CONTROL 2 10-4	Fungal	White	-	-	Fungal
79	C+C-RP CONTROL 2	Filamentous	Cream	Flat	Filamentous	Bacterial
80	G+C-RP P3	Fungal	Cream			Fungal
81	G+C-RP P3					Mixed
82	G+C-RP P3	Filamentous	Cream	Convex	Filamentous	Bacterial
84	G+C-RP P5	Rhizoid	Cream	Raised	Filamentous	Bacterial
85	G+C-RP P5	Circular				Mixed
86	G+C-RP P5	Spindle	Cream	Convex	Entire	Bacterial
88	S+S-RP CONTROL 2	Circular	Cream	Umbonate	Entire	Bacterial
90	G+S-RP P5	Circular	Translucent	Pulvinate	Entire	Bacterial

Full Length Research Paper

Genetic diversity and plant growth promoting activity of salt tolerant rhizobacteria from rice (*Oryza sativa* L.) rhizosphere

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Rhizobacteria are possible alternative to chemical fertilizers and used to manage phyto-pathogens. In this study, rhizobacteria isolated from rice rhizosphere of coastal agriculture system of South Andaman, India were evaluated *in vitro* for their plant growth promoting property (PGP), antagonistic activity and genetic diversity using PCR-RFLP. From 63 isolates, 35 were positive for salt tolerance of upto 0.5 M NaCl and 27 isolates upto 1.0 M NaCl. The biochemical characterization showed that most of the isolates were positive for one or other plant growth promoting property. Antagonistic activity against the fungi *Sclerotium rolfsii* revealed that 13 isolates have significantly inhibited mycelial growth of *S. rolfsii* as compared to the control but the isolate HMI 4, HMI 5, HMI 10, HMI 14, HMI 16 and HMI 17 were most efficient in percentage inhibition of test pathogen, caused more than 35% inhibition. RFLP of 16s rDNA showed homogenous population distributed randomly at 59% similarity. 16s rDNA sequencing result showed that most predominant population were *Pseudomonas* spp. followed by *Enterobacter* and *Bacillus* spp. Five best isolates were evaluated *in vitro* for PGP activity in rice and were found promising. The present study has resulted in the isolation of PGP and antagonistic *Pseudomonas* (HMI 16, HMI 17) and *Bacillus* spp. (HMI 14) that could be useful to design strategies to use these isolates as inoculants in sustainable agriculture of Andaman, India.

Key words: Andaman, *Bacillus*, phosphate solubilization, PCR-RFLP, siderophore.

INTRODUCTION

One of the most widespread agricultural problem in coastal areas is soil salinity, which limits plant growth and crop yield by reducing uptake of essential nutrients especially phosphorus. Intensive use of chemical fertilizers has resulted in increased soil salinity leading to deterioration of soil health. All the possible measures in use today, use of plant growth-promoting (PGP) bacteria is

an economical and environmental friendly approach (Dixon et al., 1993). Rhizobacteria inhabiting the sites exposed to frequent stress conditions are likely to be more adaptive or tolerant and may serve as better plant growth promoter under stress conditions (Lifshitz et al., 1986).

The role of microorganisms in plant growth promotion, nutrient management and disease control is eminent.

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These beneficial microorganisms colonize the rhizosphere of plants and promote growth of the plants through various direct and indirect mechanisms (Saxena et al., 2005). Soil microorganisms transform the insoluble forms of phosphorus (P) into soluble forms and thus influence the subsequent availability of P to plant roots. They also release siderophore for the uptake of Iron. The ability of bacteria to solubilize mineral phosphates and produce siderophore has been of interest to agricultural microbiologists because it can enhance the availability of phosphorus (P) and iron (Fe) for microbial and plant growth. Plant growth-promoting rhizobacteria (PGPR) has been reported not only to improve plant growth but also suppress the growth of plant pathogens by producing enzymes and secondary metabolites such as hydrogen cyanide (HCN), organic acids, etc (Dobbelaere et al., 2003). *Pseudomonas* and *Bacillus* spp. are immensely important in this regards as these are aggressive colonizers of the rhizosphere of various crops and have broad spectrum of antagonistic activity against many pathogens (Weller et al., 2002, Siddiqui; 2005). The mechanisms involved in PGPR-mediated plant growth promotion include synthesis of phyto-hormones such as indole-3-acetic acid (IAA), cytokinins and gibberellins (Idris et al., 2004) and an increased uptake of available minerals, nitrogen and phosphorus in the soil (Dobbelaere et al., 2003).

Rice (*Oryza sativa* L.) is the staple food crop of India and cultivated worldwide. In Andaman Islands, paddy is cultivated along the coastline where salinity gradient dominate. An increase in salinity led to decrease in crop productivity and rhizobacterial diversity. However, the race for producing more rice by applying more chemical fertilizers has adverse effects on the soil health and microbial community structure (Borneman et al., 1996). An understanding of structural and functional diversity of rhizobacteria is therefore essential to exploit the full potentials of these microbes for the sustained crops growth in different agro-ecosystems. With the currently available tools, the microbial community structure can be examined at several levels. The simplest analysis is based on DNA profiles, generated by PCR followed by restriction digestion of PCR product, to identify differences in the community composition. 16S rRNA gene sequences have been efficiently used for the identification of bacteria at species or strain levels (Rangarajan et al., 2001; Kumar et al., 2014). The objective of the present study was to study the genetic diversity of salt tolerant, plant growth-promoting (PGP) rhizobacterial microbes from rice rhizosphere so that a firm conclusion can be drawn on the salt-tolerant PGPR and their community structure associated with rhizosphere soil of rice in Andaman Islands.

MATERIALS AND METHODS

Collection of soil samples and isolation of bacterial isolates

The present study was conducted at Central Agricultural Research

Institute, Port Blair during 2009-10. Rice (*Oryza sativa* L.) plants were grown at agricultural field of Bloomsdale farm, Central Agricultural Research Institute (CARI), Port Blair, South Andaman, India. Soil was sandy loam and its characteristics were as follows: Organic carbon (O.C) 0.35, pH 4.9, nitrogen 336 kg/ha, phosphorus 15.1 kg/ha and potassium 43 kg/ha. Fifty days after transplanting two plants from each corner and middle of the rice plot, they were uprooted and loosely adhered soil was removed. Lower 2/3 portion of roots with tightly attached soil were used for isolation of bacteria. 63 isolates of bacteria were aseptically isolated by serial dilution (10^{-3} to 10^{-6}) method on Nutrient Agar medium (NA, Hi-Media). To ensure culture purity, each colony was zig-zag streaked thrice on new NA plate. All the pure cultures in tubes were maintained on NA slant at 4°C.

Salt tolerance and fluorescence test

All rhizobacterial isolates were tested for their NaCl tolerance by agar plate method. Rhizobacterial isolates were spot inoculated on freshly prepared agar plates amended separately with increasing concentration of NaCl (0.1M to 2.0 M). Plates were incubated at $28 \pm 2^\circ\text{C}$ for 5 days and highest concentration of NaCl sustaining the bacterial growth was defined as the maximum tolerance (Yildirim et al., 2008). The fluorescence of cultures were identified by inoculating the isolates over the King's B medium. After incubation for 2 days at $28 \pm 2^\circ\text{C}$, fluorescent bacterium was identified under UV light (Rouatt and Katznelson, 1961).

Phenotypic and biochemical characterization

Phenotypic characterization was done on the basis of Gram's staining, catalase, oxidase and utilization of 12 different substrates (Hi Media kit). The reactions tested were citrate utilization, lysine decarboxylase, ornithine decarboxylase, urease, phenylalanine deamination, nitrate reduction, H_2S production, glucose, adonitol, lactose, arabinose and sorbitol. The results were interpreted with the help of the color change after addition of the respective reagents wherever necessary.

PO_4 solubilization and siderophore production

Rhizobacterial isolates were tested for phosphate solubilization activity on Pikovaskaya's Agar (PA) media (Hi-Media) and log phase bacterial culture was spot inoculated over PA. Plates were incubated at $28 \pm 2^\circ\text{C}$ for 2 - 3 days. Appearance of a clear halo around the colonies indicates solubilization of phosphate (Mehta and Nautiyal, 2001). Production of siderophore was determined using the chrome azurol S (CAS) agar assay. Bacterial Inoculum ($10 \mu\text{l}$) was inoculated onto the center of a CAS plate and incubated at $28 \pm 2^\circ\text{C}$ for 3 days. Siderophore production was assessed on the basis of change in color of the medium from blue to orange (Schwyn and Neilands, 1987).

Indole-3 acetic acid (IAA) and hydrogen cyanide (HCN) production

The bacterial isolates were grown on LB agar supplemented with L-trptophan and a sterile filter paper was placed over the culture. After five days of incubation at $28 \pm 2^\circ\text{C}$, filter paper was treated with salkowski reagent (35% of perchloric acid and 0.5 M FeCl_3). Development of red color indicates production of IAA by bacteria. HCN was estimated qualitatively by sulfocyanate colorimetric method. The bacterial cultures were grown on King's B agar amended with glycine (4.4 g L) at $28 \pm 2^\circ\text{C}$. One sheet of Whatmann

filter paper no.1 was soaked in sodium picrate solution (2% sodium carbonate and 0.5% picric acid) for a minute and struck underneath the Petri dish lid. The plates were sealed with parafilm and incubated at 28±2°C for 48 h. Development of reddish brown color on the filter paper indicates HCN production (Vikram et al., 2007).

Production of cellulase, protease and amylase

Isolates were screened for cellulase production by plating on solid cellulose medium amended with 5 g of sodium carboxy methyl cellulose, 5 g of peptone, 20 g of agar and 10 g of yeast extract per liter. After eight days of incubation at 28 ± 2°C. Gram's iodine solution was added and colonies surrounded by clear halos were considered positive for cellulase production (Cattelan, 1999). The protease activity was determined using skim milk agar (SMA) medium (Hi-Media). Log phase cultures were spot inoculated over SMA and incubated at 28 ± 2°C for two days. Protease activity was revealed by clear zone around the culture (Smibert and Krieg, 1994). Starch agar was used to screen the isolates producing the amylase enzyme. Cultures were spot inoculated on to the starch agar and incubated at 28 ± 2°C for two days. After incubation, iodine was added on the plates and formation of clear zone depicts positive amylase isolates.

Antagonistic test

Biocontrol activity of all isolates were evaluated *in vitro* by dual culture test as described earlier (Skidmore and Dickinson, 1976) against single fungal plant pathogen which is *Sclerotium rolfsii* causing stem rot of brinjal. The pure culture of pathogen was isolated from infected part of brinjal (collar) following tissue segment method, maintained in PDA slants and preserved at 4°C. Mycelial plug (6 mm) of *S. rolfsii* was inoculated in the centre of petriplate previously poured with PDA (Hi Media), the bacterial isolates were streaked on the periphery of the plate. Plates were incubated at 28 ± 2°C for four days. The Petri plate with mycelial plug of pathogen only served as control. All isolates were tested in triplicates.

DNA extraction, RAPD and PCR amplification of 16s rRNA gene

All cultures were maintained on NA at 28 ± 2°C. For DNA extraction, pure culture of 23 best isolates were grown in nutrient broth at 28 ± 2°C, 200 rpm for 24 h. Bacterial pellets were collected by centrifugation at 10,000 rpm, 4°C, 5 min. Total genomic DNA was extracted by using the method described by Sessitsch et al. (2003) with slight modification. DNA was dissolved in 100 µl 1x TE buffer. RAPD was done with 10 different primers of OPA, OPE series obtained from OPERON TECHNOLOGIES (Inc. Alameda Calif.) (Supplementary Table 3). The PCR was performed by initial denaturation at 94°C for 5 min followed by 45 cycle of denaturation at 94°C for one min., annealing at 37°C for one min and extension at 72°C for two min, and final elongation of at 72°C for 7 min. For RFLP primer PA (5'AGAGTTTGATCCTGGCTCAG 3') and pH (5'AAGGAGGTGATCCAGCCGCA 3') designed by Edwards et al. (1989) were used to amplify a 1.5 Kb fragment of 16s rDNA. PCR amplification was performed in a total volume of 50 µl by mixing 50 ng of genomic DNA, 0.2 µM concentration of each primer (Gene[®]), 2.5 mM dNTPs (Gene[®]) and 1 unit of *Taq* DNA polymerase (Gene[®]) in 1x PCR buffer (Gene[®]). The reactions were subjected to initial denaturation of 92°C for 2.10 min followed by 45 cycle of 92°C for 1 min, 48°C for 30 s, 72°C for 2 min with a final extension of 72°C for 7 min. PCR amplified products were resolved on 1% agarose gel, prepared in 1x TAE buffer containing 0.5 µg ethidium bromide and photographed over a UV transilluminator.

RFLP and data analysis

1.5 Kb PCR product from all the 20 isolates were subjected to restriction digestion by 3 different restriction enzymes (RE) (*Msp I*, *Hae-III*, *Hinf-1*) in a final volume of 20 µl containing PCR product 17 µl, RE 1U (Gene[®]), 10x Buffer 2 µl (Gene[®]), incubated at 37°C for two hour. After two hour digested product was mixed with 2 µl of bromophenol blue dye and resolved over 1% agarose gel (Gene[®]), prepared in 1xTAE buffer containing 5 µl ethidium bromide. All the genotypes were scored for presence and absence of the RFLP bands and the data were entered into a binary matrix as discrete variables. The 0/1 matrix was used to calculate similarity as Jaccard coefficient using SIMQUAL subroutine in similarity routine. The resultant similarity matrix was employed to construct dendrogram using SAHN based UPGMA to infer genetic relationship (Rohlf, 1998).

In-vivo PGPR activity

In order to test the effective contribution to plant growth, five isolates were used for *in vivo* experiment. Pure bacterial cultures were grown in nutrient broth at 28±2°C until they reached a final concentration of 10 colony forming unit (CFU). Seeds of rice (*Oryza sativa* L) variety *C-14-8* were surface sterilized in 70% ethanol for 1 min and 1.2% sodium hypochlorite for 10 min and rinsed thrice with sterile tap water. Pots filled with a sterile soil were seeded with 10 seeds/pot. Treatment consists of inoculation of 5 ml of different bacterial cultures (HMI 4, HMI 5, HMI 7, HMI 16 and HMI 17) and fertilizer. A control without microorganism and fertilizer was also maintained. Besides, each pot was irrigated with distilled water without microorganism. 30 days after sprouting, plants were harvested and data (mean) of the root and shoot length and dry matter was recorded.

Nucleotide sequence accession numbers of 16s rRNA gene

1.5 kb PCR product of some isolates was sent to commercial gene sequencing laboratory of Bangalore Genei, Bangalore, India for sequencing. Sequence analysis of these isolates were performed using BLAST (blastn)^{2.0} search tool (<http://www.ncbi.nlm.nih.gov>) available on the NCBI homepage. Unrooted phylogram was constructed using Clustal W. The nucleotide sequences of 16s rDNA were submitted in Gene bank. Their accession numbers are presented in Table 3.

RESULTS

Isolation and screening of the salt tolerant PGPR

In the present study, we isolated 63 bacterial isolates from the rhizosphere soil of rice. All the bacterial isolates were evaluated for their salt tolerance trait and 35 were found positive at upto 0.5 M NaCl, 27 were positive at upto 1.0 M NaCl, however only 3 isolates could grow in 2.0 M of NaCl. 27 isolates which survived on 1.0 M NaCl containing NA plate were further used in the study (Supplementary Table 2)

Phenotypic and biochemical characterization of rhizobacteria

Based on the phenotypic characterization, 11 isolates

Table 1. Phenotypic and biochemical characterization of the bacterial isolates.

Biochemical test	Number of positive Isolates
Gram Staining (+)	16
Gram Staining (-)	11
Catalase	11
Oxidase	14
Phosphate Solubilization	21
IAA production	13
Siderophore production	20
Protease production	12
Amylase production	15
Cellulase production	11
Citrate utilization	8
Lysine decarboxylase	9
Ornithine decarboxylase	6
Urease	2
Phenyl alanine deamination	1
Nitrate reduction	3
H ₂ S Production	0
Glucose	7
Adonitol	1
Lactose	2
Arabinose	8
Sorbitol	2
HCN	3

Table 2. Percentage inhibition shown by rhizobacteria against *Sclerotium rolfsii*.

Isolates code	Inhibition (%)
HMI 02	25
HMI 10	37.5
HMI 11	10
HMI 13	35
HMI 14	37.5
HMI 5	50
HMI 16	37.5
HMI 18	22.5
HMI 20	24
HMI 23	25
HMI 4	42.5
HMI 7	30
HMI 17	40
CD	0.1041
SEd	0.0506

(40%) were Gram negative and 16 (59%) were Gram positive. Most of the isolates (21) showed motility over motility agar. From 27 isolates 11 (40%) were positive for

enzyme catalase and 14 (51%) for oxidase respectively. When biochemically characterized the isolates exhibited variation in utilization of different carbon source such as glucose, adonitol, lactose, arabinose as well as sorbitol, lysine and phenylalanine. Isolates also showed variability in production of the enzyme urease, nitrate reduction and H₂S production (Table 1 and Supplementary Table 1).

Test for PGPR activity and antagonism

From 27 isolates, 20, 5, 13 and 3 isolates were positive for siderophore, fluorescence, IAA and HCN test respectively, whereas 21(77%) isolates showed phosphorus solubilization on Pikovaskaya's agar. A significant reduction in pH of Pikovaskaya's agar from pH 7.4 to 4.5 was observed on 10th day of incubation. The isolates were then screened for their production of the cell wall degrading and other enzymes. From 27 isolates, 12 (44%) showed proteolytic activity by inducing clear zones on the skim milk agar medium, 15 (55%) isolates were positive for amylase enzyme and 11 (40%) were positive for cellulase enzyme (Table 1 and Supplementary Table 2). The isolates were further screened for their antagonistic potential against *S. rolfsii*. Antagonistic activity against *S. rolfsii* revealed that 13 isolates have significantly inhibited mycelial growth of phyto-pathogen as compared to the control but the isolate HMI 5, HMI 4, HMI 10, HMI 14, HMI 16 and HMI 17 were most efficient in percent inhibition of test pathogen, causing more than 35% inhibition (Table 2).

Genotypic characterization

RAPD fingerprinting

The RAPD profiles of the 23 isolates along with the markers are shown in Figure 1. All the isolates showed variation in fingerprinting pattern due to their genetic variability and distributed into different clusters. The cluster analysis based on RAPD resulted into 6 distinct clusters at a maximum similarity of 65% (Figure 6). The isolates HMI 3 and HMI 13 shows the maximum similarity of 94%.

PCR-RFLP and 16S rDNA sequence

From 27 isolates 20 were used to assess the genetic diversity by PCR-RFLP. Primer PA and PH amplifies a single band of 1.5 Kb in all the isolates and no inter or intraspecific variation among the isolates were observed. A set of three different restriction enzymes (*Hinf* I, *Hae* III and *Msp* I) were used for RFLP analysis. All the restriction enzymes have shown restriction in all the isolates. The banding pattern generated with enzyme *Hinf* I showed greater polymorphism as compared to

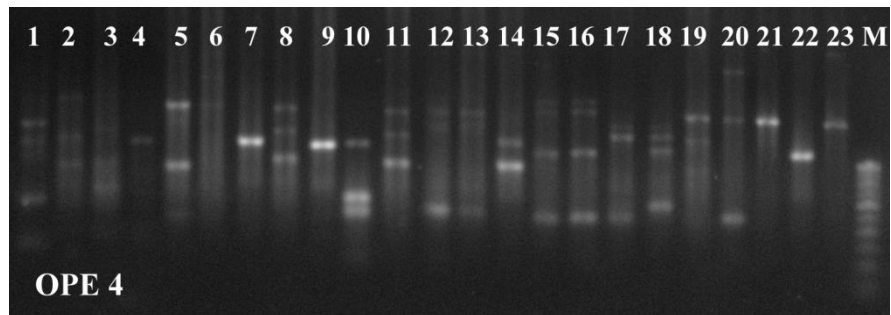


Figure 1. PCR profile of Rhizobacteria using RAPD primer OPE 4. Number (1-23) represent serial number for isolate. M is 100 bp ladder DNA.

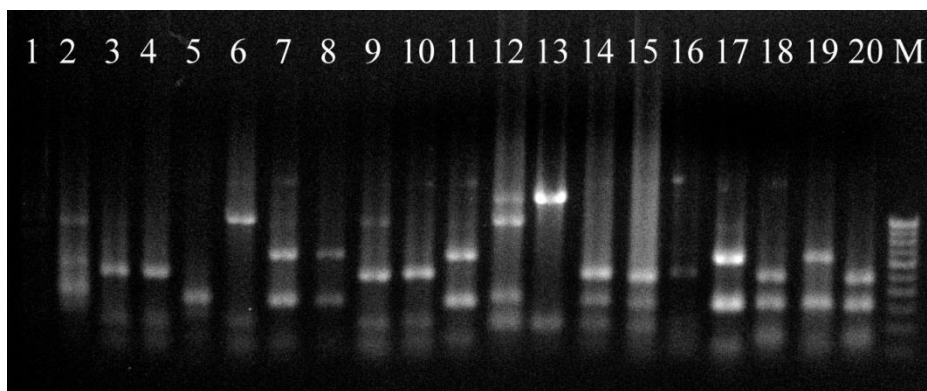


Figure 2. Restriction pattern of 16s rDNA after digestion with *Hinf* I. Number (1-20) represent serial number for isolate. M is 100 bp ladder DNA.

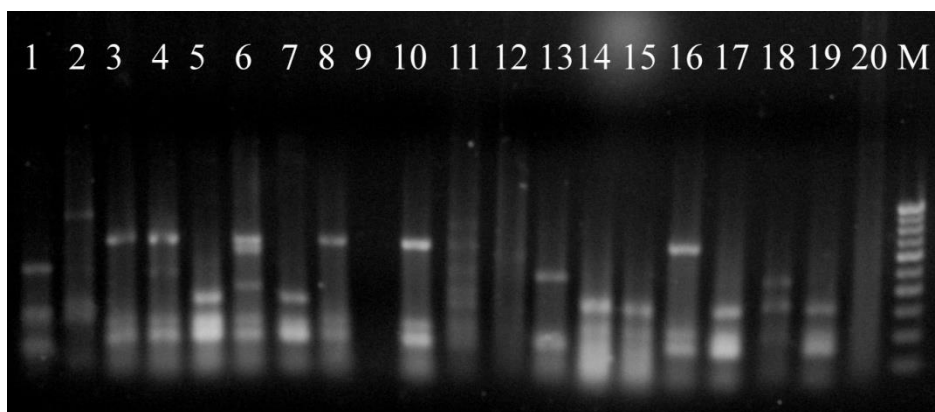


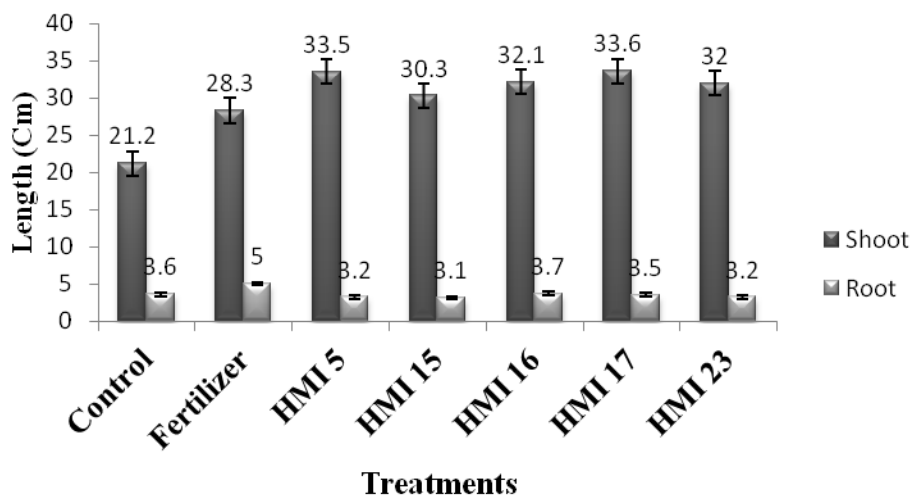
Figure 3. Restriction pattern of 16s rDNA after digestion with *Hae* III. Number (1-20) represent serial number for isolate. M is 100 bp ladder DNA.

other two enzymes (Figures 2 to 3). Banding pattern generated by enzyme *Hae* III and *Msp* I was monomorphic. Based on the combined RFLP data of all the three enzymes, a dendrogram was constructed that showed at 50% similarity, all the isolates of rhizobacterial

are randomly distributed (Figure 7). The dendrogram could be divided into five different clusters with similarity ranging from 0.59 to 0.82. Cluster one consist of HMI1, HMI 18, HMI 20, HMI 22, HMI 25 and HMI 26, cluster two consist of HMI 10, HMI 13, HMI 24 and HMI 27, cluster

Table 3. Identification of the bacteria on the basis of maximum similarity with 16s rRNA sequences.

Isolate name	Isolate code	No of base pair sequenced	Query coverage (%)	Accession	Gene bank accession number
<i>Burkholderia vietnamiensis</i>	HMI 27	581	96	JF922108	HM036111
<i>Enterobacter</i> sp.	HMI 26	745	98	JQ659764	HM036107
<i>Burkholderia vietnamiensis</i>	HMI 25	710	94	JF922108	HM036112
<i>Enterobacter</i> sp.	HMI 24	737	94	JQ659764	HM036108
<i>Enterobacter</i> sp.	HMI 18	782	99	KJ584024	HM036109
<i>Enterobacter</i> sp.	HMI 13	783	99	JQ659764	HM036110
<i>Pseudomonas putida</i>	HMI 5	725	96	GQ200822	GU937763
<i>Pseudomonas</i> spp.	HMI 4	465	95	KJ713266	GU937764
<i>Paenibacillus illinoisensis</i>	HMI 2	413	90	HF585081	GU937765
<i>Bacillus subtilis</i>	HMI14	425	99	KJ126927	GU937766
<i>Bacillus megaterium</i>	HMI 19	787	98	EF694699	HM064042
<i>Pseudomonas stutzeri</i>	HMI 7	453	98	EU741087	HM064043
<i>Pseudomonas putida</i>	HMI 16	750	98	AM184288	HM064044
<i>Pseudomonas putida</i>	HMI 17	780	100	AY714238	HM064045

**Figure 4.** Impact of rhizobacteria on seedling growth of rice cv. C-14-8.

three consist of HMI 19, cluster four consist of HMI 4, HMI 6, HMI 12, HMI 14, HMI 15 and HMI 16, cluster five consist of HMI 17, HMI 17 and HMI 21 respectively. HMI 25 and HMI 26 showed 100% similarity. The 16S rDNA sequencing data showed that most of these rhizobacteria belongs to genus *Pseudomonas* spp., *Enterobacter* spp., *Bacillus megaterium* and *Bacillus subtilis*. However, some of the isolates were from genus *Burkholderia vietnamiensis* (Table 3). An unrooted cladogram showing similarity is present in Figure 8.

In-vitro PGPR assay

In order to confirm the PGP traits, five isolates (HMI- 4, 5,

7, 16 and 17) which showed significant PGPR activities *in vitro* were further evaluated for their *in vivo* PGP assay in rice (*O. sativa* L.) varieties C-14-8. Significant difference (SEd 0.05) was observed for shoot length and biomass of plant among the culture treated, fertilizer treated and control. All isolates increased the shoot length but for root no significant difference was observed among isolates (Figures 4 and 5).

DISCUSSION

Soil salinity plays an important role in the selection of microflora as environmental stress reduces their activity,

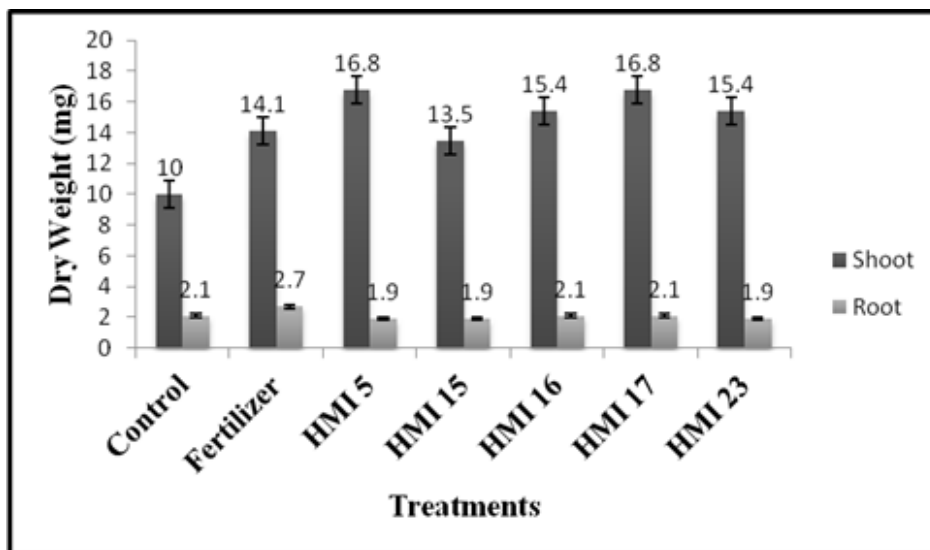


Figure 5. Impact of rhizobacteria on seedling dry weight (mg/plant) of rice cv. C-14-8.

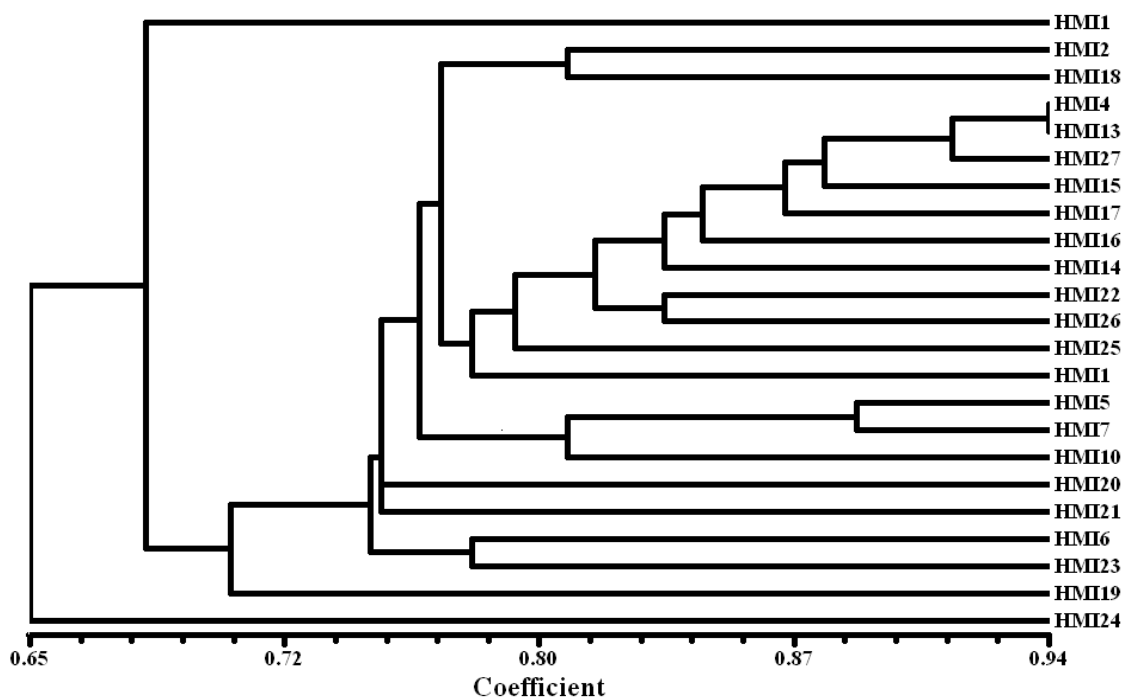


Figure 6. Dendrogram showing the relationship among 23 rhizobacterial isolates based on the RAPD.

number and diversity. Bacterial adaptation to salinity mainly depends on their genetic diversity (Sudhir et al., 2009). The present investigation revealed the salt tolerant rhizobacteria diversity with innate potential of plant growth promotion and biocontrol to *Sclerotium rolfsii*. Population of bacterial community recovered from rice rhizosphere depicts variation in their phenotypic characteristics. These differences indicate significant diversity within the cultureable population. In our study, 21 and 20 isolates were positive for phosphate solubilization and

siderophore production, respectively. The ability of bacteria to solubilize mineral phosphate and produce siderophore is a field of immense interest to agricultural microbiologists because it can enhance the availability of P and Fe for microbial and plant growth. It is believed that microbial mediated solubilization of insoluble phosphorus in soil is through the release of organic acids (Nautiyal et al., 2000). However, in addition to biotic acidification, other mechanisms such as chelation and exchange reactions can cause phosphate solubilization (Illumer

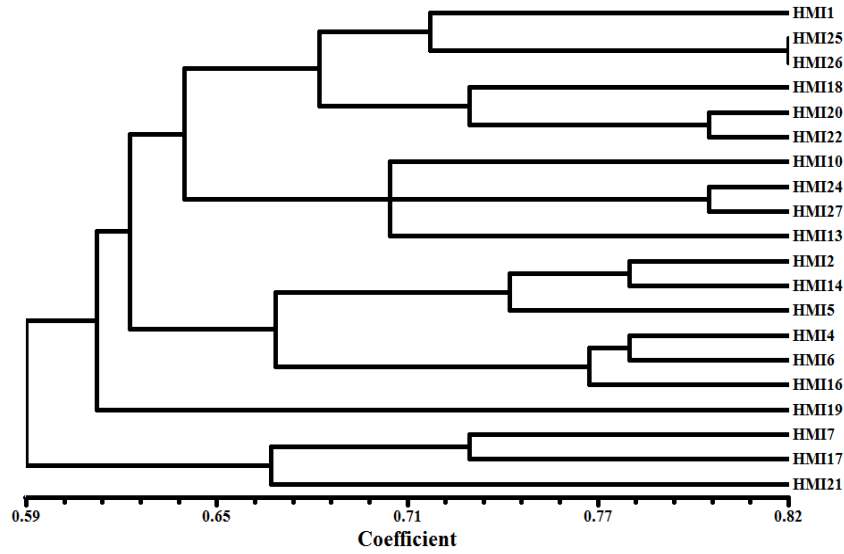


Figure 7. Dendrogram showing the relationship among 20 rhizobacterial isolates based on the PCR-RFLP profiles of 16s rDNA regions.

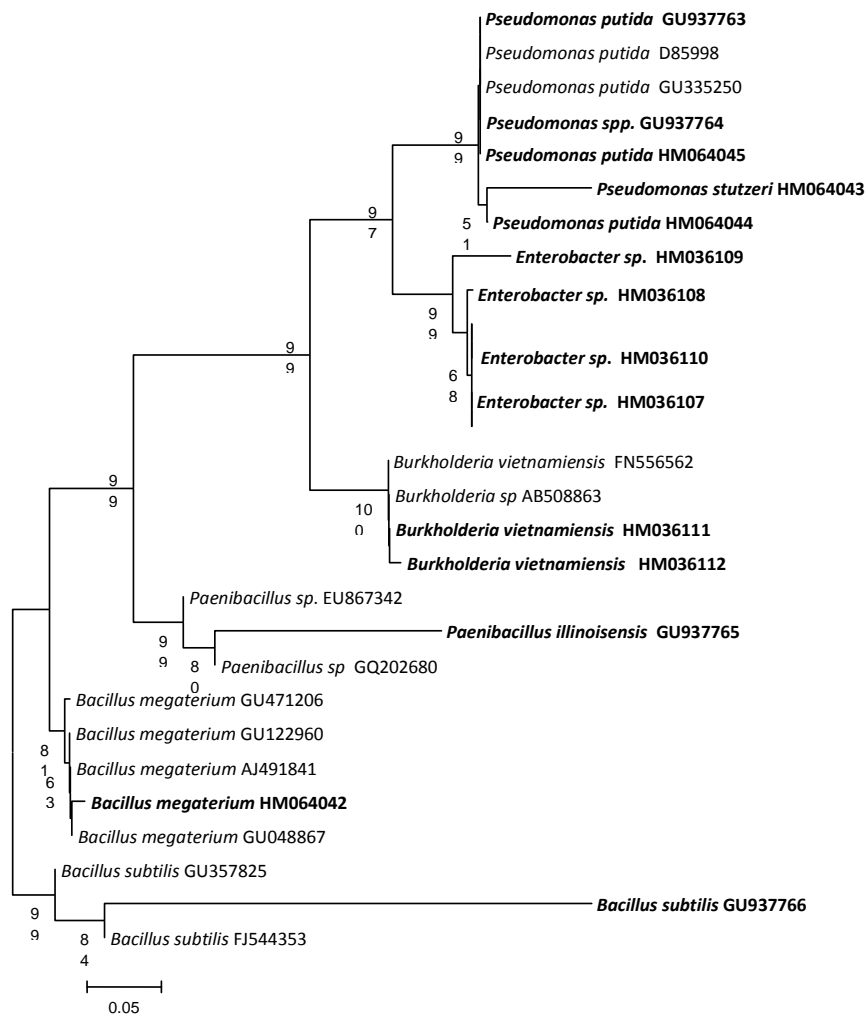


Figure 8. Unrooted phylogram constructed using 16S rRNA sequences of rhizobacteria. isolates in bold face are from the present study, along with the reference strains (in normal face) retrieved from NCBI GenBank.

and Schinner, 1995; Richardson et al., 2001; Gyaneshwar et al., 1998; Rodriguez et al., 2004). Siderophore production is another PGP feature that may influence plant growth by binding to the available iron form (Fe^{3+}) in the rhizosphere. Through this process, iron is made unavailable to the phytopathogens and at the same time siderophore protects the plant health (Siddiqui, 2005).

IAA producing bacteria are known to promote plant growth (Patten and Glick, 2002). In our study, 13 isolates produced a substantial amount of IAA, however variability in the amount of IAA was observed, suggesting that these isolates could be used for plant growth promotion. Mirza et al. (2001) showed that indolic compounds production can vary among different species and strains and is also influenced by culture conditions, growth stage and availability of the substrate.

Many *Bacillus* and *Pseudomonas* spp. are capable of producing a range of different enzymes, antibiotics and toxins, many of which can be antagonistic to plant pathogens (El-Banna, 2005; Chakraborty et al., 2006). In the present study, *Pseudomonas* and *Bacillus* isolates have revealed better antagonistic activities than other isolates. Most of the *Pseudomonas* isolates inhibited growth of plant pathogens by 37.5-50% while isolates of *Bacillus* showed 37.5%. Reduction in the growth of phytopathogen could be due to the secretion of lytic enzymes, HCN, siderophore and other secondary metabolites. Voisard et al. (1989) reported that cyanide released by *Pseudomonas fluorescens* suppresses the growth of phytopathogens sharing the same ecological niche, thereby acting as a biocontrol metabolite. Various species of *Pseudomonas* have been reported to promote plant growth and suppress disease in plants. A mixture of *Bacillus amyloliquefaciens* and *Bacillus pumilus* were found to provide a broad spectrum of protection against both soil and air-borne pathogens including cucumber mosaic virus, *Colletotrichum gloeosporioides*, *Ralstonia solanacearum*, *Rhizoctonia solani* and *Sclerotium rolfsii* (Jetiyanon et al., 2003). Adhikari et al. (2001) reported that bacterial strains isolated from the rice rhizosphere have the potential to control the seedling disease of rice. On the basis of PGPR and antagonistic activities, five *Pseudomonas* isolates were selected for the study of the effect of isolates on the growth performance of rice. An increase in plant growth was observed for all the tested isolates. This was due to the secretion of IAA, solubilization of phosphorus and secretion of other various beneficial secondary metabolites. All the tested isolates have shown enhanced shoot growth and biomass as compared to the control. However, no significant variation was observed for root length and biomass as higher concentration of IAA (auxin) is stimulatory for the shoot growth but inhibitory for root.

16S rRNA gene sequencing is a widely used standard technique in bacterial taxonomy and it is also routinely used in 'polyphasic approach' when new descriptions of

bacterial species or higher taxa are made (Ludwig and Schleifer, 1999; Rossello-Mora, 2005). Genotypic analysis by PCR-RFLP as well as 16s rRNA gene sequencing analysis revealed a low degree of diversity among the isolates. In PCR-RFLP, there was a difference in only one of the restriction pattern studied. This was with *Hinf* I which shows a common restriction pattern in all except 9 isolates. Similarity in the digestion pattern of the other enzymes used and 11 isolates that showed any variation suggests a predominant homogenous bacterial population. Homogeneity in bacterial population can be explained on the basis of bacterial ability to tolerate salt stress. The variation in the tolerance could probably be due to the fact that rhizobacteria adopt different strategies to overcome the toxic effect of the salinity. The dominance of some components of the bacterial population in the rhizosphere could be due to rhizodeposition as size and composition of the rhizospheric microflora depends on plant species (Lynch and Whipps, 1990). Rangarajan et al. (2001) analyzed population of *Pseudomonas* for their biochemical characters and genetic diversity using RAPD and RFLP and found that increased salinity caused selection of *P. pseudoalcaligenes* and *P. alcaligenes*, irrespective of the host rhizosphere.

The present work identified isolates of plant growth promoting rhizobacteria with potential for biological control. These microorganisms are of particular interest because they have revealed *in vitro* PGP and bio-control activity against tested plant pathogen. Despite *in vitro* suppression of test pathogen, an *in vivo* PGP activity was observed. These isolates (HMI 14, HMI 16 and HMI 17) may prove to be novel PGPR isolates for effective formulation of bio-fertilizer to saline affected areas. These isolates could be utilized to improve the fertility status of soil, soil health, to protect the environment and will be cost effective. Since benefits from the PGPR could be synergistic, further experiments are needed to determine the effectiveness of these isolates under different field conditions and to understand the nature of interaction with the host plant.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

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Supplementary Table 1. Growth performance of some isolates in NA containing different NaCl concentration.

NaCl conc. (M)	Isolate code																			
	HMI 2	HMI 4	HMI 5	HMI 7	HMI 8	HMI 10	HMI 11	HM I 13	HMI 14	HMI 15	HMI 16	HMI 17	HMI 18	HMI 19	HMI 20	HMI 23	HMI 24	HMI 25	HMI 26	HMI 27
0.5	+	+	-	+	-	+	-	+	+	+	+	+	+	+	-	+	+	+	+	-
1.0	-	-	-	-	-	+	-	+	-	-	-	-	-	+	-	+	+	-	+	-
1.5	-	-	-	-	-	+	-	+	-	-	-	-	-	+	-	+	+	-	+	-
2.0	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	+	-	-	-

+: Depicts isolate that can grow; - depicts isolate that cannot grow

Supplementary Table 2. Biochemical performance of some of the rhizobacterial isolates.

Substrate	Isolate code																			
	HMI 2	HMI 4	HMI 5	HMI 7	HMI 8	HMI 10	HMI 11	HM I 13	HMI 14	HMI 15	HMI 16	HMI 17	HMI 18	HMI 19	HMI 20	HMI 23	HMI 24	HMI 25	HMI 26	HMI 27
Flourescence	-	-	+	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-
Cellulase	+	+	-	+	-	-	+	+	-	-	+	+	-	-	-	-	-	-	+	-
Amylase	+	+	-	+	+	-	+	+	-	-	-	-	+	+	+	+	-	-	-	-
Protease	+	-	+	+	+	-	+	-	-	+	-	+	-	+	-	+	-	-	-	+
Siderophore	+	+	-	+	+	+	+	-	-	+	+	+	+	+	+	+	-	+	-	+
Phosphate	+	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
IAA	+	+	+	+	-	+	-	+	-	+	+	+	+	-	+	+	-	-	+	-
HCN	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-
Catalase	-	+	+	+	+	-	+	+	+	+	+	+	+	-	-	-	-	-	-	-
Oxidase	+	-	+	+	+	+	+	-	+	+	+	+	+	-	-	-	-	+	+	-
Citrate utilization	+	+	+	-	-	-	+	-	-	-	+	+	-	-	-	-	-	+	-	-
Lysine utilization	+	+	+	-	-	-	+	-	-	-	+	+	-	+	+	-	-	-	-	-
Ornithine utilization	-	-	+	-	-	-	+	-	-	-	-	-	-	+	+	-	-	-	-	-
Urease production	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-
Phe-ala deamination	-	+	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-
Nitrate Reduction	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-

Supplementary Table 2. Contd.

Glucose	-	+	+	+	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-
Adonitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
Lactose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-
Arabinose	-	+	+	+	-	-	+	-	-	-	+	+	-	-	-	+	-	+	-	-
Sorbitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-

Supplementary Table 3. Primers sequences used in RAPD analysis.

Primer name	Sequence
OPA-01	CAGGCCCTTC
OPA-04	AATCGGGCTG
OPA-07	GAAACGGGTG
OPA-11	CAATCGCCGT
OPE-02	GGTGCGGGAA
OPE-04	CCAGATGCAC
OPE-06	AAGACCCCTC
OPE-07	AGATGCAGCC
OPE-10	CACCAGGTGA
OPE-11	GAGTCTCAGG

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