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Full Length Research Paper

Evaluation of biocontrol properties of *Streptomyces* spp. isolates against phytopathogenic fungi *Colletotrichum gloeosporioides* and *Microcyclus ulei*

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South American Leaf Blight (SALB) of the rubber tree, caused by *Microcyclus ulei* and foliar anthracnose caused by *Colletotrichum gloeosporioides*, are diseases that adversely affect rubber cultivation in America. Both diseases have a significant economic impact on this agricultural subsector. The aim of the present study was to evaluate the potential as biological control agents of three *Streptomyces* species strains, namely A20, 7.1 and 5.1, against *M. ulei* and *C. gloeosporioides*. The results of analysis of variance (ANOVA) and Tukey post-hoc test of the *in vitro* antifungal activity assays evidenced the potential of the three *Streptomyces* strains to inhibit *C. gloeosporioides* growth through the production of diffusible (A20 and 5.1) and volatile compounds (7.1). Furthermore, other results indicated that strain 5.1 had a high biocontrol activity against *C. gloesporoides*, and thus such strain was selected for further evaluations as a possible biocontrol agent against *M. ulei*. *In vitro* assays suggested that active compounds produced by 5.1 inhibited *M. ulei* growth by interfering with conidia germ tube and stroma formation. Bioassay-guided fractionation with organic solvents of 5.1 fermentation broths, suggested that antifungal compounds produced by this strain were nonionic compounds of medium-polarity. Currently, studies are ongoing to elucidate the chemical structure of these antifungal compounds. These approaches aim to generate a biological control agent to provide the Colombian rubber subsector with a preventive measure for controlling *M. ulei* and *C. gloeosporioides*.

Key words: Biological control, foliar anthracnose, rubber tree, South American Leaf Blight (SALB), *Streptomyces* species.

INTRODUCTION

Hevea brasiliensis is a native plant of the Amazon basin, member of the Euphorbiaceae family used for rubber production (natural rubber). Approximately, 10 million tons/year of natural rubber are produced from this tree,

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93% of the production comes from South East Asian countries such as Thailand, Malaysia, and Indonesia, 4.5% from Africa and only 2.5% from Latin American countries (Berthelot et al., 2014; Rivano et al., 2015). Even though, Colombia is not a natural rubber exporter, it possesses an ample natural rubber cultivation tradition located in the departments of Caquetá, Putumayo, Guaviare and Córdoba and more recently Santander and Meta with a cultivation area of 44,100 ha (Castellanos et al., 2009; Confederación Cauchera Colombiana, 2015). The difference observed in productivity between Asian and Latin American countries is generated in great part by the presence of an endemic disease known as the South American Leaf Blight (SALB) of the rubber tree caused by the Ascomycete *Microcyclus ulei* fungus (Gasparotto and Pereira, 2012). This phytopathogen infects mature fruits, stems, and young leaves (stage B) of the *Hevea* genus reducing the plant's growth, causing premature leaf falling, with reduction of its photosynthetic area. Moreover, it causes death of susceptible greenhouse clones and garden clones (Chee and Holliday, 1986; García et al., 2007; Gasparotto and Pereira, 2012). In addition to SALB, foliar anthracnose caused by *Colletotrichum gloeosporioides* is the second disease of fungal origin limiting *H. brasiliensis* natural rubber production (Guyot et al., 2005; Castro, 2011; Gasparotto and Pereira, 2012). This disease has generated an important impact on Colombian and Brazilian crops, with increasing incidence (Furtado and Trindade, 2005; García et al., 2007; Castro, 2011).

Despite the efficiency obtained from traditional chemical treatments for *M. ulei* and *C. gloeosporioides* in *H. brasiliensis,* high costs and environmental impact from airplane spraying has promoted the search for new strategies of biological control. For most cases, these microbial agents are capable of self-sustained growth after an initial inoculation, with mid- and long-term pathogen suppression; and less biological impact compared with traditional chemical control (Quimby et al., 2002; Palaniyandi et al., 2013; Yuliar et al., 2015). To generate this effect, biocontrol agents can use one or more mechanisms including nutrient competition, niche exclusion (competitive exclusion), signal interference of quorum sensing (Quorum quenching), parasitism, diffusible or volatile secondary metabolite production with antimicrobial activity (antibiosis), and induced systemic resistance in plants (Bloemberg and Lugtenberg, 2001; Hibbing et al., 2010).

Among the microorganisms evaluated as potential biocontrol agents, members of the *Streptomyces* genus stand-out. These gram-positive bacteria are characterized by mycelial growth similar to that of fungi. They are commonly isolated from terrestrial (soil, rhizosphere, and endosphere of plants) or marine environments, as free-living microorganisms or in association with other organisms (Coombs and Franco, 2003; Cao et al., 2004; Kinkel et al., 2012). Furthermore, they possess a diverse secondary metabolism that allows them to produce a great array of metabolites with antibacterial, antifungal, and antiviral activity. In addition, they are bioinsecticides, antitumoral and immune suppressors, among others (Omura et al., 2001; Hopwood, 2007; Kaur et al., 2014). In agriculture, numerous *Streptomyces* species have demonstrated capability of controlling diverse fungal phytopathogens of great agroindustrial impact (Samac and Kinkel, 2001; Taechowisan et al., 2003; Tian et al., 2004; Khamna et al., 2009; Zarandi et al., 2009; Gopalakrishnan et al., 2011; Li et al., 2012; Kanini et al., 2013). However, relatively few biological inoculants have been developed for crop use, mainly based on the poor association between the efficiency determined in the laboratory compared to the one observed in the greenhouse or the field (Bonaldi et al., 2015).

For the present study, three strains namely A20, 5.1 and 7.1, previously isolated by the Bioprocess and Bioprospecting Research Group from the National University of Colombia, were selected with the aim to verify its potential to act as biological control agents against *C. gloeosporioides* and *M. ulei* and to perform a preliminary characterization of the compound(s) associated with this activity. Suarez-Moreno et al. (2016, in press), taxonomically classified all three isolates within the *Streptomyces* genus, based on their biochemical profiles, colony macro- and microscopic characterization and sequencing of the rRNA 16S gene by Supplementary Tables 1 and 2. Within the initial characterization of these three strains, isolated from symptomatic rice, carnation and yam plants, their high potentials for bacterial and fungal phytopathogen control were determined. For these reasons, this work screened all three isolates aiming to determine their potential to control rubber fungal pathogens, looking forward to develop biological inoculants for natural-rubber farmers, as an approach to integral control for SALB and foliar anthracnose.

MATERIALS AND METHODS

Isolation, characterization and isolate growth

M. ulei and C. gloeosporioides isolation and identification

M. ulei and *C. gloeosporioides* isolates were obtained from affected foliage with SALB and anthracnose symptoms respectively from Corpoica's clonal garden - La Libertad Section, located in Villavicencio in the Mavalle S.A. plantation (Department of Meta, Colombia). For *M. ulei* isolation, pure *in vitro* cultures were obtained in M4 growth media (Junqueira et al., 1984) and identified by conidial asexual morphology reported for *Fusicladium heveae* (Anamorphic form of *M. ulei*, currently known as *Pseudocercospora ulei*) (Schubert et al., 2003; Hora et al., 2014) . On the other hand, *C. gloeosporioides* isolation was performed by direct seeding on PDA of anthracnose symptomatic foliage that was previously

treated with hypochlorite (5%) and ethanol (70%) (Agostini et al., 1992; Pinzón, 2014). Obtained monosporic cultures were morphologically characterized by macro- and microscopic description of the pathogen including aspect and color of the mycelium, form, size, segmentation and conidia sporulation according to previous reports in the literature (Gunnell and Gubler, 1992; Barnet and Hunter, 1998; Pérez et al., 2003). For both isolates, internal transcribed spacer (ITS) analysis was performed using ITS1/ITS4 universal primers (White et al., 1990). Obtained amplicons were sequenced in duplicate, assembled and analyzed by BLASTN against GenBank data base.

Streptomyces spp. strains

Streptomyces spp. A20, 5.1 and 7.1 strains were isolated from rice rhizosphere soil from La Pilar (Venadillo) and El Puente (Armero) farms in the Department of Tolima (Colombia) and were identified in previous works of the research group (Suarez-Moreno et al., 2016, in press).

For antifungal activity evaluations, each *Streptomyces* strain was growth in ISP3 solid medium (Oat Meal 20.0 $g.L^{-1}$, Agar 18.0 $g.L^{-1}$, $FeSO_4.7H_2O$ 0.001 $mg.L^{-1}$, $MnCl_2.4H_2O$ 0.001 $mg.L^{-1}$, $ZnSO_4.7H_2O$ 0.001 $mg.L^{-1}$, final pH: 7.3±0.2) or M3.7 liquid medium (5 $g.L^{-1}$ glucose, 5 $g.L^{-1}$ yeast extract, 2 $g.L^{-1}$ $CaCO_3$, 2 $g.L^{-1}$ tryptose, 10 $g.L^{-1}$, starch 2 $g.L^{-1}$ $SO_4(NH_4)_2$, 2 $g.L^{-1}$ NaCl, 1 $mg.L^{-1}$ $FeSO_4$ at pH 7.2 ± 0.2) depending on the assay performed. In solid cultures, each strain was incubated for 5 days at 30°C, whereas liquid cultures were incubated for 72 h at 30°C with constant agitation (150 rpm), and were used for the antifungal assays as described in the following (Shirling and Gottlieb, 1966).

Effect of *Streptomyces* spp. isolates on *C. gloeosporioides* mycelia growth

Streptomyces spp. A20, 5.1 and 7.1 strains were evaluated by dual culture plate assay to verify their ability to inhibit growth of *C. gloeosporioides* isolates C1, C2 and C3. Each *Streptomyces* strain was seeded on PDA in a straight line at 3 cm from the Petri dish periphery. 5 mm disc containing phytopathogen mycelia from each fungus (previously obtained from a 5 days-old solid PDA culture) was placed in the center of the petri dish. Media without *Streptomyces* was used as negative control. All media were incubated for eight days at 25°C and examined to verify inhibition areas between *C. gloeosporioides* and *Streptomyces* spp. A20, 5.1 or 7.1, respectively (Yuan and Crawford, 1995).

Production of diffusible and volatile compounds with antifungal activity

To evaluate whether the observed antifungal effect was due to diffusible or volatile compounds production, an agar well diffusion test and a volatile compound production assay was performed following CLSI 2011 guide, as well as recommendations suggested by Arrebola et al. (2010), respectively.

For agar well diffusion tests, 200 µl of a conidial suspension (10^5 conidia·mL^{-1}) of *C. gloeosporioides* isolates C1, C2 or C3 was massively seeded in Petri dishes with 25 ml PDA. Wells of 7 mm in diameter were opened at a distance of 4 mm from the edges of the Petri dish. Each well was inoculated with 100 µl of A20, 5.1 or 7.1 liquid spent supernatants from liquid cultures obtained from each *Streptomyces* isolate, as described previously. 10 µl Clotrimazole (100 µg $µl^{-1}$) and 100 µl M3.7 of sterile media were used as positive and negative controls, respectively (CLSI, 2011). Plates were incubated for 48 h at 25°C, and inhibition halo diameters were recorded in triplicates for each evaluated sample (Equation 1). Assays were performed three times using three biological replicates for each *Streptomyces* culture.

$$\text{Inhibition diameter} = \text{Total inhibition diameter} - \text{Well diameter} \quad (1)$$

To test if antifungal activity of the bacterial strains was due to the production of volatile compounds, a double-dish chamber assay was carried out. Briefly, bases of two Petri dishes containing 25 ml of PDA were used. For the first Petri dish, *Streptomyces* spp. isolates A20, 5.1 and 7.1 were seeded. In the second one, a 5 mm agar disc containing *C. gloeosporioides* C1, C2 or C3 mycelium was seeded. Both plates were then confronted and sealed with Parafilm™ aiming to obtain a chamber with a shared atmosphere without direct contact between both microorganisms. As a negative control, one experiment was set without *Streptomyces* in the first Petri dish (Arrebola et al., 2010). After eight days of incubation at 25°C, radial growth of C1, C2 and C3 were measured and compared to the negative control. Inhibition percentage was calculated using Equation 2 as described by Taechowisan et al. (2012).

$$Inhibition\ (\%) = \frac{[(GDU - GDT) \times 100]}{GDU} \quad (2)$$

where GDU refers to the growth diameter in untreated control and GDT corresponds to the growth diameter in treatments.

Effect of *Streptomyces* A20 and 5.1 filtered extracts on *C. gloeosporioides* mycelial growth and conidial germination

To assess the effect of *Streptomyces* A20 and 5.1 filtered extracts on *C. gloeosporioides* mycelial growth, methods suggested by Anthony et al. (2004) were used. Briefly, *Streptomyces* A20 and 5.1 crude extracts were obtained by centrifuging at 6000 rpm for 10 min 500 ml from three independent liquid fermentation cultures of each strain in M3.7 medium. The obtained supernatant was filtered through 0.22 µm nitrocellulose membrane and subsequently used to supplement 25 ml of PDA. Increasing volumes of filtered spent-supernatants were used in order to obtain a medium with concentrations of 6, 4 or 2% (v/v) of extract per petri dish. Later, 7 mm discs from C1, C2 and C3 *C. gloeosporioides* isolates were placed in the center of each dish, and incubated at 25°C to evaluate the radial growth of each isolate every 24 h until day 17 post-inoculation. *C. gloeosporioides* growth on each treatment was compared to a negative control seeded on PDA without extract, and the growth inhibition percentage was obtained 17 days after treatment from Equation 2 (Anthony et al., 2004; Taechowisan et al., 2012).

Based on the results obtained from this assay, supernatant effect on conidia germination process of the three *C. gloeosporioides* isolates was further evaluated for strain *Streptomyces* 5.1. For this purpose, conidia germination counts were measured with treated and no-treated conidia by using lyophilized crude extracts from *Streptomyces* 5.1. To this end, 100 µl of a conidial suspension (1×10^5 conidia·mL^{-1}) was supplemented with previously lyophilized 5.1 crude extract (obtained as described previously) and seeded onto PDA previously divided into 1 cm^2 squares. Four different concentrations of crude extract were evaluated (10, 25, 50 and 100 mg ml^{-1}). Conidia germination count was performed after 10 h incubation at 25°C. Percentage of germination inhibition was calculated in relation to the total number of conidia and the present germinated conidia in the sample as defined by Equation 3 (Palaniyandi et al., 2011).

$$\%\ Germination = \left(\frac{No.of\ germinated\ conidia \times 100}{No.of\ total\ conidia}\right) \quad (3)$$

Streptomyces 5.1 antifungal activity evaluation against *M. ulei*

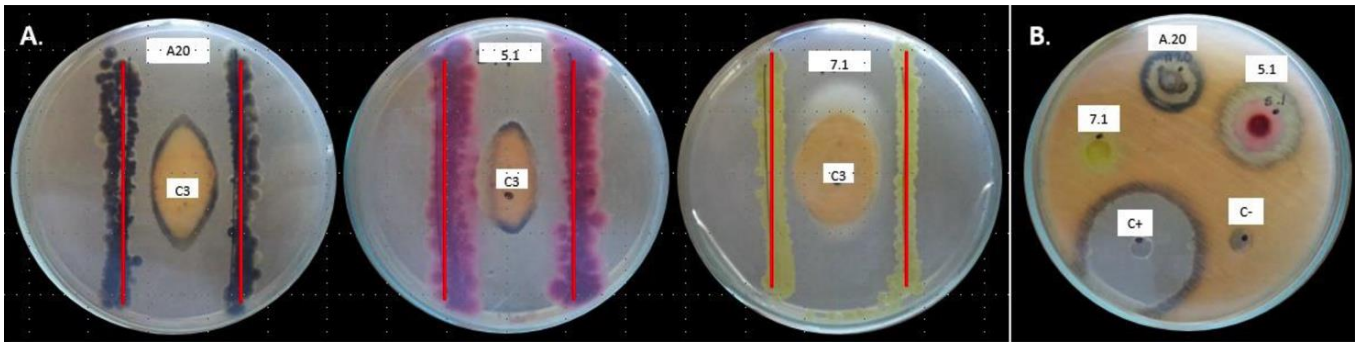

Figure 1. *C. gloeosporioides* C3 mycelial growth inhibition assay by: A. dual confrontation and B. well diffusion. C+: Clotrimazole 10 mg ml^{-1}; Media M3.7 C-: Media without inoculation.

germinated *M. ulei* conidia in suspension (1.65×10^5 conidia ml^{-1}) supplemented with four different concentrations of lyophilized *Streptomyces* 5.1 supernatant (10, 25, 50 and 100 mg ml^{-1}). 300 µl of saline solution were added to *M. ulei* conidia suspension as negative control. Conidia germination was determined as described for *C. gloeosporioides* assays (Equation 3). Moreover, the effect of 5.1 extracts on *M. ulei* stroma formation was evaluated by seeding 100 µl of treated conidia suspension on PDA with 5.1 supernatants. Stroma formation count on solid media was performed after 15 day incubation at 25°C, reporting growth as CFU ml^{-1} (Rocha et al., 2011).

Preliminary characterization of *Streptomyces* 5.1 produced active metabolites

In order to isolate and characterize antifungal compounds produced by *Streptomyces* 5.1, three independent liquid fermentations were carried out in 1 L of M3.7 medium. Culture media was inoculated and grown at 30°C, under constant agitation at 150 rpm. Each culture was then centrifuged for 10 min at 5000 rpm, and the obtained supernatants were filter sterilized by a 0.22 µm membrane. Spent supernatants were analyzed by sequential fractionation with dichloromethane and butanol (ratio solvent-supernatant 2:1 and 1:1 for each solvent, respectively) in a continuous liquid-liquid extraction system. The aqueous and organic extracts obtained were separated by decantation and dried through lyophilization or in a vacuum rotary evaporator (Labconco® Kansas City, MO USA), respectively.

All extracts were subsequently evaluated for *C. gloeosporioides* (C3) antifungal activity by using the agar well diffusion method described previously. Aqueous extracts were dissolved in distilled water to evaluate concentrations 10, 20, 50 and 100 mg ml^{-1}. Likewise, obtained organic extracts at 0.5, 0.8 and 1mg ml^{-1} were dissolved in DMSO (8% v/v). One liter of M3.7 sterile media was subjected to the same extraction process and evaluated under the same conditions as 5.1 samples in order to be used as negative control. As mentioned, this methodology was performed with three 5.1 biological replicates and the negative control, respectively.

Butanolic extracts obtained from isolate 5.1 which maintained antifungal activity against *C. gloeosporioides* were analyzed by Liquid chromatography-mass spectrometry (LC/MS) and Matrix-assisted Laser Desorption and Ionization Time of Flight mass spectrometry (MALDI-TOF). To this end, 2 mg of butanolic extract previously dissolved in ethanol (90% v/v) was injected into the HPLC VWR-LaChrom coupled to an Amazon × mass spectrometry (Bruker Daltonics, Bremen Germany) at the Universidad Industrial de Santander (Santander, Colombia). Chromatography was run in an × Terra® RP18 5 µm (4.6 × 250 mm) column with a nitrile acetate gradient with 0.075% formic acid-H_2O as mobile phase. Data were collected and analyzed using the Compass Data analysis (Bruker Daltonics®) and Mzmine 2.14.2® programs.

For MALDI TOF, an Autoflex (Bruker Daltonics, Bremen, Germany) mass spectrometry with a positive ion reflection mode was used. Identification and spec allocation were carried out automatically using a Flexanalysis software version 2.2 (Bruker Daltonics) and Mzmine. All m/z (mass to charge ratio) obtained by both methodologies were compared to those reported in the Streptome DB database (Lucas et al., 2013) and Antimarin Database in order to find compounds previously reported with the m/z ratios found in this study(Blunt et al., 2007).

Statistical analysis

All assays of the present study (either qualitative or quantitative) were performed in triplicate. To test for normal distributions for the quantitative data a Shaphiro-Wilk test was carried-out. Additionally, it was evaluated if all replicas for each assay presented similar tendencies. Tukey test for each data group was performed to find atypical data. Analysis of variance (ANOVA) was used to evaluate the effect of different treatments on phytopathogen fungal growth. Significant differences among means were compared with a Tukey post-hoc test ($p = 0.05$). GraphPad Prism© (GraphPad Software, Inc.© 2012) was used for all analyses.

RESULTS

Streptomyces A20, 5.1 and 7.1 were capable of reducing different *C. gloeosporioides* isolates mycelial growth

The potential of *Streptomyces* A20, 5.1 and 7.1 to inhibit the growth of *C. gloeosporioides* isolates was initially determined by a confrontation dual culture test. In this assay, it was evidenced that *Streptomyces* A20 and 5.1 significantly reduced *C. gloeosporioides* mycelial growth

(Figure 1). Furthermore, agar well diffusion assays suggested that A20 and 5.1 antifungal activity could be generated by a diffusible antifungal metabolite production (Figure 1B). It was determined that *Streptomyces* 5.1, presented significant differences compared with strain

Table 1. Diffusible compound production with antifungal activity by *Streptomyces* strains A20, 5.1 and 7.1.

Treatment	Inhibition halo (mm)		
	C. gloeosporioides C1	***C. gloeosporioides C2***	***C. gloeosporioides C3***
Streptomyces A20	11.33 ± 1.22[a]	9.66 ± 1.0[a]	10.89 ± 0.92[a]
Streptomyces 5.1	17.22 ± 0.97[b]	12.22 ± 1.56[b]	14.22 ± 1.48[b]
Streptomyces 7.1	0[c]	0[c]	0[c]
Clotrimazole	34.78 ± 1.4[d]	27.44 ± 2.06[d]	29.78 ± 1.64[d]
M3.7 media	0c	0[c]	0[c]

Data shown represents mean in mm ± SD of three biological replicates per treatment. Different letters in the same column represent significant differences among treatments ($p < 0.05$).

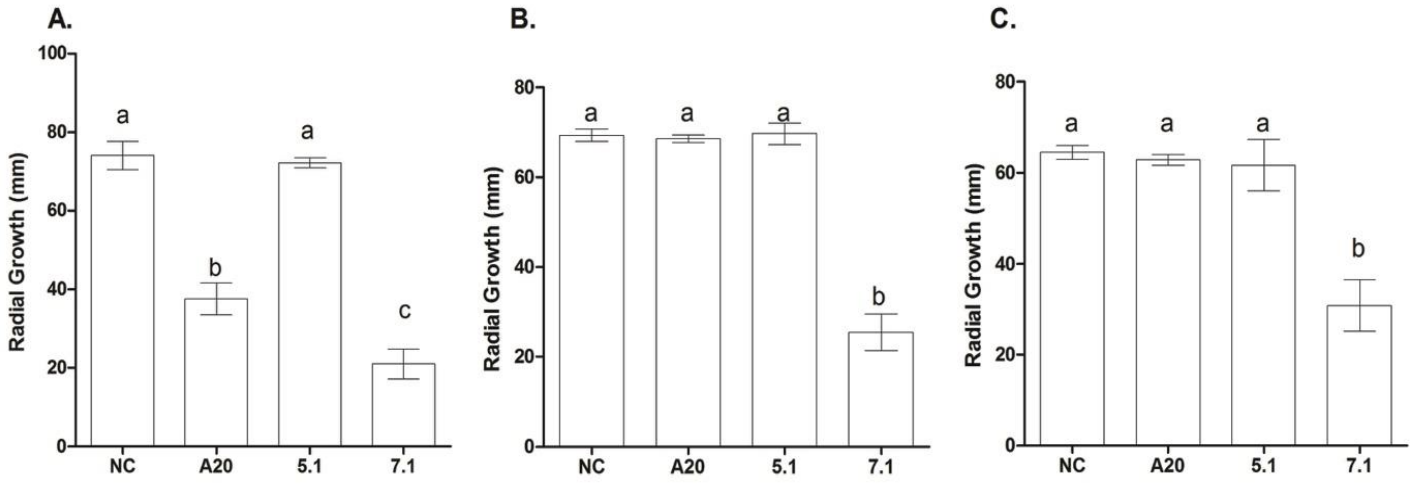

Figure 2. *C. gloeosporioides* C1, C2, and C3 mycelial growth inhibition by *Streptomyces* A20, 5.1 and 7.1 through volatile compound production. A. *C. gloeosporioides* C1, B. C2 and C. C3. Data illustrate mean (mm) ± SD of three biological replicas per treatment. Different letters indicate significant differences among treatments ($p < 0.05$).

A20 in regards to *C. gloeosporioides* mycelial growth inhibition. However, it was not comparable with the positive control (Table 1). Otherwise, strain 7.1 did not demonstrate any antifungal activity mediated by diffusible compounds. Thus to determine if *Streptomyces* 7.1 presented another means of control, it was decided to evaluate active volatile compounds against the three *C. gloeosporioides* isolates.

Through the double-dish chamber assay, it was identified that only 7.1 strain was capable to reduce *C. gloeosporioides* mycelial growth through the production of volatile compounds (Figure 2), generating growth inhibition percentages of 71.62, 63.28 and 52.27% against C1, C2 and C3, respectively. In contrast, A20 was only capable of inhibiting the growth of isolate C1 with an inhibition percentage of 49.24%. These results indicated that *Streptomyces* A20 and 5.1 characteristic antifungal activity were mediated by the production of antifungal diffusible compounds, whereas for 7.1 such effect was the result of volatile nature compounds.

Streptomyces* 5.1 diffusible compounds had a fungistatic effect on *C. gloeosporioides

Considering the aforementioned results, it was desirable to confirm the efficiency and stability of the diffusible compounds produced by strain A20 and 5.1 based on its ability to reduce *C. gloeosporioides* mycelial growth through time. It was observed that diffusible compounds produced by 5.1, retarded mycelial growth for all three *C. gloeosporioides* isolates generating a maximum growth of 61.11, 51.45 and 38.71% for isolates C1, C2 and C3, respectively at the sixth day of incubation (6% treatment) (Figure 3A to C). An inverse correlation was observed between the pathogen growth and the concentration of

the compounds produced by 5.1 against *C. gloeosporioides* C3, being 6% concentration of the most active between the treatments. Interestingly, there were no statistical differences within 4 and 6% treatments in the antagonistic assays against C1 and C2, which could be possibly associated with susceptibility differences

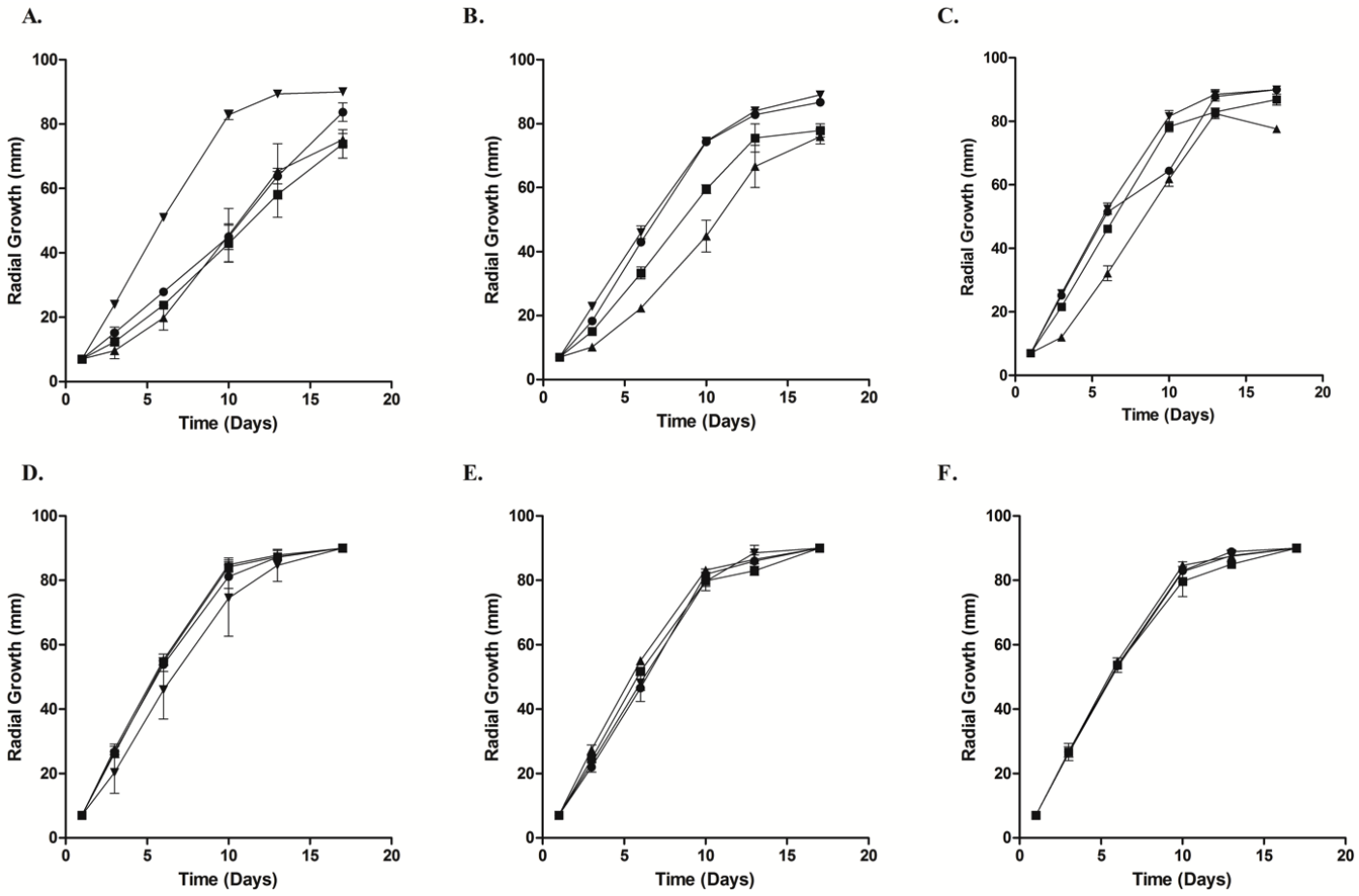

Figure 3. Effect of 5.1 and A20 produced compounds on *Colletotrichum gloeosporioides* C1, C2 and C3 mycelial growth. In the figure, sections A-C corresponds to 5.1 treatments and D-F to A20 treatments. A and D are the assays against to C1, B and E against to C2 and C and F against to C3, respectively. Data presented as mean (mm) ± Standard Deviation (SD) of six biological replicas for each treatment (2%v/v -●-, 4%v/v ■-, 6%v/v -▲, Negative control -▼-).

Table 2. Growth inhibition percentage of *C. gloeosporioirdes* isolates treated with A20 and 5.1 crude extracts.

Isolate	***Streptomyces* 5,1**			***Streptomyces* A20**		
	2%	**4%**	**6%**	**2%**	**4%**	**6%**
C. gloeosporioides C1	7.037 ± 3.195 (a, ☐)	17.96 ± 4.94 (b, ☐)	16.48 ± 2.15 (b, ☐)	0 (c, ☐)	0 (c, ☐)	0 (c, ☐)
C. gloeosporioides C2	2.622 ± 0.91 (a, ł)	12.55 ± 2.29 (b, ł)	14.61 ± 2.56 (b, ☐)	0 (c, ☐)	0 (c, ☐)	0 (c, ☐)
C. gloeosporioides C3	0 (a, ł)	3.519 ± 1.91 (b, ☐)	13.7 ± 0.90 (c, ☐)	0 (a, ☐)	0 (a, ☐)	0 (a, ☐)

Data shown represents mean percentage ± SD of three biological replicates per treatment at seventeen day of incubation. Different letters in the same row represent significant differences among treatments ($p < 0.05$). Different symbols in the same column represent significant differences among treatments ($p < 0.05$).

among the three isolates of *C. gloeosporioides* to the secondary metabolites produced by 5.1.

After ten days of incubation, the inhibitory effect for all 5.1 treatments was reduced to such extent that at the end of the evaluation (Day 17), only the 6% treatment presented inhibition percentages greater than 10% against the three *C. gloeosporioides* isolates (16.48, 14.61 and 13.7% for C1, C2 and C3 respectively) (Table

2 and Figure 3A to C). These results suggest that the effect generated by *Streptomyces* 5.1 active compounds is fungistatic and its stability after a single application has a maximum of 10 days (last measurement where the inhibitory percentage was above 30%).

On the other hand, treatments performed with three

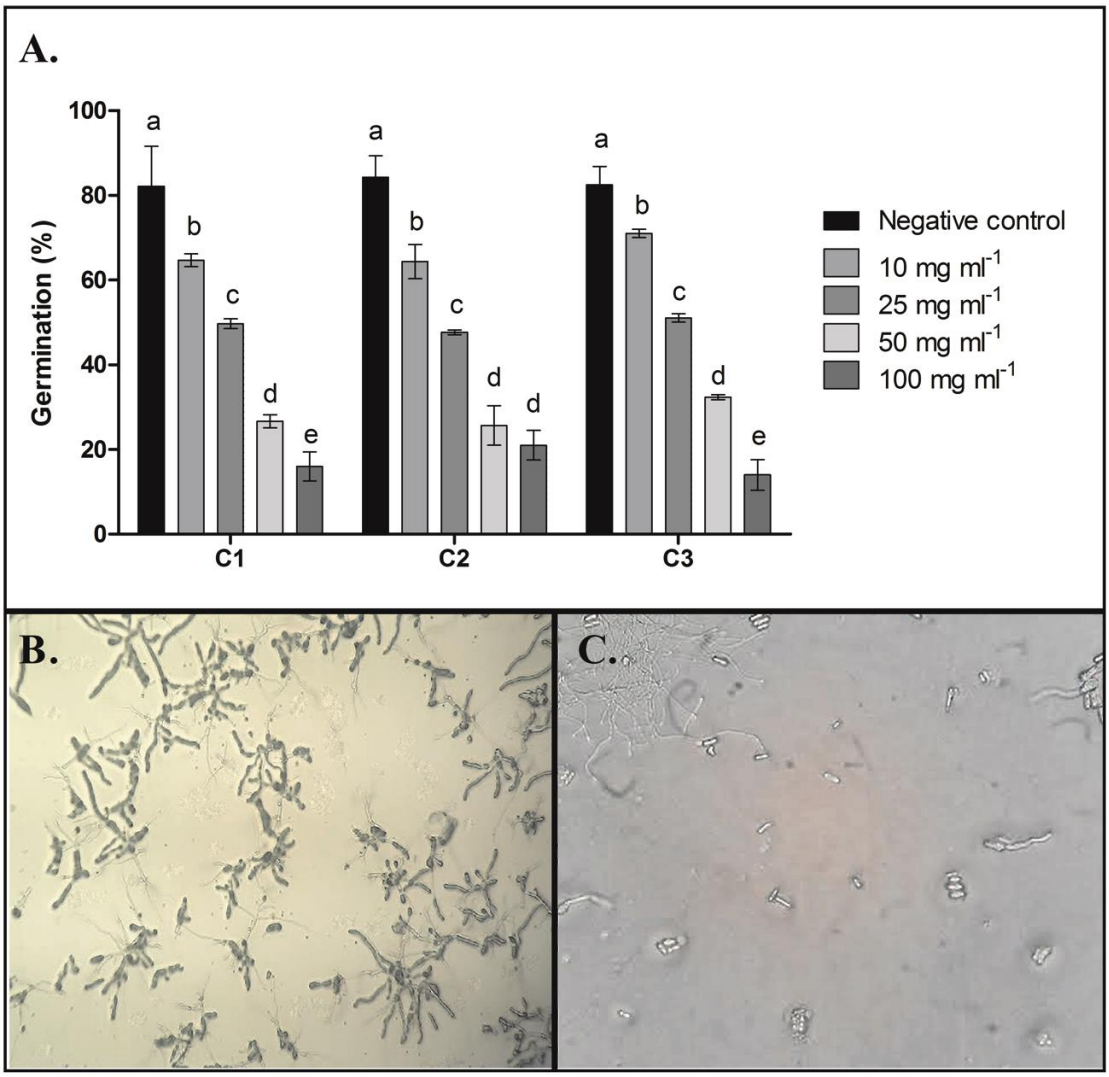

Figure 4. *Streptomyces* 5.1 active compound effect on conidia germinatio0n of three *C. gloeosporioides* isolates. A. Germination percentage of *C. gloeosporioides* isolates: C1, C2 and C3 conidia suspension with different concentrations of *Streptomyces* 5.1 lyophilized supernatant. Data presented are mean of germination (%) ± SD of six biological replicas per treatment. B. Light microscopy of non-treated conidia of *C. gloeosporioides* C3. **C.** Light microscopy of 100 mg ml^{-1} treatment conidia of *C. gloeosporioides* C3.

concentrations of the lyophilized crude extract from *Streptomyces* A20 showed no deleterious effect on the growth of the three *Colletotrichum* isolates at the end of the incubation time (Table 2 and Figure 3D to F). Suggesting that the active metabolite produced by A20 either is unstable and was degraded during the test or, it is not produced under the fermentation conditions evaluated at the concentration sufficient to inhibit phytopathogen growth. Due to these results, this isolate was excluded for the following analysis.

Treatment of *C. gloeosporioides* C1, C2 and C3 conidia with different concentrations of 5.1 lyophilized crude extracts, allowed to determine that the antifungal compounds produced by this strain are able to inhibit the phytopathogen's conidia germination (Figure 4A). As it was observed for mycelial development, the inhibitory effect was inversely proportional to the concentration of the lyophilized extract, being 100 mg ml^{-1} ^{of} the most efficient treatment with an inhibition percentage of 79.5%.

In these experiments, light microscopy evidenced that conidia presented the characteristic swelling of the first

stage of germination, in particular for 50 and 100 mg·ml^{-1} treatments, however, in these treatments conidia were unable to form the germ tube (Figure 4B and C). This result suggests that active compound(s) produced by

Streptomyces 5.1 possibly interfere with the cell wall membrane formation in *C. gloeosporioides*, which could impair the formation of germ tubes. Nevertheless, this

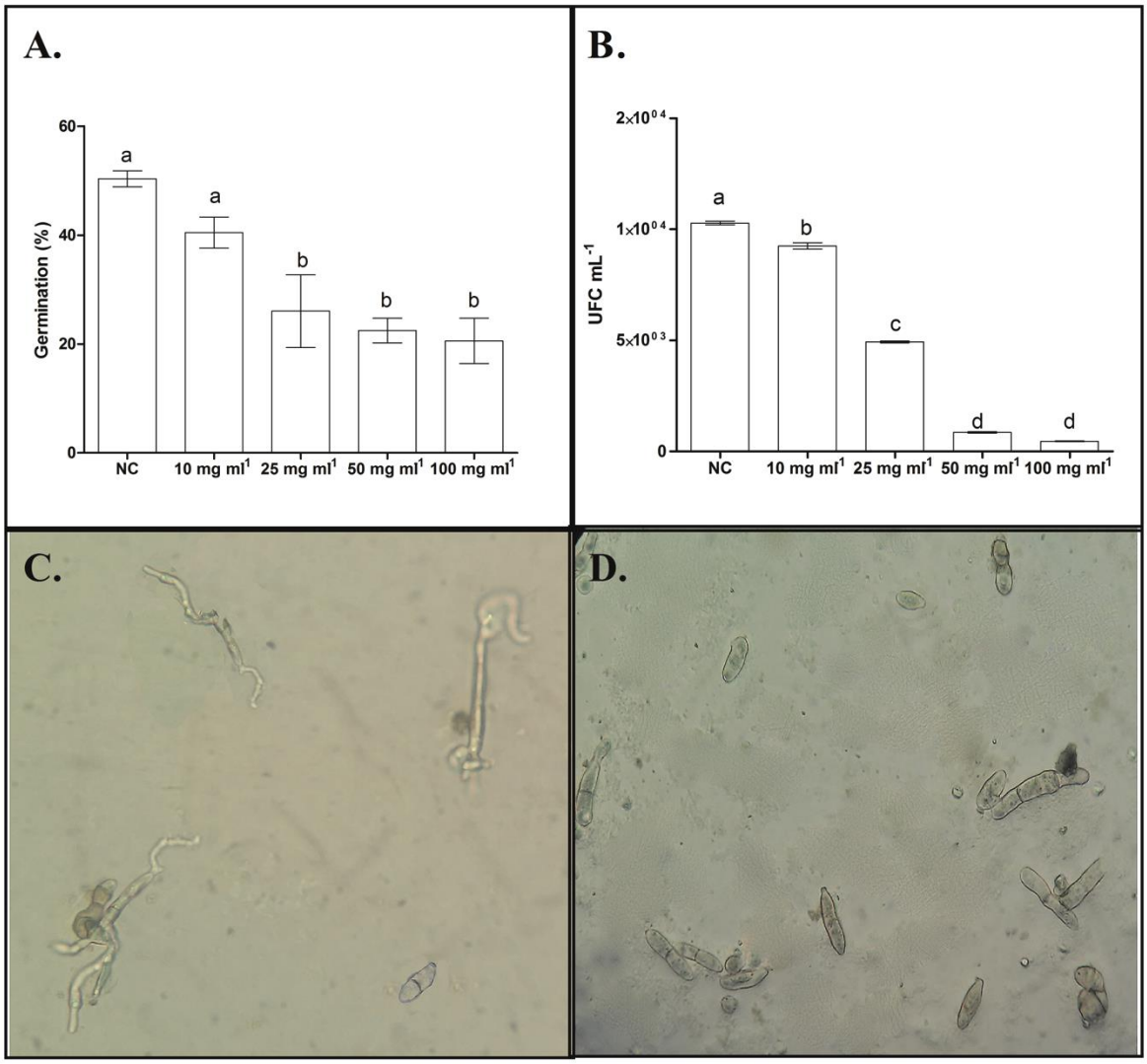

Figure 5. *Streptomyces* 5.1 supernatant effect on *M. ulei.* conidia germination and stroma formation. A. Germination percentage for conidia suspension. B. *M. ulei* stroma/ml count treated with different concentrations of lyophilized *Streptomyces* 5.1 supernatant. Data in % represent mean ± SD of three biological replicas/treatment. Different letters depict significant differences among treatments $p < 0.05$. C. Light microscopy of non-treated *M. ulei* conidia with the characteristic bipolar germination tube form. D. Light microscopy of *M. ulei* conidia treated with 100 mg ml^{-1} of 5.1 supernatant. In the figure, NC corresponds to Negative control.

hypothesis must be confirmed through cytochemical and microscopic complementary studies. In addition, it had to be established if treated conidia remained viable (fungistatic effect) or were incapable of forming new vegetative mycelium, once they lost contact with the antifungal compound (fungicidal effect).

Streptomyces* 5.1 active compound production against *M. ulei

To determine *Streptomyces* 5.1 biocontrolling potential against the phytopathogenic fungus *M. ulei*, two approaches were carried-out. First, compound capacity to inhibit *M. ulei* conidia germination process present in lyophilized supernatant from 5.1 cultures was determined. As can be observed from Figure 5, *M. ulei* conidia suspension treatment at different 5.1 lyophilized supernatant concentrations significantly reduced germination percentage at 25, 50 and 100 mg·ml^{-1}. On

the contrary, treatment with 10 mg·ml^{-1} did not present any significant difference compared to negative control (Figure 5A).

Furthermore, it was evidenced that for all 5.1 supernatant treatments (25, 50 or 100 mg·ml^{-1}) conidial germ tube formation was not completely inhibited. Nonetheless, germ tubes did not present typical *M. ulei* characteristics, where germ tube formation develops at

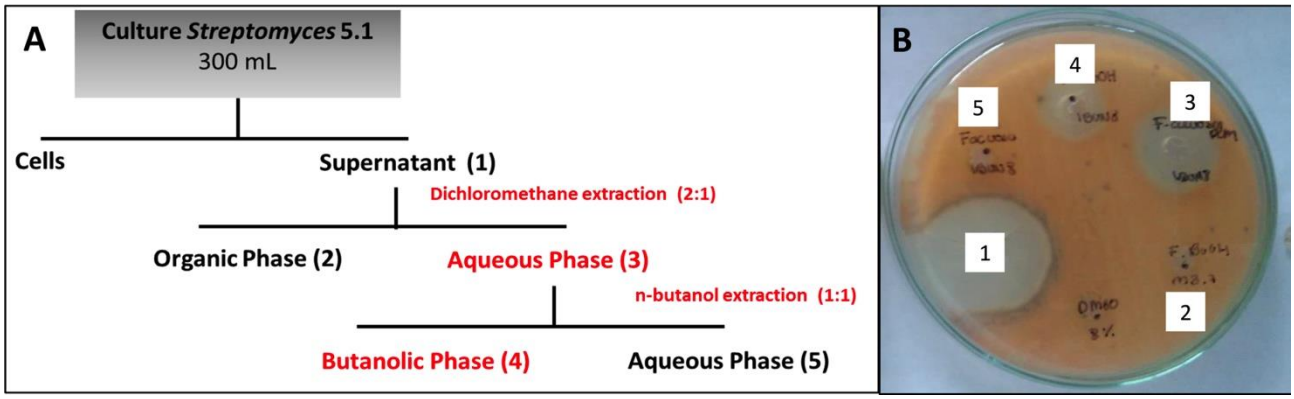

Figure 6. Bioassay guided fractionation of *Streptomyces* 5.1 active compounds. A. Flow diagram with solvents of different polarity. B. Antifungal assay against *C. gloeosporioides* C3 for fractions obtained from strain 5.1. Numbers in the figure of the right correspond to the ones presented in Figure 6A. As a solvent control DMSO (8% v/v) was used.

both poles of the conidia (Figure 5C). For the treated conidia, germination occurred only at one pole of the conidia, and it was not possible to observe the typical division of the germ tube (Figure 5D).

To determine if the *M. ulei* deficient conidia (classified as germinated conidia during germination count) had a subsequent effect on stroma formation (*M. ulei* vegetative growing stage), 100 µl of conidia suspension treated with phytopathogen were seeded in PDA to evaluate mycelia growth of the fungus after being exposed to compounds produced by Streptomyces 5.1. It was observed that all treatments with different supernatant concentrations lead to a significant reduction in the number of formed stroma (Figure 5B). This result suggested that conidia with deformed germination were incapable of generating a vegetative mycelium, thus reducing the number of stromas formed per dish. It can therefore be inferred that compounds produced by strain 5.1 have a fungicidal activity against *M. ulei.*

Streptomyces 5.1 produces different compounds with medium polarity of non-ionic nature

To preliminarily determine the nature of active compounds produced by *Streptomyces* 5.1, bioguided fractionation of *Streptomyces* 5.1 supernatant was performed with solvents of different polarity (Figure 6A), followed by antifungal activity evaluation of all obtained fractions against *C. gloeosporioides* isolate C3. Our results indicated that, antifungal compounds produced by strain 5.1 were retained in the aqueous phase of the first fractioning when dichloromethane was used as a solvent. Subsequent treatment of this aqueous phase using butanol as a solvent favored organic phase (butanolic) compound extraction (Figure 6B). The solubility profile identified for *Streptomyces* 5.1 antifungal compounds suggested that active compounds are characterized by being non-ionic, with medium polarity.

Analysis of *Streptomyces* 5.1 butanolic fractions through LC/MS and MALDI TOF evidenced the presence of six peaks with m/z values of 252,25, 309,17, 427,24, 610,06, 610,39 and 778,28. These peaks were not observed in blank media (Figure 7). Therefore, it was assumed that they were produced by *Streptomyces* 5.1. Out of these peaks, the major peak 610,39 was recognized by both techniques, thus becoming the most interesting for structure elucidation.

Search of compounds produced by different *Streptomyces* spp. presenting an antifungal activity or any antimicrobial activity, in addition to similar m/z values to those reported in this study were not found, which suggests that these compounds have not been described yet. Therefore, it is necessary to continue their purification and structural elucidation by means of complementary techniques to those utilized in the present study.

DISCUSSION

To date, Integrated system crop management of natural rubber (*H. brasiliensis*), which is a crop of agricultural importance, constitutes a main focus in its production chain. Within these systems, management with phytopathogens has a major role given its direct effect on

the crop's productivity. *C. gloeosporioides* and *M. ulei* fungi are known phytopathogens affecting *H. brasiliensis,* traditionally managed with chemicals (Castro, 2011; Berthelot et al., 2014). However, high costs, environmental impact of these practices and emergence of strains resistant to fungicides has generated a need to search for new alternatives for microorganism handling; for instance, the development of biological inoculants with antifungal capabilities (Fravel, 1998; Compant et al., 2005). In this sense, this present work evaluated the

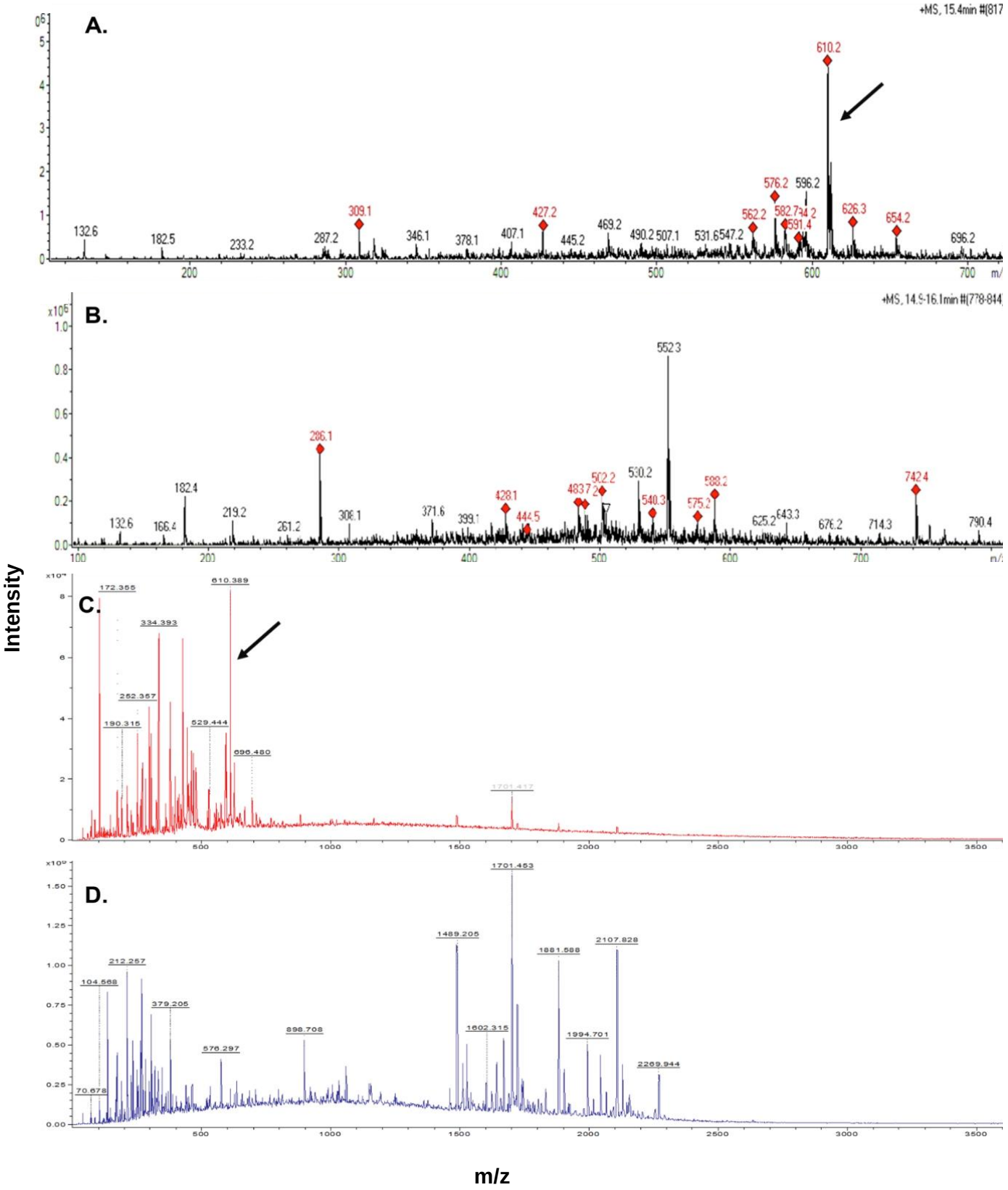

Figure 7. Mass spectrometry of *Streptomyces* 5.1 supernatant butanol fractions obtained by LC-MS and MALDI TOF. Figures A and C, correspond to the Spectrum obtained by MALDI-TOF and LC/MS of 5.1 butanol fraction, and B and D correspond to the media. Highlighted is the main peak m/z 610 present in *Streptomyces* 5.1 butanol fraction recognized by both chromatographic techniques.

possibility of three *Streptomyces* strains previously characterized for their potential to control *Burkholderia glumae* and *Pseudomonas fuscovaginae* phytopathogens in rice cultivations, as biocontrol agents of natural rubber

(*H. brasiliensis*) crops for the pathogens *M. ulei* and *C. gloeosporioides.*

Streptomyces genus is recognized for its ample secondary metabolism, which allows them to produce diverse bioactive compounds of interest for biological control including antibiotics, lytic enzymes (chitinases and glucanases, proteases, among others) and/or siderophores (Qin et al., 2011). An initial evaluation of three *Streptomyces* strains againts three *C. gloeosporioides* isolates evidenced their potential to inhibit mycelial growth of the phytopathogenic agent (Figure 1 and Table 1). Further assays permitted to establish that *Streptomyces* 7.1 was able to reduce *C. gloeosporioides* mycelial growth by producing volatile compounds with an inhibition percentage ranging from 52 to 71% (Figure 2). Antifungal activity by *Streptomyces'* genus volatile compound production for phytopathogen control has been previously evaluated by Wan et al. (2008), who evidenced *Streptomyces platensis* F1 capacity to inhibit *Rhizoctonia solani, Sclerotinia sclerotiorum* and *Botrytis cinerea* growth, and to reduce the incidence and severity of diseases caused by these pathogens in foliage tissues of rice plants, turnips, and strawberry fruits, respectively, under controlled atmosphere conditions(Wan et al., 2008).

Likewise, Li et al. (2010, 2012) developed similar studies where *Streptomyces globisporus* JK-1 capability to inhibit *B. cinerea* and *Penicillium italicum* growth in tomato plants (*Lycopersicon esculentum*) and Shatang mandarin fruit (*Citrus microcarpa*) was verified, with promising results for growth control of these pathogens through *in vivo* assays under shared atmosphere conditions (Li et al., 2010, 2012). Nonetheless, use of these types of metabolites is recommended for controlling diseases in environments that favour the presence of a microatmospheres, where a higher concentration of a volatile compound is achieved, as it is the case for control of pathogens in soil, management of foliage diseases under controlled conditions in greenhouse and use of post-crop fruit storage containers (Wan et al., 2008; Li et al., 2012). For these reason, application of microorganisms able to produce volatile compounds for management of foliage phytopathogens in *H. brasiliensis* under field cultivation conditions would not be recommended; due to possible reduction in metabolite efficiency by compound dilution effect, since there would not be a controlled atmosphere environment. Therefore, strain 7.1 was removed for posterior assays in the present study, even though its potential as a biocontrol agent should be studied in future research aimed for controlling post-crop disease control.

On the other hand, *Streptomyces* A20 and 5.1 were capable of inhibiting *C. gloeosporioides* growth by

similar results to those obtained in our study. They described *C. gloeosporioides* 50% radial growth inhibition using filtered extract of *Streptomyces hygroscopicus* during its exponential growth. Furthermore, Shahbazi et al. (2014) demonstrated antifungal activity of *Streptomyces* strains P8 and P42 isolated from chili pepper (*Capsicum annuum* L. Kulai) rhizosphere soils evaluated against *C. acutatum, C. capsici* and *C. gloeosporioides* phytopathogens. Antifungal activity was due to compounds present in supernatants from liquid culture of *Streptomyces* strains (Shahbazi et al., 2014). In both studies, lysis of the phytopathogen's hyphae cell wall by chitinase was evidenced.

Results obtained in the present study indicate that strain 5.1 had the highest antifungal activity against *C. gloeosporioides* and *M. ulei,* which was statistically significant compared to results obtained with *Streptomyces* A20 (Table 1), being the first report (as far as we know) that describe the antifungal activity of a *Streptomyces* isolate against *M. ulei*. Furthermore, results obtained from the bioassay guided fractionation suggested that enzymatic lytic activity was not produced, since antifungal activity remained without alterations after supernatant fractionation with two different organic solvents of different polarity (dichloromethane and butanol). Since, these two solvents have the capability to degrade enzymes, it can be suggested that *Streptomyces* antifungal compounds can be classified as a non-enzymatic metabolite with antifungal activity. Additionally, analysis of 5.1 filtered supernatants suggested that active compounds of this microorganism suppress *C. gloeosporioides* and *M. ulei* conidia germination process, as well as stroma formation for the later one, with a percentage inhibition of 79.5, 40.88 and 95.56%.

Light microscopy analysis, demonstrated that contact between a *Streptomyces* extract (25 to 100 $mg \cdot ml^{-1}$) and *C. gloeosporioides* or *M. ulei* conidia, reduced conidia development and germ tube elongation for each pathogen, disrupting mycelia development. This type of activity could interrupt the penetration process of both phytopathogenic fungus in rubber leaves inhibiting or delaying the infection in the plants, as it has been previously reported for others *Streptomyces* species evaluated for biological control of *C. gloeosporioides, Fusarium oxysporum f. sp. lycopersici, Verticillium albo-atrum* and *Alternaria solani.* An inversely proportional association was observed between conidia percentage germination and extract concentration, containing the active compound evaluated (El-Abyad et al., 1993; Palaniyandi et al., 2011), as it was observed in the present study (Figures 4 and 6).

However, mechanism of action against the phytopathogens for antifungal compounds isolated from

producing extracellular diffusible metabolites, as suggested by the antifungal activity retained in liquid culture filtered supernatants from both microorganisms (Figure 1 and Table 1). Prapagdee et al. (2008) reported

Streptomyces 5.1 is still un-known. To elucidate such activity, first of all it is necessary to establish the type of metabolite produced. The present study evidenced *Streptomyces* 5.1 produced at least two non-ionic

compounds. Analysis through LC/MS and MALDI TOF of 5.1 fermentation butanol extractions revealed six unique strain peaks; with the highest peak at a molecular mass of 610.2 m/z. Data obtained from these experiments did not correlate with compounds reported in StreptomeDB or Antimarin Database, therefore, these compounds could be assumed as new. However, it is necessary to perform a discrimination process among the six peaks to determine which of them is responsible for the observed antifungal activity. Moreover, complementary assays must be carried out to reveal the compounds and verify the new molecule hypothesis.

Last, despite our results it is necessary to establish additional *in vitro* and *in vivo* experiments focused on (i) evaluating *Streptomyces* 5.1 and *C. gloeosporioides* and *M. ulei* population behavioral dynamics in association with rubber plants (*H. brasiliensis*), taking into account the plant's defense response to *Streptomyces* 5.1. inoculation, (ii) comparisons between incidence and severity of the disease treated with 5.1, and (iii) determining its efficiency with respect to the traditional chemical control.

This study evidenced that *Streptomyces* 5.1 strain competence to produce extracellular metabolites with antifungal activity to inhibit mycelial growth in addition to impede *C. gloeosporioides* and *M. ulei* germination process under *in vitro* conditions. Obtained results demonstrated *Streptomyces* 5.1 potential to be utilized as a biological control agent destined for rubber plant (*H. brasiliensis*) protection against foliar anthracnose and SALB. To the best of our knowledge this is the first report establishing *Streptomyces* genus for important phytopathogen fungi control of the natural rubber.

Conflict of Interests

The authors have not declared any conflict of interests.

Abbreviations

DMSO, Dimethyl sulfoxide; **ISP,** International *Streptomyces* Project; **ITS,** internal transcribed spacer; **LC-MS,** liquid chromatography–mass spectrometry; **Mbp,** megabase pairs; **PDA,** potato dextrose agar; **ppm,** parts per million; **SALB,** South American Leaf Blight.

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Supplementary Table 1. Biochemical characterization of *Streptomyces* A20, 5.1 and 7.1.

Strain	Catalase	Oxidase	Glucose	Sucrose	Fructose	Rhamnose	Arabinose	Inositol	Xylose	Mannose
A20	+	-	+	-	-	-	-	-	-	-
7.1	+	-	+	+	+	-	-	-	-	-
5.1	+	-	+	-	-	-	-	+	-	-

Single colonies were characterized by their colony morphology in ISP2, ISP3, ISP4, Nutrient agar and Mueller Hinton Agar. Catalase, oxidase and their carbon source utilization were tested, as suggested for *Streptomyces*-like bacteria, as described previously (Shirling and Gottlieb, 1968; Goodfellow, 2012).

Supplementary Table 2. 16S rRNA phylogenetic analyses.

Strain	***Streptomyces* species**	Similarity (%)
A20	*S. racemochromogenes*	99.93
	S. polychromogenes	99.86
	S. flavotricini	99.66
5.1	*S. angustmycinicus*	98.77
	S. abikoensis	98.69
	S. sioyaensis	98.64
7.1	*S corchorusii*	99.86
	S. canarius	99.79
	S. olivaceoviridis	99.73

PCR amplification, sequencing and analysis of the entire 16S rRNA locus was performed as described by Wang et al. (2013). The cleaned PCR products were directly sequenced using universal primers 27F, 500F, 818R and 1492R by Macrogen (Korea). Closely related sequences were obtained from EZTaxon (Kim et al., 2012) and are listed. *16S rRNA* sequencing results showed that our strains display high similarity to those of the *Streptomyces* species, locating strains A20, and 7.1 a closelly related to *S. racemochromogenes*, and *S. corchorusii*, whereas strain 5.1 was related to the *S. sioyaensis* clade, respectively.

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Endogenous fungi isolated from three locoweed species from rangeland in western China

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Leguminous locoweeds cause toxicosis to grazing animals in western China and western USA. Swainsonine, a toxic alkaloid, is produced by the endophytic fungus *Alternaria* section *Undifilum* sp. living within the locoweed plants. Nothing is known of the other endogenous fungi associated with locoweed and it is unknown if the presence of *Alternaria* sect. *Undifilum* sp., a potential mutualist, in a locoweed influences the fungal microbiome associated with the plant. To help address these questions, endogenous fungi associated with three locoweed species (*Oxytropis glabra, Sphaerophysa salsula,* and *Astragalus variabilis*) collected from grasslands from western China were evaluated. Fungi were isolated from the tissues and identified by morphological features and sequencing of the internal transcribed spacer (ITS) regions. A total of 1209 fungal isolates were obtained from 1819 tissues for an isolation rate of 66.5%. *Alternaria* sect. *Undifilum oxytropis*, *Alternaria* spp. and *Fusarium* spp. were most commonly isolated. Plant host species, plant part, and environment influenced the endogenous fungal communities isolated from the locoweed plants. There were significant differences in the diversity of fungal species isolated from *O. glabra* from two sites, and no differences between the diversity of fungi isolated from *A. variabilis* from two sites. *Alternaria* sect. *Undifilum* was found most frequently associated with toxic locoweeds. Plants or plant parts that did not yield this endophyte had more plant pathogenic fungi associated with them. This is the first report of the diversity of fungi associated with these locoweeds and the first to suggest a beneficial role for *Undifilum*.

Key words: Fungal endophytes, *Undifilum* sp., locoweeds.

INTRODUCTION

Locoweeds are perennial, herbaceous, poisonous legume plants containing the fungal-produced toxic alkaloid, swainsonine, which causes the neurological disorder locoism in grazing animals (Dorling et al., 1980;

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James et al.,1981; Allred, 1991; Cook et al., 2009b). Locoweeds are primarily species of *Astragalus* and *Oxytropis* genera and have been reported from the Americas (western USA and South America) and Asia (Allred, 1991; Robles et al., 2000; Yu et al., 2010). Swainsonine is an indolizidine alkaloid that was first identified from *Swainsona*, a toxic legume that is found in Australia (Colegate et al., 1979). Swainsonine inhibits alpha-mannosidase and mannosidase II causing a lysosomal storage disease and neurologic impairment (Dorling et al., 1980). Locoism symptoms include a lack of muscular coordination and inability to eat or drink (James et al., 1970; Stegelmeier et al., 1999). Losses due to locoism depend on the severity of poisoning, but have been estimated at $300 million annually in the western United States due to poisoned cattle, sheep, and horses (Torell et al., 2000; Turner et al., 2012).

A large diversity of locoweeds is found in China. There are 46 species of locoweeds reported in China, including 23 species of *Oxytropis*, and 23 species of *Astragalus* (Lu et al., 2012b). Thirteen of the 46 species have been reported to cause severe damage to animal husbandry. Toxic locoweeds are primarily distributed over an area of 11 million ha in arid and semi-arid grasslands of Tibet, Inner Mongolia, Qinghai, Gansu, Xinjiang, Ningxia, Shanxi, and Sichuan provinces (Zhao et al., 2003; Lu et al., 2012b).

Toxic locoweeds from North America and China, and *Swainsona* from Australia, contain an endophyte, *Alternaria* section *Undifilum* spp. that produces the swainsonine (Braun et al., 2003; Wang et al., 2006; Pryor et al., 2009; Yu et al., 2010; Lu et al., 2012a; Creamer and Baucom, 2013; Grum et al., 2013; Woudenberg et al., 2013). Although not every plant in a population is toxic or contains the endophyte, all toxic plants that contain swainsonine have been shown to contain a swainsonine-producing fungal endophyte (Cook et al., 2011, 2012; Achata et al., 2012).

A fungal endophyte is defined as a fungus that lives within a plant. Fungal endophytes include saprophytic fungi, pathogenic fungi, and mutualistic fungi (Petrini, 1991). Fungal endophytes are widely found in roots, stems, and leaves of higher and lower plants. The diversity of fungal endophytes includes species diversity in colonization of host plants and diversity in the distribution of fungal endophytes within different tissues of host plants. This diversity could be due to differences in the host plants and natural factors, such as geographical location and climate, in addition to presence of other organisms (Tiina et al., 2013).

Different locoweed species have been associated with different *Alternaria* sect. *Undifilum* sp. *Oxytropis lambertii* and *O. sericea* harbor *A. U. oxytropis*, *Astragalus mollissimus* contains *A. U. cinerea*, and *Astragalus lentiginosus* contains *A. U. fulva* (Pryor et al., 2009; Baucom et al., 2012). *Alternaria* sect. *Undifilum* spp. can readily be cultured from dried plant tissue and most produce conidia in culture. The cultures are very slow growing, expanding less than an average of 0.2 mm/day (Pryor et al., 2009). They can be readily isolated from leaves, petioles, stems, flowers and seed (Braun et al., 2003). The fungi are vertically transmitted through seed, but found only in the seed coat and underlying layer (Oldrup et al., 2010).

Alternaria sect. *Undifilum* spp. can usually be cultured from locoweed samples with swainsonine concentrations greater than 0.01% swainsonine, and not from plants with lower swainsonine levels (Ralphs et al., 2008). While the endophyte has been found in all plant parts, it is highest in the crown, and has been difficult to isolate from roots (Cook et al., 2009a; Cook et al., 2011). Endophyte amounts change seasonally in above ground parts of plants until the plants reach maturity (Achata et al., 2012; Cook et al., 2012).

Gao et al. (2012) cultured *Alternaria* sect. *Undifilum oxytropis* from *Astragalus variabilis* and *Oxytropis glabra* plants collected from Inner Mongolia that contained swainsonine concentrations greater than 0.01%. Swainsonine was detected in only 2 of 50 samples of *Sphaerophysa salsula*, and at concentrations less than 0.01% and *A. U. oxytropis* was not cultured from any of the samples.

Only fungi have been demonstrated to produce swainsonine. In addition to *Undifilum* sp., fungi such as *Slafractonia* (*Rhizoctonia*) *leguminicola*, *Metarhizium anisopliae*, and an undescribed species of Chaetothyriales, have also been reported to produce swainsonine (Sim and Perry, 1997; Cook et al., 2014; Alhawatema et al., 2015).

While the role of *Undifilum* sp. in swainsonine production has been clearly established, the ecological role of *A.U. oxytropis* is not quite so clear. The fungus does not cause any obvious disease symptoms on the plant hosts and the plant host does not show a resistance response toward the fungus even at the cellular level (Reyna et al., 2012). The fungus does not deter invertebrate feeding, or aid in plant growth under heat stress or nitrogen deficiency (Thompson et al., 1995; Oldrup et al., 2010). Does *Alternaria* sect. *Undifilum* provide benefit to the locoweed plant, or does it function primarily as a commensal (Creamer and Baucom, 2013)? Perhaps it could help deter disease from pathogenic fungi. An ecological role for *Alternaria* sect. *Undifilum* could be suggested if it influenced the fungi associated with its host plant.

Highly stringent isolation procedures developed to culture the slow-growing *Alternaria* sect. *Undifilum* preclude the growth of other fungi (Braun et al., 2003). As a result, there is little known about the fungi, other than *Alternaria* sect. *Undifilum* sp., that is associated with locoweeds. Therefore, using lower stringency culture techniques, fungi were isolated from two toxic locoweeds,

O. glabra, *A. variabilis*, and a rarely-toxic locoweed, *S. salsula,* that were collected from semi-arid meadows in Ningxia Hui Autonomous Region and Inner Mongolia Autonomous Region in north central China. Fungi were identified based on morphological characteristics and phylogenetic analyses of rDNA ITS sequences and the diversity of the fungi were compared between plant species, locations, and plant parts. This study is the first to identify the diverse fungi associated with locoweeds and the first to suggest a beneficial role for *Alternaria* sect. *Undifilum*.

MATERIALS AND METHODS

Sample collection

Samples were collected from four areas, Yinchuan city in Ningxia Hui Autonomous Region and BayanHot, AoLun Prague, and Jilantai towns, in the Inner Mongolia Autonomous Region, which were selected to be representative of different environments (Table 1). *O. glabra* was collected from Yichuan city and Bayan Hot, *A. variabilis* was collected from AoLun Prague and Jilantai, and *S. salsula* was collected from BayanHot. One whole healthy-looking locoweed plant was collected from each of three sites within each of the four areas. At sampling, the type of vegetation, landscape, habitat, altitude, latitude, and climate were recorded and the location determined by GPS (Global Position System) (Table 1). The samples were dried with allochroic silica gel and maintained at-20°C.

Isolation of fungal endophytes

The dried plants were washed thoroughly with running tap water, then twice more with double distilled water to remove debris. The plants were cut into separate parts, i.e. leaves and petioles, roots, and seed pods were removed. Stems, leaves, and seeds of *O. glabra*, *A. variabilis* and *S. salsula* were assessed. Roots from *A. variabilis*, and petioles from *S. salsula* were also tested. Similar tissues from plants of the same species were combined, that is, *O. glabra* leaves harvested from the three plants collected from BayanHot were pooled for testing to maximize diversity from each plant species and location. The tissues were treated with a minimal cursory treatment of 30 s in 75% ethanol, followed by 1-3 min in 2%NaClO, and then 3-5 times in sterile water. Tissues were dried on sterile paper towels, and stems, petioles, and roots were cut into 5 mm sections, leaves were cut into 5 mm x 5 mm sections, and seeds were quartered. The tissues (6 to 7 sections) were placed in the same potato dextrose agar (PDA, Qingdao High-tech Industrial Park Haibo Biotechnology Co., Ltd, Qingdao, Shandong, China) plate at 28°C for 7 to 30 days. To determine if loosely associated microbes had been removed from the plant tissue surface, tissue prints were done with sterile water on control media. No bacterial contamination was observed, suggesting that cursory surface sterilization was effective. When hyphae grew from the cut tissues, the hyphae were removed to new media, and transferred 2 to 3 times. The hyphae were harvested and stored at 4°C.

Morphological identification of fungal endophytes

Hyphal tips of all fungi were plated onto PDA, and cultured in an inverted position at 28□C. Colonies were observed daily and size, color, texture, growth rate, edge, and shape were recorded. Subsequently, a third of a coverslip was inserted into the edge of the colonies in the medium at a 45° angle, and fungal mycelium, conidiophore structure, spore morphology, color, and ontology, were observed using light microscopy. Primary identification was carried out based on the taxonomic guides of Barnett and Hunter (1977), Shao et al. (1996), and Wei (1979). Ecological roles for fungi were determined using the USDA Fungal Databases from the Systematic Mycology and Microbiology Laboratory (http://nt.ars-grin.gov/fungaldatabases). Fungi were classified as pathogens, mutualists, or saprophytes based on the majority of reports for the fungus. Fungi not identified to species were not classified.

Identification of rDNA ITS sequences

Hyphae grown on PDA were collected and weighed. Genomic DNA was extracted from the fungi, with cetyltrimethylammonium bromide (CTAB) lysis buffer (Sun et al. 2006). Universal primers that amplify fungal rDNA, ITS1 (5'-TCCGTAGGTGAACCTGCGC-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'), were used as upstream and downstream primers (White et al., 1990; Deng et al., 2006) to amplified the conserved ribosomal rDNA-ITS sequences using the genomic DNA as template. PCR reactions were carried out in a volume of 25 µL, containing 3 µL of DNA, 10 $\mu mol \cdot L^{-1}$ of ITS1 and ITS4 (1 µL each), 12 µL of 2X EasyTaq PCR SuperMix (TransBionovo, Beijing) and 8 µL of ddH_2O. Amplification was carried out in a Bio-RAD Gene Cycler™ PCR with an initial denaturation at 95°C for 30 min, 35 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 45 s, and a final extension of 72°C for 10 min. Amplification products (5 µL) were separated by electrophoresis on a 1.2% low-melting agarose gel, and the bands were visualized using a gel imaging system. Selected DNA fragments were purified from the agarose gel using a DNA extraction kit and sequenced (Sangon Biotech Co., Ltd., Shanghai). Species identification was based on sequence analysis of approximately 500 bp from the nuclear ribosomal internal transcribed spacer region (rDNA-ITS), (Arnold and Lutzoni 2007).

Phylogenetic relationship between different fungi

After sequencing, homology comparison was done using Blast and 5.8S rDNA-ITS sequence in Gen Bank, then the selected high similarity sequences were used to produce a phylogenetic tree by applying ClustalX 1.83 version and Neighbor-Joining method (MEGA5.0) with bootstrap support based on 1,000 replicates, and evolutionary distances computed using the Maximum Composite Likelihood method.

Data analysis

The isolated fungal communities were characterized for diversity by plant and by site. The α-diversity of the fungal community associated with locoweeds was analyzed using Shannon-Wiener diversity index ($H' = -\sum_{i=1}^{k} P_i \times \ln P_i$) and Simpson index ($D = -\sum (P_i)^2$) (*k*= the total species of fungi in certain plants, *Pi*= the proportion of a specific fungus out of all isolated fungi) (Gazis and Chaverri, 2010; Kharwar et al., 2011). The Evenness index (J=H/lnS) was used to estimate the evenness of species distribution

Table 1. Location and environment of locoweed collection sites.

Species/Locations	GPS (No.)	Altitude/m	Phenological condition	Land Forms and Soil
Oxytropis glabra (BayanHot, Inner Mongolia)	(1) 38°49' 805", 105°42' 021"	1593	Fruiting	*Ixeris denticulate, Herba taraxaci,* L. *Plantago depressa* Willd*., Phleum pratense, Agropyron cristatum* (Linn.) Gaertn, brown calcic soil
	(2) 38°49' 810", 105°41' 988"	1574	Flowering and early fruiting	*Medicago falcate* Linn, *Oxytropis aciphylla,* pine needle grass, brown calcic soil
	(3) 38°49' 803", 105°42' 015"	1593	Fruiting	*Ixeris denticulate, Agropyron cristatum* (Linn.) Gaertn. *Phleum pratense, L. Plantago depressa* Willd., brown calcic soil
Oxytropis glabra (Yinchuan, Ningxia province)	(1) 38°29' 870", 106°08' 288"	1108	Flowering and early fruiting	Lawn*, Agropyron cristatum* (Linn.) Gaertn*, Chenopodium album* Linn*, Ixeris denticulate, Thermopsis lanceolata* R. Br, gray soil
	(2) 38°29' 867", 106°08' 289"	1101	Flowering and early fruiting	Lawn, *Agropyron cristatum* (Linn.) Gaertn. *Ixeris denticulate. Thermopsis lanceolata* R. Br, *Medicago sativa* Linn*,* gray soil
	(3) 38°29' 979", 106°08' 268"	1116	Flowering and early fruiting	Lawn*, Agropyron cristatum* (Linn.) Gaertn, *Salix babylonica, Chenopodium album* Linn*,* Black spongy soils.
Astragalus variabilis	(1) 38°49' 812", 105°41' 988"	1584	Full bloom	*Ixeris denticulate, Agropyron cristatum* (Linn.) Gaertn*.,* pine needle grass, brown calcic soil
(Jilantai, Inner Mongolia)	(2) 38°49' 808", 105°42' 003"	1593	Full bloom	*Ixeris denticulate, Agropyron cristatum* (Linn.) Gaertn*.,* pine needle grass, brown calcic soil
	(3) 38°49' 813", 105°41' 983"	1599	Flowering and early fruiting	Desert*, Agropyron cristatum* (Linn.) Gaertn*., Phleum pratense*, grit, brown calcic soil
Astragalus variabilis	(1) 40°26' 874", 106°12' 878"	1036	Fruiting	Desert*, Agropyron cristatum* (Linn.) Gaertn*., Phleum pratense,* grit, brown calcic soil
(Aolun Prague, Inner Mongolia)	(2) 40°26' 930", 106°12' 763"	1038	Fruiting	Desert*, Agropyron cristatum* (Linn.) Gaertn*., Phleum pratense,* grit, brown calcic soil
	(3) 40°31' 593", 106°27' 083"	1066	Full bloom	Desert*, Agropyron cristatum* (Linn.) Gaertn. *,Peganum nigellastrum* Bunge*,* pebble beach, brown calcic soil
Sphaerophysa salsula (BayanHot, Inner Mongolia)	(1) 38°49' 803", 105°42' 015"	1593	Flowering and early fruiting	Lawn*, Agropyron cristatum* (Linn.) Gaertn.. *Ixeris denticulate. Thermopsis lanceolata* R. Br, *Medicago sativa* Linn*, Chenopodium album* Linn*.*
	(2) 38°49' 813", 105°42' 011"	1596	Flowering and early fruiting	*Agropyron cristatum* (Linn.) Gaertn., *Oxytropis glabra DC, Oxytropis coerulea,* pine needle grass*,* basic brown calcic soil
	(3) 38°49' 813", 105°41' 983"	1599	Flowering and early fruiting	*Ixeris denticulate, Herba taraxaci,* L. *Plantago depressa* Willd*., Phleum pratense, Agropyron cristatum* (Linn.) Gaertn., brown calcic soil

of fungi in the biotic community (*H*=the Shannon-Wiener diversity index, *S*=the total species) (Kharwar et al., 2011). The β-diversity of the fungi associated with locoweed species and sites was determined using two similarity indices, Sorenson Index (C_S = 2/(a+b)) and Jaccard Index (C_J =j/(a+b-j)), which were used to compare the similarity of fungal species composition between the two sites (*j*= the common species of fungi between the two sites, *a* and *b*= the species of fungi in the two sites, respectively) (Gazis

Table 2. Isolation of fungi from locoweeds through surface sterilization.

Locoweeds	Location	Parts	Number of tissues	Number of isolates	Isolation rate (%)
Sphaerophysa salsula	BayanHot, Inner Mongolia	Leaf	49	26	53.1
		Petiole	38	16	42.1
		Stem	71	23	32.4
		Seed	57	29	50.9
Oxytropis glabra	BayanHot, Inner Mongolia	Leaf	36	25	69.4
		Stem	52	35	67.3
		Seed	123	108	87.8
	Yinchuan, Ningxia	Leaf	126	85	67.5
		Stem	174	96	55.2
		Seed	342	236	69.0
Astragalus variabilis	Jilantai, Inner Mongolia	Leaf	46	26	56.5
		Stem	38	30	79.0
		Seed	60	46	76.7
		Root	253	183	72.3
	AoLun Prague, Inner Mongolia	Leaf	95	75	79.0
		Stem	85	75	88.2
		Seed	48	35	72.9
		Root	126	60	47.6

and Chaverri, 2010).

RESULTS

Isolation and identification of fungi associated with locoweeds

Fungi were isolated from all tissues using all combinations of bleach and ethanol treatments tested. Generally, fewer fungi were isolated from toxic locoweeds with increasing times of bleach and ethanol. Although only tested for *A. variabilis*, roots yielded the highest fungal isolation rates, 87%. Isolation rates from the nontoxic *S. salsula* were generally lower and more variable, ranging from a high of 41.6% from leaves to a low of 3.9% from seed.

One hundred sixty-eight fungal isolates were obtained from 211 tissues (leaves, stems, seeds) from *O. glabra* from BayanHot with an isolation rate of 79.6%, and 417 fungal isolates were obtained from 642 tissues from *O. glabra* from Yinchuan with an isolation rate of 65.0%. Fungal isolation rates from BayanHot and Yinchuan were 87.8 and 69.0% from seeds, 69.4 and 67.5% from leaves, and 67.3 and 55.2% from stems, respectively (Table 2). The fungi isolated from *O. glabra* differed between the two areas. Only five fungal species were isolated from plants from BayanHot, while 21 species were isolated from plants from Yinchuan.

Two hundred eighty five fungal isolates were obtained from 397 tissues (leaves, stems, seeds, roots) from the *A. variabilis* from Jilantai. The isolation rate was 71.8%. Two hundred forty five fungal isolates were obtained from 354 tissues of *A. variabilis* from AoLun Prague. The isolation rate was 69.2%, with higher isolation rates from stems and seeds than from leaves or roots from both locations (Table 2). The number of fungi isolated from *A. variabilis* was somewhat similar between

the two areas. Fifteen fungi were isolated from plants from Jilantai and 19 fungi were isolated from plants from AoLun Prague.

Ninety-four fungal isolates were obtained from 215 tissues of *S. salsula* (leaves, petioles, stems, and seeds) from BayanHot. The isolation rate was 43.7%, from leaves, 53.1% from seeds and 50.9% from stems (Table 2). The 94 cultures of fungi that were isolated included 19 species, distributed in 4 classes, 5 orders, 8 families, (2 families undetermined) and 10 genera on the basis of morphological characteristics and rDNA-ITS sequence analyses (Figure 1).

Overall there were 10 orders of fungi isolated, with the largest number of fungi within the Pleosporales and Hypocreales (Table 3). There were 9 species of *Alternaria*, 7 species of *Fusarium*, and 4 species of *Aspergillus* isolated. *Alternaria porri* was the only fungus isolated from all locations, while *Alternaria* sect. *U. oxytropis*, *Alternaria alternata*, and *Fusarium tricinctum* were each isolated from 4 locations.

Community composition and diversity of isolated fungi

The distribution of fungi isolated from *O. glabra* differed between BayanHot town and Yinchuan city. Only 5 fungal species were isolated from *O. glabra* from BayanHot, while 21 were isolated from the plants collected from Yinchuan. The endophyte, *A. U. oxytropis,* was distributed in all tissues of *O. glabra* from both locations except for the leaves in Yinchuan city. Relative isolation rates of *A. U. oxytropis* were all higher than 65.0% in *O. glabra* from BayanHot, with a relative isolation rates in seeds, leaves, and stems of 80.6, 80.0 and 68.6%, respectively. In Yinchuan, however, *A. U. oxytropis* was isolated only from the seeds and stems of *O. glabra*, and the relative isolation rates were 58.9 and 44.8%, respectively. Additionally, *Alternaria* spp., *Aspergillus* spp., and *Fusarium* spp. were isolated from most tissues of *O. glabra*, from both locations, and relative isolation rates were also higher (Table 4). The α-diversity (number of unrelated species of fungi isolated) for *O. glabra* (Figure 3) and *A. variabilis* (Figure 2) was similar and lower than for *S. salsula* (Figure 1).

Alternaria sect. *Undifilum* spp. were distributed in all the tissues of *A. variabilis* collected from both locations and was the dominant species in most tissues. The total relative isolation rates of *Alternaria* sect. *Undifilum* sp. from *A. variabilis* in Jilantai were 46.2, 60.0, 58.7 and 33.9% from leaves, stems, seeds, and roots, respectively, and in AoLun Prague was 62.7, 38.7, 68.6 and 20.0%, from leaves, stems, seeds, and roots, respectively. Hence, *A. U. oxytropis* was the dominant fungal species isolated from *A. variabilis* and *O. glabra. Alternaria* sect. *Undifilum gansuense*, instead of *A. U. oxytropis*, was isolated from roots of *A. variabilis* collected from AoLun Prague, and the dominant species was *Alternaria alternata*, which was similar to that isolated from leaves of *O. glabra* in Yinchuan (Table 4, Figure 2).

For the four tissues of *S. salsula*, *Alternaria* spp. were dominant in the stems, leaves, and seeds, while *Fusarium chlamydosporum* was dominant in petioles. The relative isolation rates of *Alternaria* spp. from stems, leaves, and seeds were 65.2, 46.2, and 44.8%, respectively, while the rate for *F. chlamydosporum* from petioles was 25.0% (Table 4).

Fungal diversity indices

According to the Shannon-Wiener index for the fungi isolated from the 3 locoweeds in the 4 ecological communities, the diversity of fungi varied among plants and locations (Table 5). The diversity of fungi from *O. glabra* in BayanHot was quite low (1.0) compared to that of *O. glabra* in Yinchuan (2.2). Enriched diversity of fungi from *A variabilis* was the same (2.0-2.1) in both sampling sites. The Shannon-Wiener index of fungi from the three tissues sampled from all plants (leaves, stems, seeds) for *S. salsula* was the highest at 2.5, followed by *A. variabilis* at 2.0-2.1, and *O. glabra* at 1.0-2.2.

The Simpson index (range 0 to 1) reflects the dominance in a community, so increases as diversity decreases. The Simpson index values for fungi isolated from locoweed tissues gave similar results as those calculated for the Shannon-Wiener index. The Simpson index of *S. salsula* was the lowest, and that for *O. glabra* was the highest, reflecting the highest and lowest diversity, respectively.

Evenness reflects the uniformity of distribution of different species in the community. The evenness of distribution of fungi was not homogenous in the sampling sites. The evenness index of fungi was highest for the community isolated from *A. variabilis* and lowest for the community from *S. salsula*. However, the evenness of distribution of fungal endophytes in *O. glabra* differed between the two sampling sites (Table 5).

The Jaccard and Sorenson indices provide an estimate of similarity or shared species between samples. The Jaccard indices were 0.4 or less comparing fungi isolated from *S. salsula* to those from *O. glabra* or *A. variabilis*, suggesting low similarity. However, the Sorenson indices were 0.6 for fungi from stems and leaves of *O. glabra* from Bayan Hot and leaves, stems, and leaves of *A. variabilis* from Jilantai compared to the fungi from *S. salsula* leaves, suggesting more similarity among those populations.

For fungi isolated from *O. glabra* collected from BayanHot, the Sorenson and Jaccard indices among stems and leaves, seeds and leaves, and seeds and

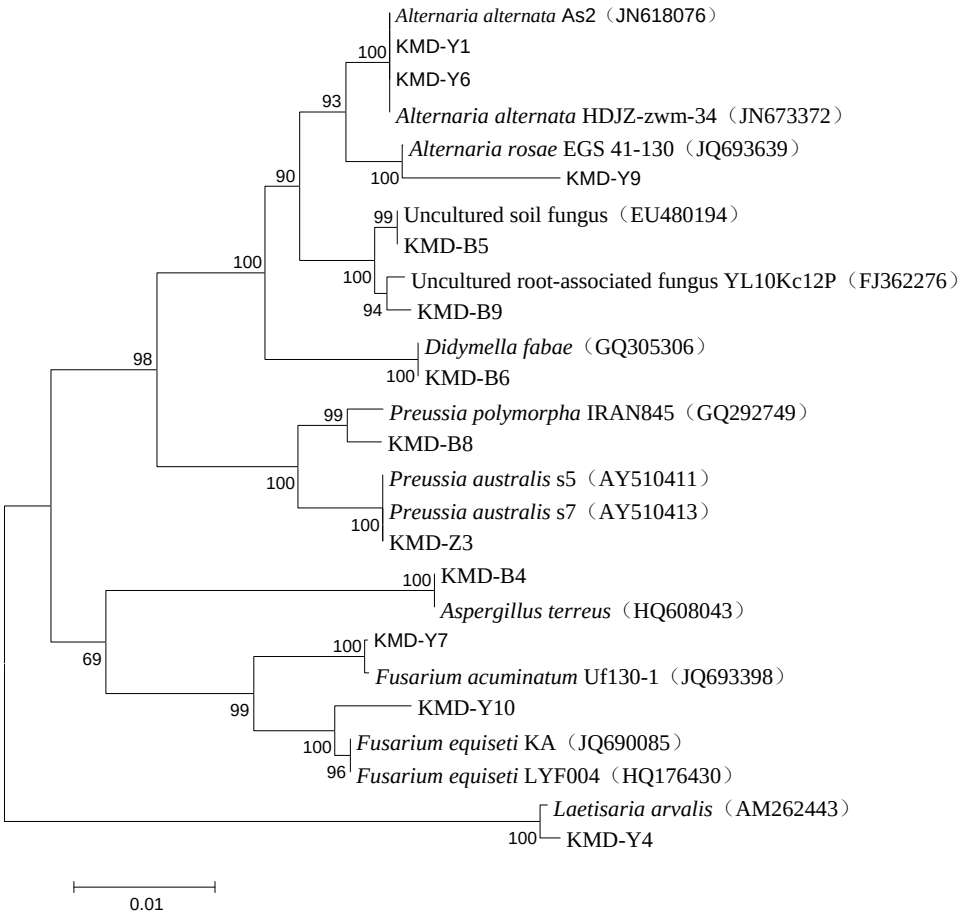

Figure 1. Phylogenetic tree constructed using Neighbor-joining (NJ) based on 5.8S rDNA-ITS sequences of fungi isolated from *Sphaerophysa salsula.*

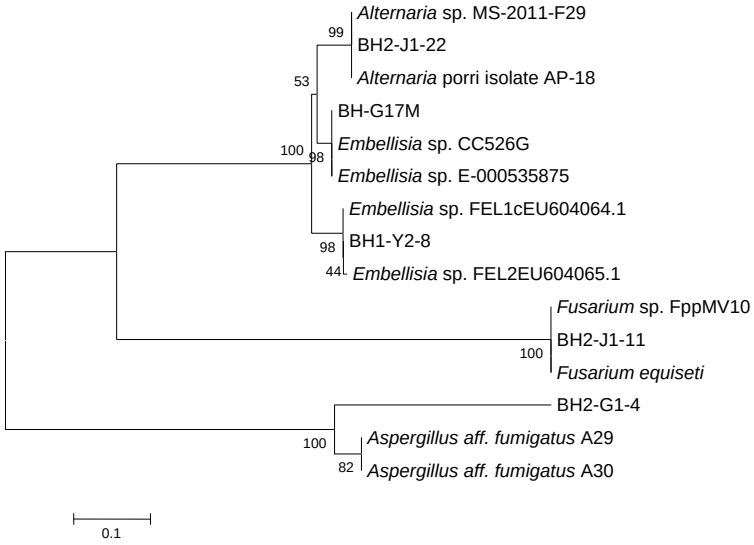

Figure 2. Phylogenetic tree constructed using Neighbor-joining (NJ) based on 5.8S rDNA-ITS sequences of fungi isolated from *Astragalus variabilis.*

Table 3. Species of fungi isolated from Locoweeds.

Species	Order	Number of isolations from aerial plant parts/roots					Ecological Role - Host
		O. glabra		*A. variabilis*		*S. salsula*	
		BayanHot	Yinchuan	Jilantai	AoLun Prague	BayanHot	
Acremonium sp.	Hypocreales		4				S
Alternaria alternata	Pleosporales		58	14	21/14	22	P- bean/many hosts[1]
A. brassicae	Pleosporales		1				P- many hosts
A. infectoria	Pleosporales			2			P/S – wheat
A. porri	Pleosporales	17	61	6/25	4	4	P-onion
A. rosae	Pleosporales			6		3	P - rose
A. solani	Pleosporales			2		3	P- tomato
A. tenuissima	Pleosporales		13		7	10	P/S-pigeonpea[2]
A. sect. *Undifilum gansuense*	Pleosporales				0/12		P–*Astragalus adsurgens*[3]
A. sect. *Undifilum oxytropis*	Pleosporales	131	182	57/62	100		M- locoweeds
Aspergillus candidus	Eurotiales		3	2			S
A. fumigatiaffinis	Eurotiales	6	3		4/6		M
A. lentulus	Eurotiales		3				S
A. terreus	Eurotiales			0/17	2/3	2	S
Bipolaris neergaardii	Pleosporales			4			S
Chaetomium globosum	Sordariales		5				S
Chloridium sp.	Sordariales					3	S
Cladosporium sp.	Capnodiales					5	P/S - mixed
Cochliobolus lunatus	Pleosporales				5		P - sugarcane
Colletotrichum cereale	Glomerellales			0/10			P-wheat
C. gloeosporiodes	Glomerellales		5				P - citrus
Coniolariella hispanica	Xylariales		6				S
Curvularia coicicola	Pleosporales	I		0/13			S
Didymella fabae	Pleosporales					2	P-beans[4]
Diplodia sp.	Botryosphaeriales					1	P-mixed
Fusarium acuminatum	Hypocreales					4	P-pine
F. brachygibbosum	Hypocreales				7		P-mixed
F. chlamydosporum	Hypocreales			0/23			P-lentil/many hosts[5]
F. equiseti	Hypocreales	11		0/10	5/8	19	P- many hosts[5]
F. oxysporum	Hypocreales			0/23			P-alfalfa/many hosts[5]
F. proliferatum	Hypocreales		15				P/S-locoweed[6]
F. tricinctum	Hypocreales	3	30	12	9/10		P-many hosts[5]
Gibberella moniliformis	Hypocreales				1		P-cereals
Humicola fusocoatra					1		S

Table 3. Cont'd.

Laetisaria arvalis	B - Corticiales				3	M
Microascus sp.	Microascales	3				S
Myrothecium verucaria	Hypocreales				2	P-mixed
Paecilomyces lilacinus	Eurotiales		2	3		S
Phaeosphaeria nodorum	Pleosporales	2				P-wheat
Phoma glomerata	Pleosporales	5				S
Preussia australis	Pleosporales				3	S
P. polymorpha	Plesoporales			3	1	S
Xylaria sp.	Xylariales	6				S

P=pathogen, S=saprophyte, M=mutualist; [1] Tu (1985), [2]Sharma et al. (2012), [3]Liu et al. (2016), [4]Hernandez-Bello et al. (2006), [5]Asan (2011) and [6]Zhou et al. (2012).

Table 4. Quantitative distribution of fungi isolated from locoweeds (ME=mutualist endophyte, P=pathogen, S=saprophyte).

Locoweed	Location/# species	Tissue type	Fungi isolated（number）	Ecological Roles/ # species	Dominant species
Oxytropis glabra	BayanHot (5 species)	Leaf	*Alternaria sect. Undifilum oxytropis* (20) M, *Alternaria porri* (3) P, *Aspergillus fumigatiaffinis* (2) M	88% ME 12% P 3 species	*A. U. oxytropis* (80.0%)
		Stem	*Alternaria sect. Undifilum oxytropis* (24), *Alternaria porri* (7) P, *Fusarium equiseti* (4) P	68% ME 31% P 3 species	*A. U. oxytropis* (68.6%)
		Seed	*Alternaria sect. Undifilum oxytropis* (87), *Alternaria porri* (7) P, *Fusarium equiseti* (7) P, *Aspergillus fumigatiaffinis* (4) M, *Fusarium tricinctum* (3) P	84% ME 15% P 5 species	*A. U. oxytropis* (80.6%)
	Yinchuan (19 species)	Leaf	*Alternaria porri* (54) P, *Alternaria alternata* (17) P, *Xylaria* sp. (6) S, *Alternaria tenuissima* (4) P, *Aspergillus candidus* (3) S, *Alternaria brassicae* (1) P	89% P 11% S 6 species	*Alternaria porri* (63.5%)
		Stem	*Alternaria sect. Undifilum oxytropis* (43), *Alternaria alternata* (15) P, *Fusarium tricinctum* (11) P, *Alternaria porri* (7) P, *Dothideomycetes* sp. (7), *Chaetomium globosporum* (5) S, *Aspergillus lentulus* 7(3) S, *Phoma glomerata* (5) S,	34% P 19% S 8 species	*A. U. oxytropis* (44.8%)
		Seed	*Alternaria sect. Undifilum oxytropis* (139), *Alternaria alternata* (26) P, *Fusarium tricinctum* (19) P, *Fusarium proliferatum* (15) P, *Alternaria tenuissima* (9) P, *Coniolariella hispanica* (6) S, *Colletotrichum gloeosporioides* (5), P, *Dothideomycetes* sp. (5), *Acremonium* sp (4) S, *Microascus* sp. (3) S, *Aspergillus fumigatiaffinis* (3) M, *Phaeosphaeria nodorum* (2) P	60% ME 32% P 6% S 12 species	*A. U. oxytropis* (58.9%)

Table 4. Cont'd.

Astragalus variabilis	Jilantai (16 species)	Leaf	*Alternaria sect. Undifilum oxytropis* (12), *Alternaria porri* (6) P, *Fusarium tricinctum*(5) P, *Alternaria solani* (2) P, *Paecilomyces lilacinus* (1) S	50% P 46% ME 4% S 5 species	*A. U. oxytropis* (46.2%)
		Stem	*Alternaria sect. Undifilum oxytropis* (18), *Alternaria rosae* (6) P, *Alternaria alternata* (3) P, *Alternaria infectoria* (2) PS, *Fusarium tricinctum* (1) P	60% ME 33% P 7% S 5 species	*A. U. oxytropis* (60.0%)
		Seed	*Alternaria sect. Undifilum oxytropis* (27), *Alternaria alternata* (11) P, *Fusarium tricinctum* (6) P, *Ascomycota* sp. (2)	59% ME 37% P 4 species	*A. U. oxytropis* (58.7%)
		Root	*Alternaria sect. Undifilum oxytropis* (62), *Alternaria porri* (25) P, *Fusarium oxysporum* (23) P, *Fusarium chlamydosporum* (23) P, *Aspergillus terreus* (17) S, *Curvularia coicicola* (13) S, *Fusarium equiseti* (10) P, *Colletotrichum cereale* (10) P	50% P 34% ME 16% S 8 species	*A. U. oxytropis* (33.9%)
	AoLun Prague (20 species)	Leaf	*Alternaria* sect. *Undifilum oxytropis* (47), *Fusarium tricinctum* (9) P, *Alternaria alternata*(7) P, *Paecilomyces lilacinus* (3) S, *Preussia polymorpha* (3) S, *Dothideomycetes* sp. (2), *Aspergillus candidus* (2), *Gibberella moniliformis* (1) P, *Humicola fuscoatra* (1 S)	63% ME 23% P 12% S 9 species	*A. U. oxytropis* (62.7%)
		Stem	*Alternaria* sect. *Undifilum oxytropis* (29), *Fusarium brachygibbosum* (7) P, *Alternaria tenuissima* (7) P, *Alternaria alternata* (6) P, *Cochliobolus lunatus* (5) P, *Fusarium equiseti* (5) P, *Bipolaris neergaardii* (4) S, *Alternaria porri* (4) P, *Dothideomycetes* sp. (3), *Ascomycota* sp. (2), *Aspergillus terreus* (2) S, *Aspergillus fumigatiaffinis* (1) M	45% P 40% ME 8% S 12 species	*A. U. oxytropis* (38.7%)
		Seed	*Alternaria* sect. *Undifilum oxytropis* (24), *Alternaria alternata* (8) P, *Aspergillus fumigatiaffinis* (3) M	77% ME 23% P 3 species	*A. U. oxytropis* (68.6%)
		Root	*Alternaria alternata* (14) P, *Alternaria* sect. *Undifilum gansuense* (12) P, *Fusarium tricinctum* (10) P, *Fusarium equiseti* (8) P, *Phoma chystallifera* (7) S, *Aspergillus fumigatiaffinis* (6) M, *Aspergillus terreus* (3) S	73% P 17% S 10% ME 7 species	*Alternaria alternata* (23.3%)
Sphaerophysa salsula	BayanHot (17 species)	Leaf	*Alternaria tenuissima* (10) P, *Fusarium equiseti* (7) P, *Fusarium acuminatum* (4) P, *Laetisaria arvalis* (3) M, *Alternaria rosae* (2) P	88% P 11% ME 5 species	*Alternaria tenuissima* (38.5%)
		Petiole	*Fusarium chlamydosporum* (4) P, *Aspergillus terreus* (2) S, *Didymella fabae* (2) P, Uncultured soil fungus (1), *Preussia polymorpha* (1) S, *Myrothecium verrucaria* (2) P, Uncultured root-associated fungus (2), *Alternaria solani* (1) P, *Alternaria rosae* (1) P	63% P 25% S 9 species	*Fusarium chlamydosporum* (25.0%)

Table 4. Cont. Cont.

	Stem	*Alternaria alternata* (9) P, *Alternaria porri* (4) P, *Alternaria solani* (2) P, *Cladosporium* sp. (2) PS, *Fusarium equiseti* (5) P, *Chloridium* Lk. (1) S	91% P 9 % S 6 species	*Alternaria* (39.1%)	*alternata*
	Seed	*Alternaria alternata* (13) P, *Fusarium equiseti* (7) P, *Preussia australis* (3) S, *Cladosporium* sp. (3) PS, *Chloridium* Lk. (2) S, *Diplodia* sp.(1) P	78% P 22% S 6 species	*Alternaria* (44.8%)	*alternata*

stems were not less than 0.6, suggesting that the similarity of fungi among leaves, stems, and seeds at this location was very high. For fungi isolated from *O. glabra* collected from Yinchuan, the Sorenson indices for stems and leaves and seed and leaves was less than 0.5, suggesting that there was lower similarity in fungal species.

Comparing the fungi isolated from tissues of *O. glabra* at two sampling sites and *A. variabilis* at two sampling sites gave generally low similarity, with a few exceptions. The Sorenson index was 0.6from stems of *A. variabilis* collected from AoLun Prague compared with *O. glabra*, and 0.6 for stems of *O. glabra* collected from Yinchuan compared with *A. variabilis* (Table 4).

Among the fungi isolated from leaves, stems, and seeds of *A. variabilis* collected in Jilantai, both Sorenson and Jaccard indices were higher than 0.6. In contrast, the Sorenson and Jaccard indices were lower for AoLun Prague. The Jaccard index was less than 0.5 among seeds, leaves and stems of *A. variabilis* collected in AoLun Prague. The Sorenson index was more than 0.5, indicating that similarity of fungi was higher in *A. variabilis* collected from AoLun Prague (Table 6).

Ecological roles of isolated fungi

The relative rate of isolation of mutualist endophytes significantly impacted recovery of pathogenic fungi (Table 4). There was an inverse relationship between isolation of *A. U. oxytropis* and plant pathogenic fungi. *O. glabra* from BayanHot yielded very high levels of *A. U. oxytropis* (68.6- 80.6% of total fungal isolations) and very low levels of plant pathogens (12-15% of total fungal isolations). In the absence of *A. U. oxytropis*, the pathogen levels were very high; *O. glabra* leaves from Yinchuan yielded 89% pathogens. Overall the relative pathogen and saprophyte levels from *O. glabra* from Yinchuan were higher and *A*. *U. oxytropis* levels lower than from *O. glabra* from BayanHot. The leaves from Yinchuan had 89% pathogens and 11% saprophytes, the stems 34% pathogens and 19% saprophytes, and the seeds 32% pathogens and 6% saprophytes. *A. variabilis* yielded moderate pathogen levels at both locations. Pathogen levels from leaves, stems, seeds, and roots from Jilantai were 50, 33, 37 and 50%, respectively, and at AoLun Prague were 23, 45, 23 and 73%, respectively. Saprophyte levels were higher in roots than other tissues from both locations, 16% from Jilantai and 17% from AoLun Prague. *S. salsula* yielded high levels of pathogens from all tissues, 88, 63, 87 and 72% from leaves, petioles, stems, and seeds, respectively. Higher levels of saprophytes (25%) were only isolated from petioles.

DISCUSSION

Plant host species, plant part, and environment influenced the endogenous fungal communities isolated from the locoweed plants. Diverse fungi were found associated with the locoweed plants. While one fungal species (*Alternaria porri*) was found associated with all three plants tested, generally different species of plants were colonized by different fungi. There were a few dominant species found, particularly *Alternaria* sect. *U. oxytropis* from *O. glabra* and *A. variabilis.* There were also abundant rare fungal species found in small numbers from a single plant species, for example 12 for *O. glabra* from Yinchuan, 8 from *S. salsula* from BayanHot and 7 from *A. variabilis* from AoLun Prague. Since there are very few reports of fungi associated with locoweeds, for many of the fungi isolated, this constitutes the first report of most of the association of those fungal species with these plant species. Only *A. U. oxytropis*, *A. U. gansuense*, and *Fusarium proliferatum* have been previously reported to be associated with locoweeds in China (Gao et al., 2012; Zhou et al., 2012; Liu et al., 2016). Other than these three fungal species, only 8 species found in this study have even been reported to be a pathogen of any legume (Tu, 1985; Hernandez-Bello et al., 2006; Asan, 2011; Sharma et al., 2012).

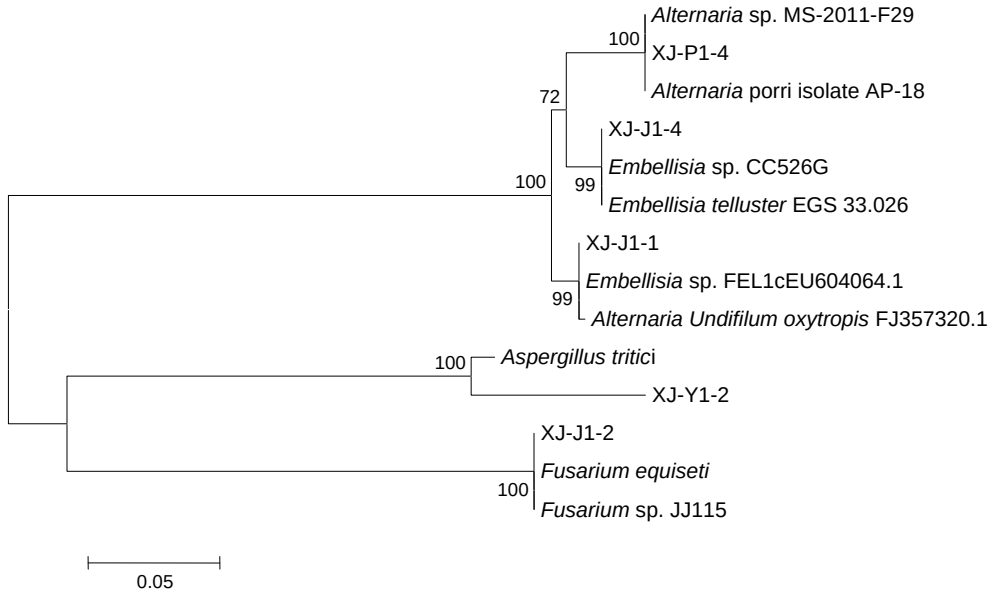

Figure 3. Phylogenetic tree constructed using Neighbor-joining (NJ) based on 5.8S rDNA-ITS sequences of fungi isolated from *Oxytropis glabra*

Table 5. Diversity index of fungi from locoweeds and sampling sites.

Locoweed	Location	Diversity index		Evenness
		Shannon-Wiener (H')	Simpson (D)	
Sphaerophysa salsula	BayanHot	2.5	0.1	0.5
Oxytropis glabra	BayanHot	1.0	0.5	0.6
	Yinchuan	2.2	0.2	0.7
Astragalus variabilis	Jilantai	2.0	0.2	0.8
	AoLun Prague	2.1	0.2	0.8

Table 6. Similarity index of fungi isolated from different locoweed tissues and species.

Locoweed tissues		*Sphaerophysa salsula*			*Oxytropis glabra*			*Astragalus variabilis*		
		Leaf	Stem	Seed	Leaf	Stem	Seed	Leaf	Stem	Seed
S. salsula	Leaf		0.4	0.3	0.2 0.2*	0.4 0.2*	0.4 0.2*	0.3 0.2*	0.3 0.2*	0.3 0.2*
	Stem	0.6		0.8	0.3 0.2*	0.3 0.3*	0.3 0.2*	0.3 0.2*	0.3 0.2*	0.3 0.2*
	Seed	0.4	0.8		0.3 0.1*	0.3 0.4*	0.3 0.3*	0.3 0.3*	0.3 0.3*	0.3 0.1*
O. glabra	Leaf	0.3 0.5*	0.5 0.3*	0.4 0.1*		0.6 0.2*	0.6 0.2*	0.2 0.2*	0.2 0.2*	0.2 0.5*
	Stem	0.6 0.4*	0.5 0.5*	0.4 0.4*	0.8 0.3*		0.6 0.5*	0.3 0.5*	0.3 0.5*	0.3 0.3*
	Seed	0.6 0.3*	0.5 0.3*	0.4 0.4*	0.8 0.3*	0.8 0.6*		0.3 0.3*	0.3 0.3*	0.4 0.3*

Table 6. Contd.

A. variabilis	Leaf	0.6 0.3*	0.5 0.3*	0.4 0.4*	0.3 0.3*	0.5 0.6*	0.4 0.4*		0.6 0.5*	0.6 0.3*
	Stem	0.6 0.3*	0.5 0.3*	0.4 0.4*	0.3 0.3*	0.5 0.6*	0.4 0.4*	0.8 0.6*		0.6 0.3*
	Seed	0.6 0.3*	0.5 0.3*	0.4 0.3*	0.3 0.5*	0.5 0.5*	0.6 0.5*	0.8 0.5*	0.8 0.5*	

"*" means the second sampling site (Yinchuan or AoLun Prague); the number represents Jaccard index (above diagonal) and Sorenson index (below diagonal).

Interestingly, most of the pathogens found have not been reported to infect legumes, including *A. porri*, a pathogen of onion, which was found associated with all three plant species. Most of the saprophytic fungi isolated are considered ubiquitous, found in soil or on decaying plant material of many different plant types and environments (Petrini, 1991). Our experimental objective was to screen for culturable strains of fungi associated with locoweeds. It is likely that other nonculturable fungi are associated with the locoweeds, but not identified in this study.

Environment had an influence on the endogenous fungal communities, particularly for the two toxic locoweeds, for which location and environment appeared to strongly influence the fungal community. The isolation rates and species of fungal endophytes differed within *O. glabra* collected from different locations, in which, five species of fungi were isolated from *O. glabra* in BayanHot town, while 21 species were isolated from Yinchuan city and 12 of those 21 species were unique to that site. BayanHot is in Inner Mongolia and Yinchuan is in Ningxia province, thus, providing different locations and environmental factors. The environmental factors that differed between the two sites include surrounding flora, habitat, soil type, rainfall, temperature, and altitude. Because of the limited number of sampling sites, the quantitative relationship between a particular environmental factors and fungal endophyte species could not be definitively determined. In contrast, the numbers of fungi isolated from *A. variabilis* from Jilantai (14, with 4 unique species) were similar to that isolated from AoLun Prague (17, with 7 unique species). Both collection sites for *A. variabilis* are located in Inner Mongolia. The distribution of fungi within a plant species can be influenced by location, season, age, climate, and geographical conditions. Arnold and Lutzoni (2007) analyzed the distribution of communities of fungal endophytes in forests between the Canadian arctic to the central part of Panama, and found that the colonization rate of fungal endophytes is significantly influenced by latitude. However, within a narrow scope of latitude, fewer fungal endophyte species were found in arid areas than in semi-deciduous forests (Hoffman and Arnold, 2008). Siles and Margesin (2016) found that the relative size fungal communities associated with alpine forests increased with altitude and the composition changed as well. The authors noted that changes in fungal richness and diversity and community structure were primarily influenced by pH and carbon/nitrogen in the soil, which was the result of environmental factors at the sites tested.

For α-diversity index, Shannon-Wiener and Simpson indices were used in this study. Two factors influence the Shannon-Wiener index: diversity (variety and amount) and uniformity of fungal localization among species. In this study, the Shannon-Wiener index of *S. salsula* collected from BayanHot of the Inner Mongolia Autonomous Region was the highest, while that of *O. glabra* was the lowest. These results could be because the presence of the fungal endophyte, *Alternaria* sect. *U. oxytropis* has an impact on other fungi infecting the same plant tissues. The Simpson index is also an indicator of diversity, with a larger value indicating greater diversity, but is a dominance index giving more weight to common species, such that a few rare species do not affect the diversity value. The Simpson diversity index of fungal endophytes in *O. glabra* in the two locations was significantly different, but that of *A. variabilis* was not significantly different between locations. In contrast, the Shannon-Wiener index of fungal endophytes isolated from *A. variabilis* collected in AoLun Prague was higher than in Jilantai. Compared to forage plants in similar ecological environments in other countries, the diversity index we found was slightly low (Porras-Alfaro et al., 2008; Khidir et al., 2009). This may be due to the small number of samples.

β-diversity, as measured through the Jaccard and Sorenson indices, is the difference in the composition of species between different communities in different habitats, or the replacement rate of species along environmental gradients, which is also called between-habitat diversity. The main ecological factors controlling β-diversity are thought to be soil, topography, and interference of external factors. Fewer common species are found in different communities or different sites in the same environmental gradient and hence, β-diversity is

larger. The β-diversity index can indicate the degree to which species are isolated by habitat, which can be used to compare habitat diversity in different areas, and constitutes the overall diversity or the biological heterogeneity in certain locations together with the α-diversity index. For β-diversity index, Jaccard and Sorenson diversity indices were used to compare the degree of similarity of composition of species of fungal endophytes between two locations. In this study, the species and quantity of distribution of fungal endophytes differed between hosts and tissues. Compared to the results calculated with Jaccard and Sorenson indices, there were uniform patterns of distribution of fungal endophytes in different tissues from the same plant, and the same plant at different sample sites. Stems and seeds showed the highest index (0.8) in similarity of flora in *S. salsula* collected in BayanHot, followed by petioles and seeds, indicating a similarity in flora of fungal endophytes among stems, petioles, and seeds in *S. salsula*. The Sorenson and Jaccard indices between the tissues colonized by fungi, in *O. glabra* and *S. salsula* and between all the tissues colonized by fungi in *A. variabilis* and *S. salsula*, were low, suggesting that there are no similarities in the community of fungi between *O. glabra* or *A. variabilis* and *S. salsula*.

The fungal communities differed somewhat by plant part. Although roots were only sampled from *A. variabilis*, they yielded higher proportions of saprophytes than the aerial portions of the plants. Similar results were demonstrated for roots and aerial parts of wheat (Comby et al., 2016). David et al. (2016) demonstrated that the influence of location and host species on fungal endophyte community composition also depended on plant part, in that environment and host species were very important in endophyte community composition from leaves of beach grasses, but only the sand dune environment was important for determining the endophyte community from roots.

Alternaria sect. *U. oxytropis* was consistently isolated from the seeds of the toxic locoweeds and less consistently with the leaves and stems. This colonization is consistent with transmission of *A.U. oxytropis*, which is maternally transmitted through seed (Oldrup et al. 2010). Braun et al. (2003) found that slow growing *Alternaria* sect. *Undifilum* spp. were isolated from *A. mollissimus*, *O. lambertii*, and *Oxytropis sericea* locoweed populations collected from New Mexico. They found that the infection rates of fungal endophytes in stems, leaves, flowers, and seeds were 97.2, 72.9, 100 and 93.1%, respectively. Fast-growing fungi were also isolated from roots when sterilization was minimal, but they were not characterized. Cook et al. (2009a) quantified fungal endophytes in 10 plants each of *O. sericea*, *A. mollissimus*, and *A. lentiginosus*, using quantitative PCR, and showed that the amount of *Alternaria* sect. *Undifilum* sp. differed significantly among the plant species and between individual plants within a species.

The isolation rate of *Alternaria* sect. *U. oxytropis* from plant sections coincided with fewer plant pathogens isolated from the same sections. Fewer plant pathogens and a higher isolation rate of *A. U. oxytropis* were obtained from *O. glabra* from BayanHot than from Yinchuan. Comparing plant species collected from BayanHot, the non-toxic *S. salsula*, which did not yield *A. U. oxytropis*, had a much higher relative proportion of pathogens, than did *O. glabra*. The diversity of the fungi was also much lower from *O. glabra* than *S. salsula* from BayanHot. Together this suggests that *A. U. oxytropis* may influence the decrease in pathogen isolations. This could be due to competition for space or nutrients or it might be related to its swainsonine toxin production. Since swainsonine data was not obtained from these plants, this can't be verified. Infection with fungal endophytes has been shown to change microbial species composition and reduce pathogens (Clay, 1990; Arnold et al., 2003; Pan and May, 2009; Busby et al., 2016). Infection with *E. coenophiala*, a shoot-specific fungal endophyte of tall fescue influenced soil fungal communities, decreasing the relative abundance of Ascomycota and increasing the abundance of Glomeromycota (Rojas et al., 2016). Fungal endophyte infection in mature leaves of the tropical tree *Theobroma cacao* prevented infection by *Phytophthora* sp. pathogens (Arnold et al., 2003). The nature of the beneficial interaction needs to be assessed with other locoweeds and species of *Alternaria* sect. *Undifilum* to determine if this is a universal characteristic or specific only to this situation.

Conclusion

The diversity of fungi associated with locoweeds, the sampling sites, plant part and isolation rates were considered in this study. Location/environment, plant species, and presence/absence of the fungal endophyte *Alternaria* sect *Undifilum* all had strong impacts on the diversity of fungi associated with the locoweed plants. This initial catalog of fungi associated with locoweeds lays the foundation for future research on the microbial community associated with these toxic plants. The endogenous fungal community associated with locoweeds in other locations such as the western USA and associated with other plant species containing different *Alternaria* sect. *Undifilum* spp. should be examined to determine if the community profile is ubiquitous. This is the first report of a beneficial role for this fungal endophyte. The beneficial role for *Alternaria* sect. *Undifilum* sp. found here has implications for management strategies, since this endophyte has been found associated with a variety of plants in the western USA and Australia, as well as China. Current efforts to

manage the toxicosis associated with grazing plants infected with the fungus have ranged from using herbicide to kill the plant, to avoiding grazing on infected (toxic) plants, and using fungicide to cure locoweeds of the toxic endophyte. If the beneficial role for the endophyte is linked to its production of swainsonine, then efforts to develop endophyte-free plants should be pursued. If the beneficial role for the endophyte is not tied to swainsonine production, then development of a fungal mutant that did not produce swainsonine might possibly help its plant host survive pathogen infection.

Conflict of Interests

The authors have not declared any conflict of interests.

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Microbial community structures of an offshore and near-shore oil production facilities after biocide treatment and the potential to induce souring and corrosion

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Microbial communities of six samples from Escravos and Meren oil production facility in Nigeria were examined after biocide treatment using cultural and molecular approaches. Sulfate reducing bacteria (SRB) count and activity were the highest in skimmer pit samples (1N1) which was affiliated with Euryarchchaeota (44%) and Proteobacteria (39%). Treatment of Meren injection water (1N2) with biocides significantly reduced SRB population (10^2 cell/ml). Sample 1N3 (produced water discharge area) and 1N5 (treated produced water) were dominated by 70.79 and 52% Euryarchaeota, respectively. 1N4 (partially treated produced water) was dominated by Euryarchaeota (51.78%) and Proteobacteria (38%), while 1N6 (produced oil) was dominated by Proteobacteria (28%) and Euryarchaeota (62.6%). Metabolic activities in 1N1, 1N4 and 1N5 were dominated by sulfate reduction and methanogenesis; however, that of 1N2 was dominated only by sulfate reduction, while 1N3 and 1N6 were dominated by methanogenesis. Anaerobic incubation of 1N1 samples gave relatively higher corrosion rates (0.065 mm/year) while 1N2, 1N3 and 1N5 gave moderate corrosion rates (0.018 to 0.037 mm/year). This investigation in a Nigerian oil facility have substantially expanded our knowledge on the extent of microbial diversity in these fields after routine biocide treatment and have also shown the abundance and complexity of microbial communities in these fields that have potential to initiate souring and corrosion despite regular biocide treatment.

Key words: Sulfate reduction, methanogenesis, corrosion, souring, microbial diversity.

INTRODUCTION

Nigeria presently ranks about the 6th position in the world in terms of crude oil production with a maximum daily production capacity of 2.5 million barrels per day and it is also the world's 10th largest oil reserves estimated at

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about 25 billion barrels (OTC, 2002). Known in the energy circle as a “Gas province with a little pool of oil”, the country’s gas reserve is put at over 166 trillion standard cubic feet and the current gas production is put at 2 billion standard cubic feet (OTC, 2002).

The constant demand for oil has driven more interest in the study of petroleum reservoir microbial diversity as microbial activity in an oil reservoir can have significant implications on oil quality and recovery (Head et al., 2003). For example, microbial activity can have costly negative effects such as hydrocarbon degradation, clogging, souring and corrosion of pipelines (Magot et al., 2000; Nimati et al., 2001; Li and Hendry, 2008; Voordouw, 2011) or beneficial effects on the rate or extent of oil recovery and productivity (Lake, 1989; Youssef et al., 2009). In the recent years, an assessment of microbial diversity and habitat conditions within a petroleum reservoir is being increasingly recognized as an important component of reservoir management (Li et al., 2012). A comprehensive assessment of the diversity, metabolic processes and habitat conditions for petroleum microorganisms is therefore of practical importance for assessing the economic potentials of oil fields. It will also help in the understanding of how *in-situ* biotic factors may affect oil production operations (Pham et al., 2009). A study by Van der Kraan et al. (2009) have also indicated how the presence of specific microbial communities could present information on some characteristics of oil reservoir such as temperature, acidity, salinity, and redox-potential, etc.

In Nigeria, Meren field is located south east of Lagos, Nigeria with an estimated oil reserves of 1.8 billion barrels, out of which about 750,000 barrels has been produced as at 2002 (Lumley et al., 2000). The current production capacity is about 85,000 barrels per day. Sea water picked up to 25 ft below sea level is treated with continuous injection of sodium hypochlorite with residual chlorine maintained at 1.5 mg/L. Apart from continuous chlorination, two other biocides (amine and quaternary ammonium compound and a gluteraldehyde) have been applied alternatively for 6 h weekly at a concentration of 200 pm. Even with the biocide application, it has been reported that total elimination of sulfate reducing bacteria (SRB) from Meren field is difficult (Adetoba, 1985; Lumley et al., 2000). SRB are mostly anaerobes and they have the ability to reduce sulfate ions in injection water to sulfide ion which can result to souring and pitting corrosion (Voordouw, 2011; Hubert et al., 2005). The insoluble sulfide formed can also cause plugging in filters and formation (Hubert et al., 2005). Meren sea water is a semi-closed system so both aerobic and anaerobic microorganisms co-exists and the de-aerated sea water provides an almost oxygen free environment for sulfate reducing bacteria (SRB) to thrive.

Escravos processing facility on the other hand is a sharp contrast from Meren. The facility is located nearshore in the Niger Delta, about 100 km south east of Lagos, Nigeria. It is comprised of a tankfarm where escravos crude oil is stored and loaded, a processing facility for raw crude coming from Abiteye, Okan and Olero creek (with a capacity of about 70,000 barrels of crude/day) and a gas to liquid facility (EGTL) that processes about 325 million cubic feet per day of natural gas (http://www.chevron.com). Within the Escravos facility is an oily waste storage pit (Skimmer pit) where oily waste that drains from the facility is stored temporarily before treatment and disposal. With an average temperature of about 60°C, it is expected that skimmer pit microbal flora will be dominated by moderate thermophiles. There is no sea water reinjection at Escravos facility as it is done at Meren but the same routine of biocide application is applied at Escravos as it is done at Meren. Produced water is mechanically treated to knock down the hydrocarbon level below 50 ppm before being disposed at nearby Escravos river. Considerable concentrations of SRB has been detected in Escravos produced water and the receiving sea water (Okoro et al., 1999, 2010) despite regular biocide treatment.

In characterization of microbial communities, culture independent 16S rRNA gene pyrosequencing approach which has been proven to be very efficient and reliable as opposed to the culture dependent methods which greatly underestimates the microbial diversity associated with an environment (Pham et al., 2009; Voordouw et al., 1996, Orphan et al., 2000; Grawbowski et al., 2003; Li et al., 2007; Nazina et al., 2007; Dahle et al., 2008; Okoro et al., 2014). Culture independent 16S rRNA gene based surveys are extremely valuable in providing an overall view of the community composition in a specific ecosystem regardless of the metabolic activities of the community members (Youssef et al., 2009; Gittel et al., 2009). These studies also provide preliminary information on the relative abundance of different groups of organisms within the ecosystem and how this could be used in monitoring temporal and spartial changes within the ecosystem.

Unfortunately, information on microbial diversity and habitat conditions in Nigerian oil fields is scantly in literature and this has provided strong incentives for a detailed study of microbes present in Nigerian oil fields and in production operations. To the best of our knowledge, there has been no recorded study on the microbial communities of these fields that persist after routine biocide treatments and with potential to induce souring and corrosion. The main objective of the present study therefore is to characterize the microbial communities associated with corrosion and souring in Chevron’s Meren offshore field and Escravos production and processing facility after routine biocide treatment. The present study will therefore provide some useful information that can be used to mitigate potential cases of souring, plugging, corrosion and fouling after biocide treatment and can also provide information on the

Table 1. Description of samples obtained from Nigerian oil fields and their water chemistry.

Sample code	Field	Description	pH	Site Temp.(°C)	Sulfate (mM)	$N0_3$ (mM)	$N0_2$ (mM)	NH_4^+ (mM)	Ace[a] HPLC	Pro[a] HPLC	But[a] HPLC
1N1/CR3[b]	Escravos facility	Waste water from Skimmer pit	6.9	40-60	0.04	0.05	0	1.17	18.8	1.8	0
IN2/CR5[b]	Meren Offshore	Injection water	6.2	28-35	24.5	0	0	0.02	0	0	0
1N3/CR8[b]	Escravos facility	Mixture of sea water and produced water at produced water discharge area.	7.2	40-50	0.03	0.05	0	1.16	20.9	2.2	0
1N4/CR7[b]	Meren Offshore	Partially treated produced water	7.4	30-40	0.02	0	0	1.56	0	0	0
IN5	Meren Offshore	Fully treated produced water	7.1	30-40	9.0	0.03	0	1.39	4.4	1.5	0
1N6	Meren Offshore	Produced oil/water mixture from production well	6.9	28-32	17.8	0.01	0	0.71	8.8	1.4	0

[a]Concentrations (mM) of the volatile fatty acids acetate (Ace), Propionate (Pro) and Butyrate (But) determined by HPLC. [b]Samples labeled CR were used for corrosion rate measurements.

efficacy of biocides currently used in these facilities to mitigate souring and corrosion.

MATERIALS AND METHODS

In the present study, samples were collected from two Nigerian oil production facilities (Meren and Escravos facility) after routine biocide treatment and analyzed both chemically and microbiologically.

Site and process description

Six samples, 1N1, 1N2, 1N3, 1N4, 1N5 and 1N6 from Escravos and Meren oil production facilities (Table 1) were collected in sterile 500 ml Nalgene sample bottles, filled to the brim to exclude air and shipped to the University of Calgary, Alberta Canada for further analysis within one week of collection. From Escravos facility, samples were collected from skimmer pit which is a temporary storage pit for oily wastes arising from hot water flushing and cleaning of the oil facility. Its temperature is usually elevated ranging from 45 to 60°C. Another point of sample collection at Escravos is the point of produced water discharge where sea water mixes with produced water. At Meren offshore facility, both treated and partially treated produced water samples and samples from wellhead were collected. Injection sea water which was picked from 25 ft below sea level, filtered and treated with biocides were also collected. On arrival at the University of Calgary, samples were transferred to Coy anaerobic hood with an atmosphere of90% (v/v) N_2 and 10% (v/v) CO_2.

Chemical analysis

The pH of the samples was measured using an Orion pH meter. Aqueous sulfide was analyzed by using the diamine method (Trüper and Schlegel, 1964) and NH_4^+ by the indophenol method (APHA, 1992). Sulfate, NO_3^-, NO_2^- and the volatile fatty acids (VFA) acetate, propionate and butyrate were analyzed by high-performance liquid chromatography (HPLC), as described previously (Grigoryan et al., 2008). For analysis of inorganic anions, 100 µL of sample was combined with 400 µL HPLC anion buffer, while for analysis of VFA, 300 µL of the sample were combined with 20 µL 1 M phosphoric acid.

Measurement of microbial activities and most probable numbers

The activities of SRB, as well as of heterotrophic nitrate-reducing bacteria and sulfide-oxidizing nitrate-reducing bacteria (hNRB and soNRB) were measured in Coleville synthetic brine (CSB-K) medium (Nemati et al., 2001). Medium was anaerobically dispensed in 70 ml aliquots in 125 ml serum bottles with a gas phase of 90% N_2 and 10% CO_2, and closed with sterile butyl rubber stoppers. Medium was amended with 40 mM lactate and 20 mM sulfate or 3 mM VFA and 20 mM sulfate for measurement of SRB activity, with 3 mM VFA and 10 mM nitrate for measurement of hNRB activity, and with 5 mM sulfide and 10 mM nitrate for measurement of soNRB activity. The 3.5 ml of each sample was injected into the medium in the bottle and those were incubated at 37°C on shaker. Using a sterile syringe needle, 1 ml was taken periodically to determine the sulfide, sulfate, nitrate and nitrite concentrations. Microbial activities were calculated as $100/t_{1/2}$ units/day, where $t_{1/2}$ is the time (days) needed to reduce half of the sulfate (SRB activity) or nitrate concentration (hNRB and soNRB activities), or oxidize half of the sulfide concentration (so-NRB). The most probable number (MPN) of lactate-utilizing SRB was determined, using vials with 9 ml of anaerobic API RP-38 broth. These were inoculated with 1 ml of sample and with 10-fold dilutions derived thereof. Formation of a black iron sulfide precipitate was used to score the presence of SRB. The vials were incubated at 37°C for 30 days after which the MPN was determined. For acid-producing bacteria (APB), ZPRA-5 phenol red-dextrose medium with a salinity of 5000 ppm was used. Change in colour from orange to yellow showed the presence of acid producers.

Iron corrosion testing

Iron coupons (2×1×0.1 cm^3) were cut from ASTM A366 carbon steel with 0.015% (w/w) carbon (ASTM international designation A1008/A) and cleaned according to a standard protocol (NACE RP0775-2005) in which the coupons were polished with 400 grit sandpaper and then placed in a dibutyl-thiourea-HCL solution for 2 min. The coupons were then neutralized in a saturated bicarbonate solution for 2 min, rinsed with dH_2O and then with acetone and finally dried in a stream of air. The coupons were weighed 3 times and the average weight was recorded as the starting weight. Duplicate coupons in small plastic

holders to prevent them from contacting the glass wall or each other were placed in 120 ml serum bottles containing 70 ml of sample under anaerobic conditions (headspace of 90% v/v N_2 and 10% CO_2; N_2-CO_2). One set contained unfiltered sample, whereas another set contained sample filtered through 0.2 µm acrodisc syringe filters. Serum bottles with 70 ml of filtered Milli-Q purified water were used as a control. The samples were incubated at 32°C while being shaken at 60 rpm. Samples (1 ml) were withdrawn weekly for chemical analysis with a sterile syringe flushed with N_2-CO_2. After the incubation period, the coupons were cleaned and dried according to the NACE standard protocol and weighed again. The corrosion rate (CR) was determined from the metal weight loss (ΔW in g) as:

$$CR = 87{,}600 \times \Delta W/(A \times D \times T) \text{ mm/year}$$

Where A, D and T represent the coupon area (4.6 cm^2), the density of the steel (7.85 g/cm^3) and the incubation time (h), respectively. (Okoro et al., 2014).

DNA extraction, amplification, sequencing and bioinformatic analysis

Genomic DNA was extracted from 40 ml of samples 1N1 to 1N6 using the MP Biomedical FastDNA kit.. Extracted DNA (2 ng μL^{-1}) was then amplified through 25 PCR cycles. The PCR products were purified and subjected to a second round of 10 PCR cycles with pyrosequencing primers 454T-RA-X (which is barcoded) and 454T-FB, which have 926f and 1392r (Park et. al., 2011) as their 3' ends. PCR product quality was verified on an 0.7% agarose gel and PCR products were purified with a QIAquick PCR Purification Kit (Qiagen) following which their concentrations were determined on a Qubit Fluorometer (Invitrogen), using a Quant-iT dsDNA HS Assay Kit (Invitrogen). Detailed procedures have been described (Park et al., 2011). PCR products (typically 20 µL of 5 ng μL^{-1}) were sent to the Genome Quebec and McGill University Innovation Centre for pyrosequencing with an FLX Instrument, using a GS FLX Titanium Series Kit XLR70 (Roche Diagnostics Corporation). Data analysis was conducted with Phoenix 2, a 16S rRNA data analysis pipeline, developed in house (Park et al., 2011; Soh et al., 2013). High quality sequences that remained following quality control and chimeric sequence removal were clustered into operational taxonomic units at 3% distance by using the average linkage algorithm (Schloss and Westcott, 2011). A taxonomic consensus of all representative sequences from each of these was derived from the recurring species within 5% of the best bitscore from a BLAST search against the SSU Reference data set SILVA102 (Pruesse et al., 2007). Amplicon libaries were clustered into a Newick-formatted tree using the UPGMA algorithm with the distance between libraries calculated with the thetaYC coefficient (Yue and Clayton, 2005) as a measurement of their similarity in the Mothur software package (Schloss et al., 2009). The Newick format of the sample relation tree was visualized using Dendroscope (Huson et al., 2007). The entire set of the raw reads is available from the Sequence Read Archive at the National Center for Biotechnology Information (NCBI) under accession numbers; SRR631214, SRR631215, SRR631216, SRR631217, SRR631828, and SRR631829.

RESULTS

Chemical characterisation of samples

Results for chemical analysis of samples are indicated in Table 1. Partially treated produced water samples from Meren offshore, water samples from produced water discharge area at Escravos and samples from Escravos skimmer pit had low sulphate concentrations which ranged between 0.02 and 0.04 mM while samples from treated produced water, produced oil and injection water from Meren offshore had relatively high sulphate concentration which ranged between 9 and 24.5 mM. Nitrate concentrations were low in all samples (0-0.05 mM). Ecsravos skimmer pit samples and water samples from produced water discharge area had relatively high acetate concentrations which ranged between 18.8 and 20.9 mM; the rest of the samples had relatively low acetate concentrations. Propionate was present at relatively low concentrations in samples 1N1, IN3, 1N5 and 1N6 (1.4-1.8 mM) while butyrate was not detected in any of the samples. Ammonium ion was present in all the samples (0.02-1.39 mM). Meren offshore injection water samples recorded the lowest concentration of ammonium ion (0.02 mM) while partially treated produced water from Meren offshore recorded the highest (1.56 mM).

Microbial activities and counts

Detailed microbial activities are indicated in Figures S1 to S6 (Supplementary material) while microbial counts are shown in Table 2. Partially treated produced water, treated produced water and oil/water mixture from Meren offshore showed no SRB counts (Table 2), consequently there were no activities of lactate utilising and VFA utilising SRB as well as VFA utilising hNRB and so-NRB (Figure S4 to S6). Injection water samples from Meren offshore had relatively low SRB counts (10^2 cell/ml) with moderate activities of lactate utilizing and VFA utilizing SRB (Figure S2); however, significant concentrations of sulphide were generated in lactate media. There were no activities of hNRB and so-NRB. The highest concentration of SRBs were detected in samples from Escravos skimmer pit (10^6 cell/ml) followed by samples from produced water discharge area at Escravos (10^5 cell/ml), consequently activities of lactate utilizing and VFA utilizing SRB were high (Figures S1 and S3) with considerable generation of sulphide in lactate media. There were no activities of hNRB and so-NRB in skimmer pit water samples but considerable activities of hNRB and so-NRB were recorded in samples from produced water discharge area at Escravos with significant reduction of sulphide by the so-NRB. It will be recalled from the chemical analysis data that samples from produced water discharge area at Escravos had some residual nitrate concentration and possibly presence of indigenous so-NRB.

Microbial communities in Escravos and Meren offshore samples

Bacterial DNA was successfully extracted and purified

Table 2. Most probable number (MPN) results of samples.

Sample code	Sample description	# of SRBs per ml	SRB	hNRB	so-NRB
1N1	Waste water from skimmer pit	10^6	+	+	+
1N2	Injection water from Meren offshore field	10^2	+	+	-
1N3	Mixture of sea water and produced water at produced water discharge point	10^5	+	+	+
1N4	Partially treated produced water from Meren offshore field	BD	-	+	-
1N5	Treated produced water from Meren offshore oil field	BD	-	+	-
1N6	Oil/Water Mixture from Meren production well	BD	-	-	-

BD, Below detection; -, absence of bacteria; +, presence of bacteria.

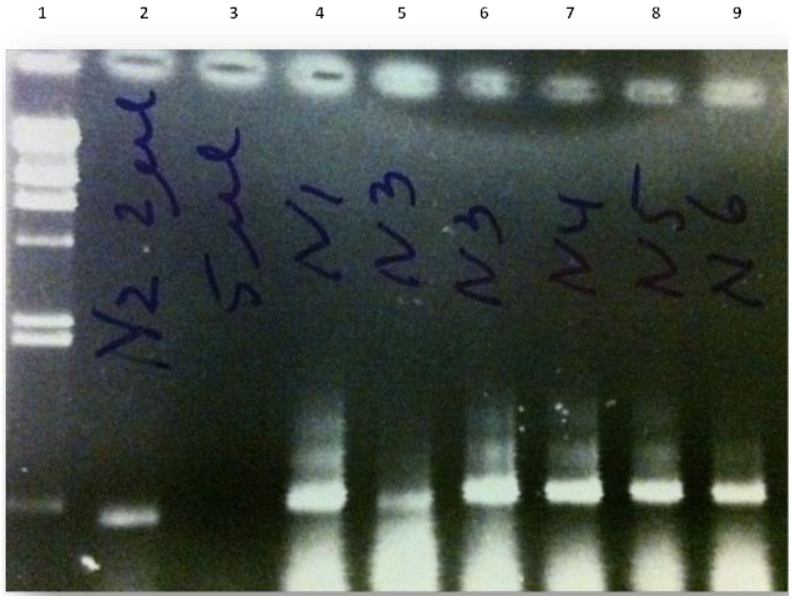

Figure 1. Agarose gel picture showing the bands verifying presence of 16S rRNA gene in all the samples. Lane (1) Hind III ladder, (2) 1N2, (3) negative control, (4) 1N1, (5) 1N3, (6) 1N3 duplicate, (7) 1N4, (8) 1N5, and (9) 1N6.

from all samples including those with zero SRB counts and microbial activity. This was done to show further proof of the usefulness of culture independent methods in identifying more diverse group of organisms that could not be identified with culture dependent methods. Following PCR amplification and purification by agarose gel electrophoresis which showed bands that verified the presence of 16S rRNA gene in all the samples (Figure 1); the 16S rRNA amplicons of the samples were subjected to pyrosequencing. The sequences clustered into 191 to 218 OTUs (groups of sequence with 95% sequence identity) for Escravos samples and 152-359 OTUs for Meren samples. The relationship between the number of identified OTUs and the number of sequences is shown in refraction curves (Figure 2) and the relationship three is shown in Figure 3. These indicated that microbial diversity was on average higher at Meren offshore than at Escravos neareshore. The normalised shanon index on the average was also higher at Meren than Escravos (Table 3).

Sequence analysis indicates the presence of 90 to 185 taxa in microbial communities in these fields (Table 3). Microbial community diversity analysis showed that the Escravos skimmer pit samples (1N1) were dominated by methanogenic archeae with genus *Methanosaeta*, an acetotrophic methanogen and another predominant microbial flora is the proteobacteria with genus *Marinobacterium*. Samples from Escravos skimmer pit (1N1) showed some similarity in microbial composition and diversity with those from produced water discharge area (IN3) suggesting that there might be some form of relationship between them. For instance, the same methanogenic archeae that dominated skimmer pit water samples also dominated produced water discharge area

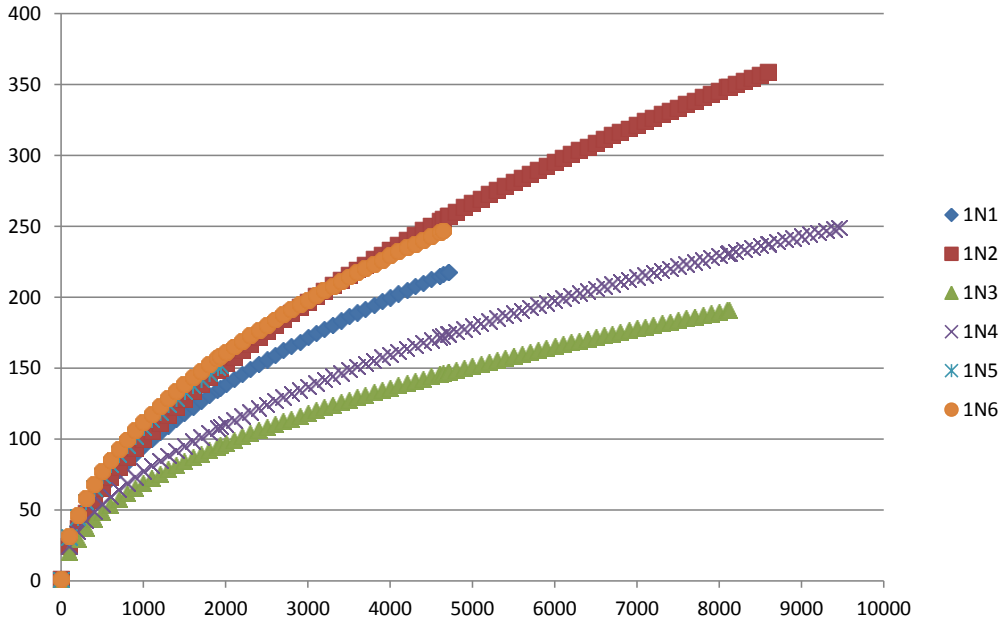

Figure 2. Refractive curves for the 16S rRNA sequence libraries indicated in Figure 3 and Table 3. The data indicate that Meren samples have average more OTUs than Escravos samples (Y= Number of OUT'S, X = Number of sequence reads).

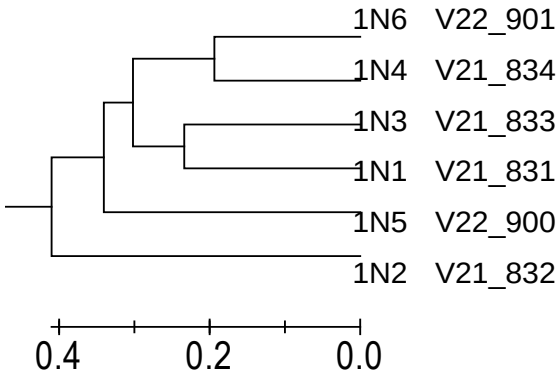

Figure 3. Relationship tree for microbial community composition derived by pyrosequencing of Escravos and Meren samples.

samples). The presence of moderate acetate concentration from Escravos (18.8 to 20.9 mM) may be the reason for observable presence of acetotrophic methanogens in these samples. Comparatively, microbial composition and diversity from Meren samples are considerably different from those from Escravos. For instance, Meren injection water were dominated by Proteobacteria (Deltaproteobacteria) with genus *Delsufuromonas* detected followed closely by Gammaproteobacteria with genus *Marinobacterium* and *Oceanobacter* present). Generally, the partially treated produced water (1N4) and fully treated produced water (IN5) showed some similarity in microbial diversity as shown in Table 3; however, the composition of the microbial flora was differed. While the genus *Marinobacter* was present in sample IN4, *Holomonas* was present in 1N5.

Microbial diversity and composition of oil water mixture (produced oil from well head) from Meren were similar with those of Meren produced water (partially treated and treated). They were dominated by the presence of methanogenic archeae with genus *Methanocalculus*, *Methanolinea*, *Methanomicrobiacea*, *Methanosaeta* and *Methanobolus*. It should be noted that these samples (1N4-1N6) recorded no SRB counts and activities of SRB, hNRB and so-NRB. Methanogenic activities which were not part of the initial culture dependent analysis carried out and which is significant in corrosion and souring related issues was revealed by the culture independent analysis as indicated in Table 3.

Corrosion rates of coupons exposed to Escravos and Meren samples

The corrosivity of four samples (1N1, IN2, IN3 and IN4) which showed the considerable bacterial and archaeal activities was examined as part of a larger experiment in which incubations were labelled CR1 to CR8. Escravos samples are referred to as CR3/1N1 and CR8/1N3 whereas Meren samples are referred to as CR5/1N2 and CR7/IN4 as shown in Figure 4. General corrosion rates were determined by weight loss of coupons suspended in unammended samples under anaerobic conditions with

Table 3. Phylogenetic classification of pyrosequencing reads for samples from Escravos facility and Meren offshore oil field.

Sample name		1N1	1N2	1N3	1N4	1N5	1N6
Type of sample		Skim. Pit	Inj. Wat.	PW/SW	PW	PW	OIL/WAT.
Sequence Code		V21_831	V21_832	V21_833	V21_834	V22_900	V22_901
Total Reads		4711	8597	8119	8454	1937	4640
Number of OTU'S		218	359	191	249	152	247
Number of Taxa		94	185	90	151	112	144
Estimated OTU's (Chao)		461	615	334	410	276	481
Normalized Shanon Index		2.91	2.71	1.90	2.82	3.25	3.27
Entry	**Taxon (Phylum; Class; Order; Family; Genus)**						
1	Actinobacteria; Actinobacteridae; Actinomycetales; Corynebacterineae; Dietziaceae; Dietzia	0.02	2.43	0.07	**1.40**	**3.51**	**2.78**
2	Bacteroidetes	0.30	0.07	0.37	0.11	0.10	0.28
3	Bacteroidetes; Bacteroidia; Bacteroidales; Porphyromonadaceae	0.15	0.02	0.42	0.03	0.10	0.04
4	Bacteroidetes; Bacteroidia; Bacteroidales; Rikenellaceae	0.02	0.04	0.37	0	0	0
5	Candidate_division_OP9	0	0.04	0.53	0.02	0.05	0.09
6	Firmicutes	2.12	1.69	0.54	**2.04**	**2.22**	**4.03**
7	Firmicutes; Clostridia; Clostridiales	0.17	0.65	0.12	0.11	0.21	0.24
8	Firmicutes; Clostridia; Clostridiales; Clostridiaceae; Tindallia	0.55	0.23	0.06	0.09	0	0.19
9	Firmicutes; Clostridia; Clostridiales; Family_XII_Incertae_Sedis; Fusibacter	**2.14**	**3.40**	**1.32**	0.44	0.93	0.75
10	Firmicutes; Mollicutes; Acholeplasmatales; Acholeplasmataceae; Acholeplasma	0.40	0.05	**1.76**	0.49	0.31	0.73
11	Planctomycetes; Phycisphaerae; vadinBA30	0.66	0.01	0.09	0.90	**1.29**	**1.64**
12	Proteobacteria	0.23	0.42	0.04	0.01	0	0.02
13	Proteobacteria; Betaproteobacteria; Burkholderiales; Alcaligenaceae; Castellaniella	0	0.42	0.03	0.20	0.72	0.50
14	Proteobacteria; Betaproteobacteria; Hydrogenophilales; Hydrogenophilaceae; Petrobacter	0.26	0.42	0.21	0.11	0.10	0.24
15	Proteobacteria; Betaproteobacteria; Rhodocyclales; Rhodocyclaceae	**2.40**	0.01	0.51	**1.22**	**2.12**	**2.46**
16	Proteobacteria; Deltaproteobacteria; Desulfobacterales; Desulfobulbaceae; Desulfobulbus	**4.03**	0.66	**3.20**	**1.86**	**1.24**	**4.01**
17	Proteobacteria; Deltaproteobacteria; Desulfovibrionales; Desulfomicrobiaceae; Desulfomicrobium	0.45	**1.47**	0.25	0.14	0.36	0.67
18	Proteobacteria; Deltaproteobacteria; Desulfovibrionales; Desulfovibrionaceae; Desulfovibrio	0.30	0.92	0	0.11	0.05	0.22
19	Proteobacteria; Deltaproteobacteria; Desulfuromonadales	0.45	0.94	0.01	0.03	0.10	0.19
20	Proteobacteria; Deltaproteobacteria; Desulfuromonadales; Desulfuromonadaceae; Desulfuromonas	**4.54**	**30.48**	0.71	**1.08**	**1.03**	**2.05**
21	Proteobacteria; Gammaproteobacteria; Alteromonadales; Alteromonadaceae; Marinobacter	0.26	0.06	0.83	**26.29**	0.26	0.58
22	Proteobacteria; Gammaproteobacteria; Oceanospirillales	0.53	0.50	0.07	0.01	0.05	0
23	Proteobacteria; Gammaproteobacteria; Oceanospirillales; Halomonadaceae	0.02	0.01	0.37	**3.89**	0.57	0.07
24	Proteobacteria; Gammaproteobacteria; Oceanospirillales; Halomonadaceae; Halomonas	**3.40**	0.05	0.01	0.88	**15.02**	0
25	Proteobacteria; Gammaproteobacteria; Oceanospirillales; Oceanospirillaceae; Marinobacterium	**18.70**	**22.02**	**9.96**	0.74	0.67	**1.06**
26	Proteobacteria; Gammaproteobacteria; Oceanospirillales; Oceanospirillaceae; Oceanobacter	**1.95**	**19.65**	**2.08**	0.53	0.41	0.86
27	Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas	0.93	**1.44**	0.01	0.01	**3.67**	0.15

Table 3. Contd.

28	Spirochaetes; Spirochaetes	0.06	0.13	**1.26**	0.02	0.21	0.07
29	Spirochaetes; Spirochaetes; Spirochaetales; Spirochaetaceae; Spirochaeta	**6.22**	0.52	0.60	**1.80**	**3.87**	**5.80**
30	Bacteria	0.76	0.31	0.62	0.50	0.41	0.91
31	Euryarchaeota; Methanobacteria; Methanobacteriales; Methanobacteriaceae; Methanobacterium	0.09	0.07	0.12	0.93	**1.70**	**1.60**
32	Euryarchaeota; Methanobacteria; Methanobacteriales; Methanobacteriaceae; Methanothermobacter	0	0.44	0.01	0.44	**1.29**	0.63
33	Euryarchaeota; Methanomicrobia; Methanomicrobiales; Candidatus; Methanoregula	0.04	0.01	0	0.29	0.67	0.39
34	Euryarchaeota; Methanomicrobia; Methanomicrobiales; Methanocalculus	**8.47**	0.28	**2.78**	**11.45**	**12.44**	**13.49**
35	Euryarchaeota; Methanomicrobia; Methanomicrobiales; Methanolinea	0.06	0.19	0.01	**4.80**	**4.18**	**5.88**
36	Euryarchaeota; Methanomicrobia; Methanomicrobiales; Methanomicrobiaceae	0.36	0.69	0.80	**11.88**	**12.24**	**14.38**
37	Euryarchaeota; Methanomicrobia; Methanosarcinales; Methanosaetaceae; Methanosaeta	**32.97**	**2.80**	**65.38**	**18.42**	**16.88**	**21.68**
38	Euryarchaeota; Methanomicrobia; Methanosarcinales; Methanosarcinaceae; Methanolobus	**1.36**	0.14	**1.02**	**2.57**	**1.86**	**2.61**
	Sum	**95.37**	**93.66**	**96.55**	**95.80**	**90.87**	**91.25**

head space of 90% (v/v), N_2 and 10% (v/v) CO_2. Following incubation for 4 weeks, observable precipitate indicating FeS formation was evident in samples CR3/1N1 and and CR8/1N3 as shown in Figure 4. Filtration of samples through a 0.2 µm filter decreases sulphide formation and associated corrosion in sample CR8/1N3 but not in sample CR3/1N1. Corrosion rates obtained from the measured weight loss of the carbon steel coupons were 0.065 ± 0.0078 (CR3/1N1), 0.037 ± 0.003 (CR5/1N2), 0.026 ± 0.0025 (CR7/1N4), and 0.060 ± 0.0018 (CR8/1N3).

DISCUSSION

It is a well-established scientific fact that oil reservoirs harbour and sustain diverse bacterial and archaeal communities (Zhu et al., 2003; Grabowski et al., 2005; Youssef et al., 2009, Li et al., 2006 and 2007) and multiple groups of microorganisms with diverse physiological and metabolic activities and phylogenetic affiliations have routinely been recovered from oil reservoirs (Voordouw et al., 1996; Orphan et al., 2000; Green and Voordouw, 2003; Jan-Roblero et al., 2004; Magot, 2005; Li et al., 2006, 2007, Dahle et al., 2008; Pham et al., 2009; Okoro et al., 2014). A comprehensive assessment of the diversity, metabolic processes and habitat conditions within a petroleum reservoir is therefore an important component of reservoir management because the knowledge gained can be useful in mitigating ahead of time potential cases of souring and bio-corrosion and can also be used to improve oil production and recovery processes (Li et al., 2012; Youssef et al., 2009).

In the present study, we have used both cultivation based and molecular methods to characterise microbial communities with potential for souring and corrosion after routine biocide treatment in two Nigerian oil production facilities that has been producing oil for the past 20 years with a combined production capacity of about 155,000 barrels per day. Among the six samples that were analysed, we found the highest SRB counts (10^6 cell/ml) and activities in sample 1N1 which was collected from the waste water of skimmer pit where all the liquid wastes from Escravos facility drains into there/it after washing and high temperature flushing of the facility. The physicochemical analysis indicated very low sulphate concentration in the skimmer pit (0.04 mM) suggesting that high SRB population may have depleted the available sulphate with possible generation of hydrogen sulphide which can result to souring (Vance and Trasher, 2005). The pyrosequencing survey indicated that this sample contained about 94 anaerobic taxa including the dominant acetotrophic methanogen, *Methanosaeta*. Another prominent methanogen present is the methylotrophic methanogen, *Methanolobus*. Among the SRB are the predominant *Marinobacterium*, *Oceanobacter*, *Holomonas*, *Desulfuromonas* and *Desulfobolus*. Escravos facility processes crude from Abiteye (Swamp location), Okan (Offshore) and Olero creek (swamp) and the microbial community of the skimmer pit where oily waste drains into is expected to reflect those of Abiteye, Okan and Olero. Compared with samples collected from treated produced water discharge area at

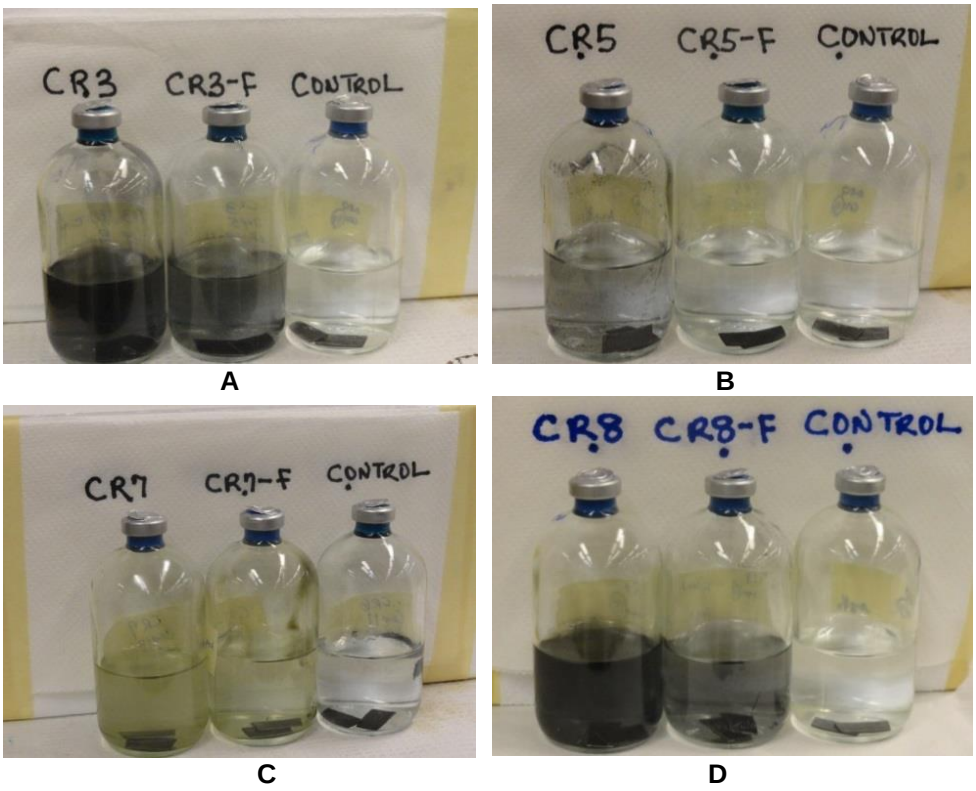

Figure 4. Weight loss corrosion tests with Escravos and Meren samples. A: CR3/1N1 (Escravos skimmer pit); B, CR5/1N2 (Injection water, Meren); C, CR7/1N4 (Produced water, Meren); D. CR8/1N3 (Mixture of produced and seawater). Duplicate iron coupons were incubated under anaerobic conditions (90% N_2, 10% $C0_2$) with 70 ml of sample either used as it is or passed through a 0.2 µm filter (F). The control contained 70ml of filtered deionized water.

Escravos which is represented in sample 1N3, we also observed high SRB counts (10^5 cell/ml) and activity and most microorganisms that were found to be predominant in skimmer pit samples were also found to be predominant in samples collected from produced water discharge area. Some examples include Acetotrophic methanogen, *Methanosaeta* and Methylotrophic methanogen, *Methanolobus*. The observed SRB from the samples were *Oceanobacter*, *Marinobacterium* and Desulfobulbus. The data suggest that the microbial communities of skimmer pit and produced water discharge area at Escravos are similar and possibly represent indigenous microbial communities of Abiteye, Okan and Olero creek. Youssef et al. (2009) advanced that the global presence of specific microbial lineages in geographically isolated oil reservoirs is a good indication of their indigenous nature, on that note we state that most of the indigenous microbial communities from the present study are truly indigenous because they have been isolated elsewhere in oil field environments from different geographical locations around the world for example; *Methanobacteriales* (Faisal et al., 2013), *Methanosaeta* (Dahle et al., 2008), *Methanolobus* (Pham et al., 2009), *Desulfobulbus* (Wilms et al., 2006), *Spirochaeta*, *Desulfomicrobium* (Dahle et al., 2008), *Marinobacter*, *Halomonas* (Van der Kraan et al., 2009), and *Marinobacterium* (Li et al., 2012). These microorganisms have always been associated with corrosion and souring and may have been responsible for the corrosion of metal coupons and subsequent production of hydrogen sulfide under laboratory conditions (Figure 2).

Treatment of Meren injection water with continuous chlorination and alternate application of an amine and gluterldehyde biocide at a concentration of 200 ppm (6 h weekly) significantly reduced SRB population (10^2cell/ml) and activity but pyrosequencing survey reveal that some SRB such as *Desulfuromonas*, *Marinobacterium*, and *Oceanobacter* with potential for souring and corrosion still persists confirming earlier assertion that SRB was difficult to eliminate at Meren field despite continuous biocide treatment (Adetoba, 1985; Lumley et al., 2000). The pyrosequence data also suggests that the major metabolic processes in Meren injection water is sulphate reduction as opposed to methanogenesis that seem to dominate microbial activities in Meren production well and produced water. Grabowski et al. (2005) observed

that methanogenesis is a dominant terminal process in a low temperature biodegraded oil reservoir which Meren facility is close to. The diversity and composition of the microbial community in Meren production well and produced water samples (1N4, IN5 and IN6) are similar. The predominant microbial flora includes an actinobacteria of the genus *Dietzia;* this genus seems not to be popular in oil fields and as such its originality cannot be verified. Others include the family Rhodocyclaceae, genus *Desulfobulbus*, *Desulfuromonas* and Spirochaeta among the bacterial community and Methanocalculus, *Methanolinea*, *Methanomicrobiaceae*, *Methanosaeta* and *Methanolobus* among the archaeal community as indicated in Table 3. Most of these organisms are indigenous to oil fields and some have been associated with souring and corrosion in the past (Dahl et al., 2008; Pham et al., 2009; Wilms et al., 2006; Li and Hendry, 2008; Okoro et al., 2014).

In summary, the pyrosequencing survey of the two oil facilities investigated indicate that sample IN1 was dominated by sequences affiliated with Euryarchaeota (44%), and Proteobacteria (39%) indicating methanogenesis and sulphate reduction while 1N2 was dominated by sequences affiliated with Proteobacteria (82%) indicating only sulphate reduction. Other samples and their respective dominant phyla include; 1N3 (Euryarchaeota, 70.79%), IN4 (Euryarchaeota, 51.78% and Proteobacteria, 38%), IN5 (Euryarchaeota, 52% and Proteobacteria, 28%), and IN6 (Euryarchaeota, 62.6%). It has been advanced that both Proteobacteria and Euryarchaeota have capabilities for sulphate reduction (Youssef et al., 2009; Magot et al., 2000); therefore, it is expected that the major metabolic activities in these fields where Proteobacteria and Euryarchaeota are present will be sulphate reduction where sulphate is present but in the absence of sulphate or nitrate, water mediated fermentation of oil organics to methane and carbon dioxide becomes a dominant process (Grabowski et al., 2005; Gray et al., 2011; Pham et al., 2009; Youssef et al., 2009; Zengler et al., 1999). Laboratory investigation using weight loss method and microbial activity showed that the microbial communities from both Escravos and Meren samples were able to induce corrosion and souring.

In conclusion, we advance that this investigation on microbial diversity and metabolic processes of Escravos oil processing and loading facility and Meren offshore fields after routine biocide treatment have expanded substantially our knowledge on the extent of microbial diversity in these fields with potential to induce souring and corrosion despite regular biocide treatments and have also high lightened the abundance and complexity of microbial community that have potential to tolerate biocides and initiate souring and bio-corrosion in these fields. Future research efforts should therefore focus on the biocidal efficacy of the biocides currently used to mitigate corrosion and souring related problems in these fields.

Conflict of Interests

The authors have not declared any conflict of interests.

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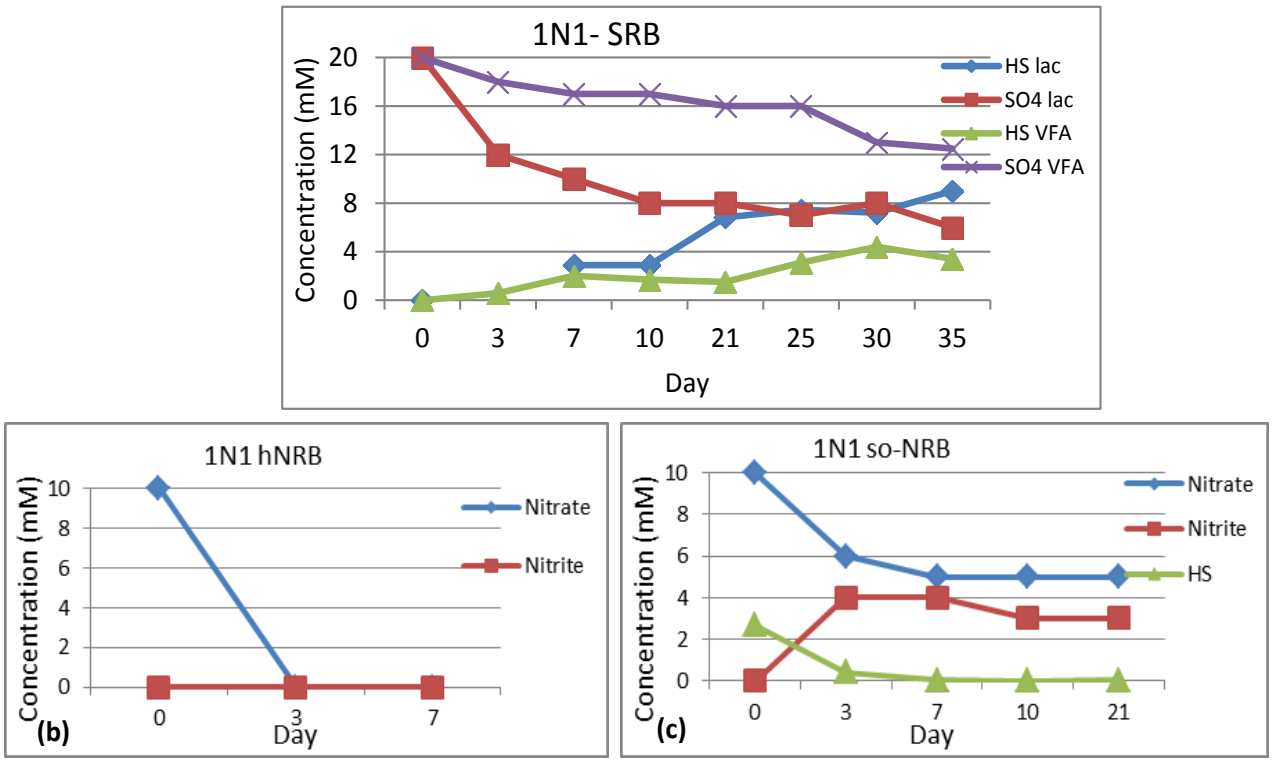

Figure S1. Microbial activities in sample 1N1 (Skimmer Pit) for (a) SRB activities showing sulfide and sulfate concentrations in both lactate and VFA media; (b) heterotrophic, nitrate-reducing bacteria (hNRB) showing nitrate and nitrite concentrations; (c) Sulfide-oxidizing, nitrate-reducing bacteria (so-NRB) showing sulfide, nitrate and nitrite concentrations.

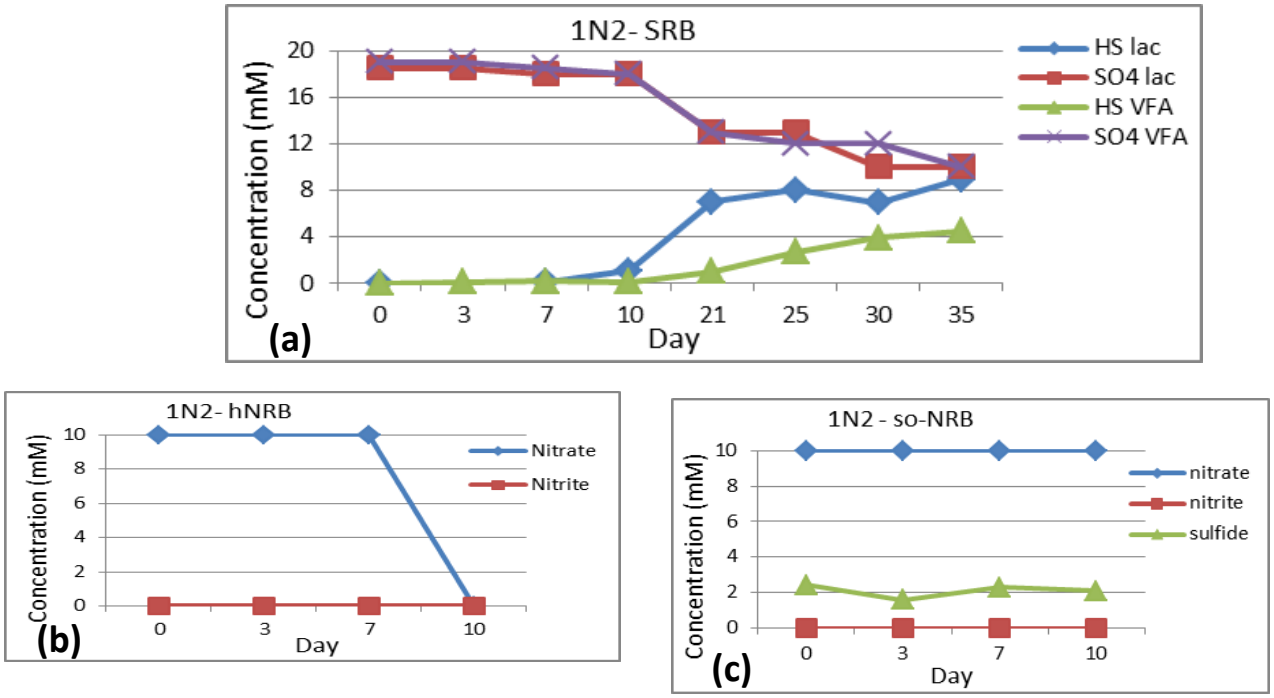

Figure S2. Microbial activities in sample 1N2 (Injection water) for (a) SRB activities showing sulfide and sulfate concentrations in both lactate and VFA media; (b) heterotrophic, nitrate-reducing bacteria (hNRB) showing nitrate and nitrite concentrations; (c) Sulfide-oxidizing, nitrate-reducing bacteria (so-NRB) showing sulfide, nitrate and nitrite concentration.

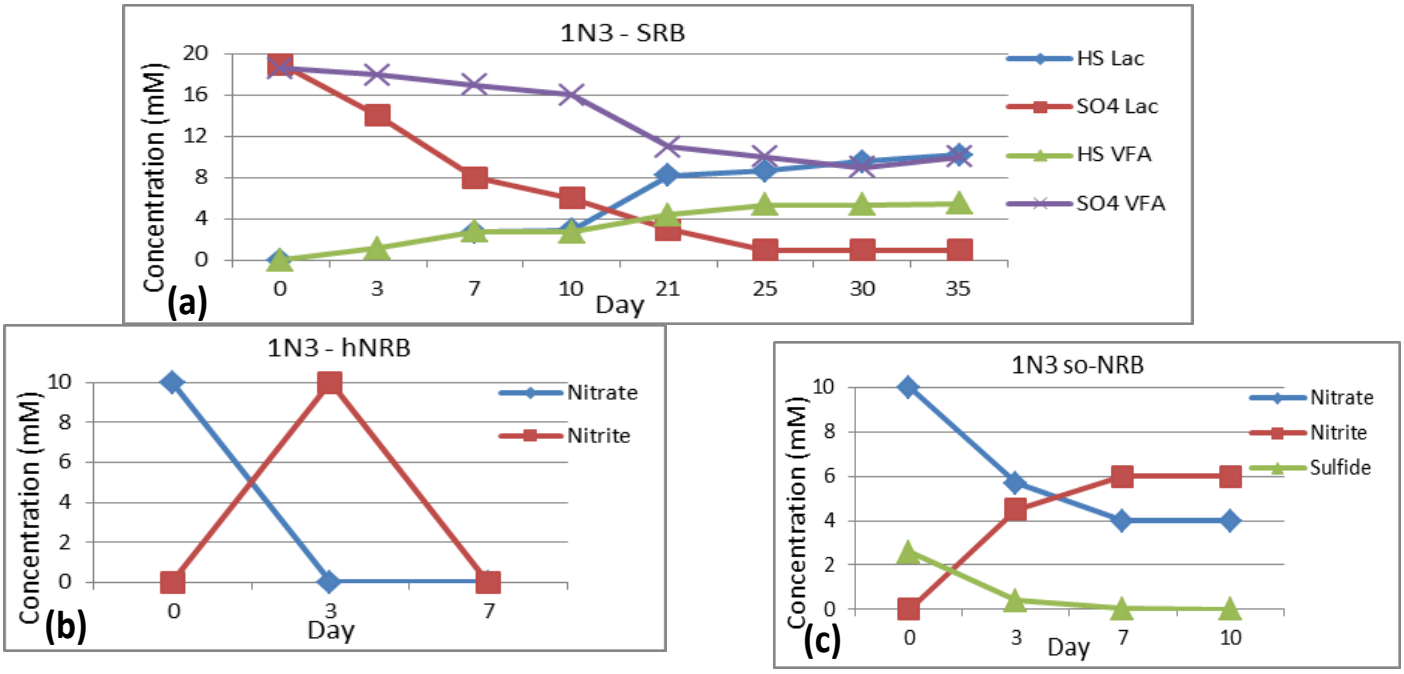

Figure S3. Microbial activities in sample 1N3 (Escravos sea water/Produced water mixture) for (a) SRB activities showing sulfide and sulfate concentrations in both lactate and VFA media; (b) heterotrophic, nitrate-reducing bacteria (hNRB) showing nitrate and nitrite concentrations; (c) Sulfide-oxidizing, nitrate-reducing bacteria (so-NRB) showing sulfide, nitrate and nitrite concentrations.

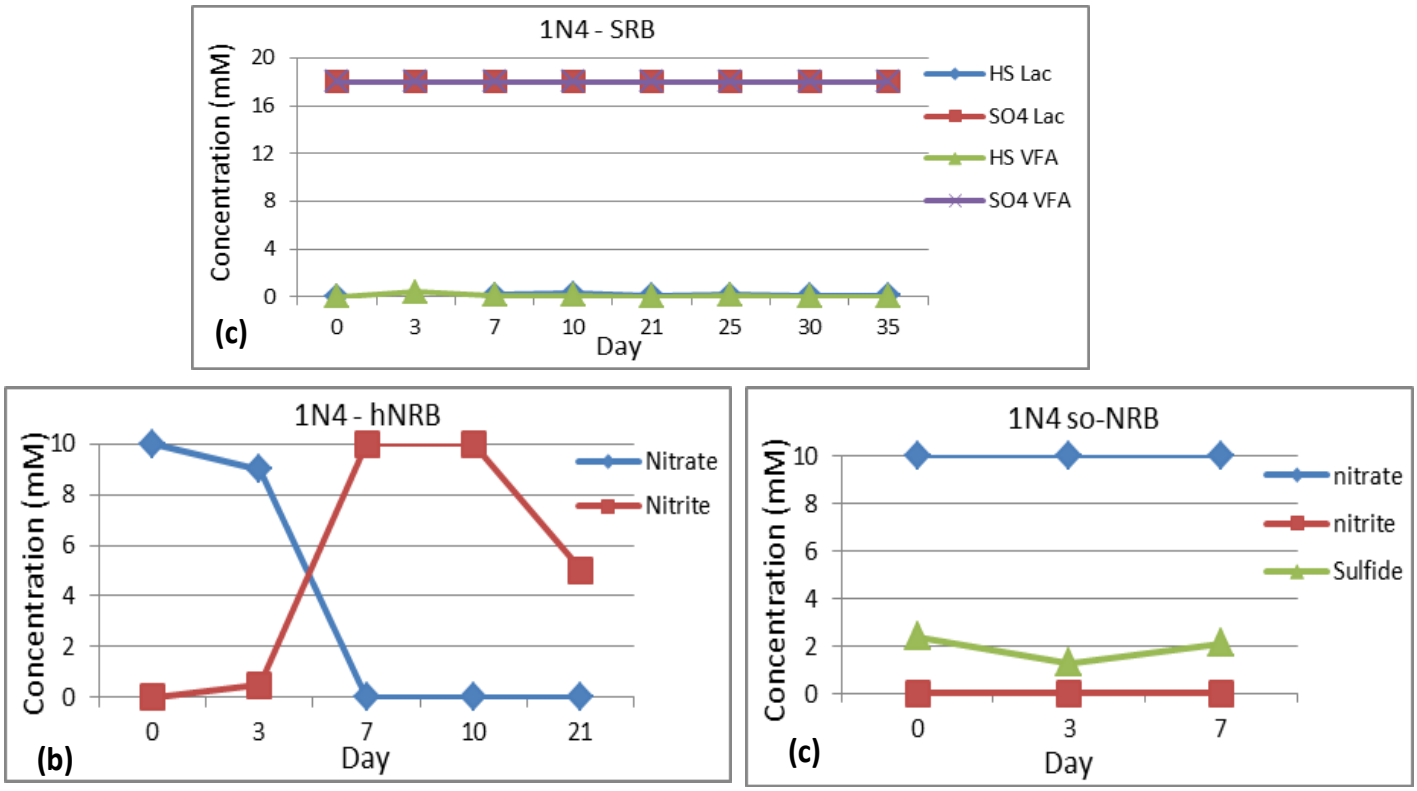

Figure S4. Microbial activities in sample 1N4 (Partially treated produced water) for (a) SRB activities showing sulfide and sulfate concentrations in both lactate and VFA media; (b) heterotrophic, nitrate-reducing bacteria (hNRB) showing nitrate and nitrite concentrations; (c) Sulfide-oxidizing, nitrate-reducing bacteria (so-NRB) showing sulfide, nitrate and nitrite concentrations.

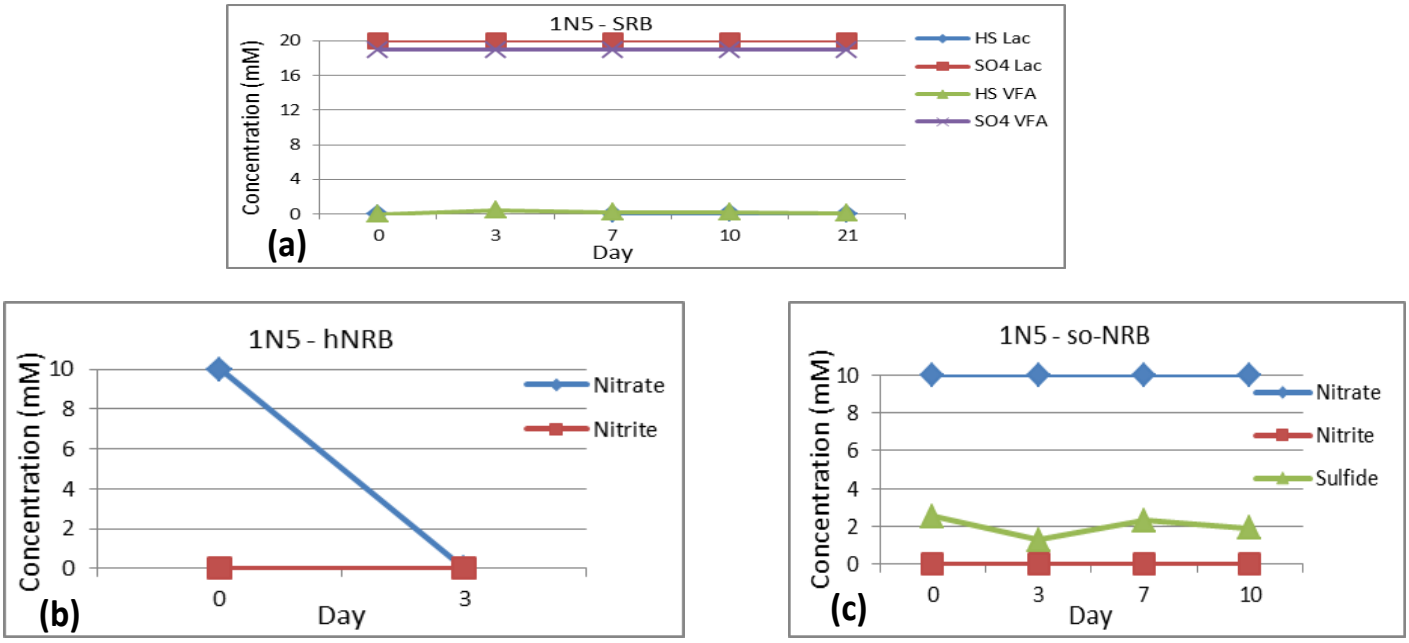

Figure S5. Microbial activities in sample 1N5 (Treated produced water) for (a) SRB activities showing sulfide and sulfate concentrations in both lactate and VFA media; (b) heterotrophic, nitrate-reducing bacteria (hNRB) showing nitrate and nitrite concentrations; (c) Sulfide-oxidizing, nitrate-reducing bacteria (so-NRB) showing sulfide, nitrate and nitrite concentrations.

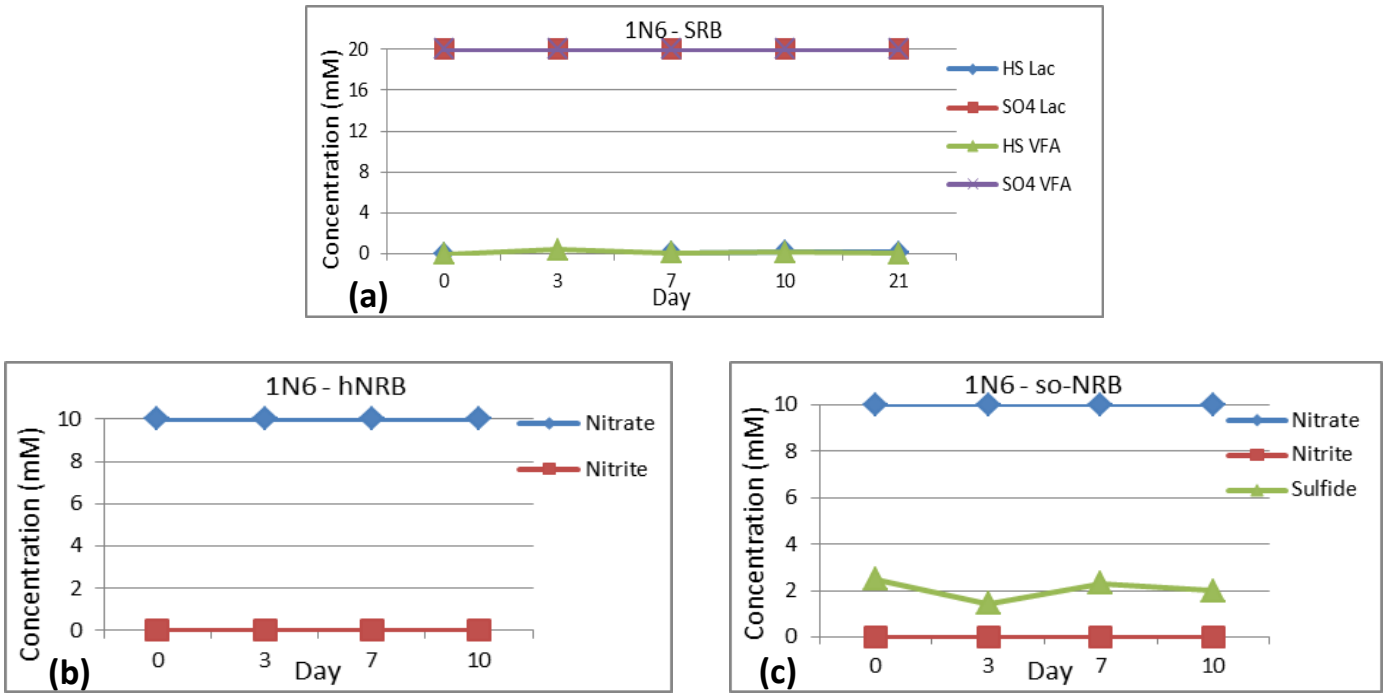

Figure S6. Microbial activities in sample 1N6 (Oil/water mixture from Meren production well) for (a) SRB activities showing sulfide and sulfate concentrations in both lactate and VFA media; (b) heterotrophic, nitrate-reducing bacteria (hNRB) showing nitrate and nitrite concentrations; (c) Sulfide-oxidizing, nitrate-reducing bacteria (so-NRB) showing sulfide, nitrate and nitrite concentrations.

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Microbiological quality of selected dried fruits and vegetables in Maseru, Lesotho

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Food safety is a global issue, affecting food production and processing. The study assessed the microbiological quality of commercially dried fruits and home dried fruits and vegetables in Lesotho. Moisture content, pH and water activity of the fruits and vegetables were determined using standard methods. Nine different growth media were used for microbial evaluation. Moisture content and water activity were within World Health Organisation guidelines for dried fruits and vegetables. Fungi counts ranged from $2.0x10^2$ to $8.7x10^5$ CFU g^{-1}, and dried pumpkin leaves recorded the highest. More than 45% and 38% of the samples exceeded the fungal and total aerobic counts recommended by WHO, respectively. Possible pathogens of the genera *Salmonella*, *Shigella*, *Bacillus* and other Enterobacteriaceae were isolated from home dried samples. Faecal coliforms were detected in 55% of the home dried food products. More than 60% of the samples recorded higher microbial levels than recommended. While half of commercially dried fruits exceeded international standards, all home dried fruits and vegetables recorded unacceptably high levels of fungal contamination. The presence of possible pathogenic organisms in these foodstuffs suggest a potential public health hazard to consumers. Sanitation and personal hygiene, especially during home-based food processing, needs improvement.

Key words: Dried fruits, food-borne illness; food safety, guideline, low moisture foods, microbial quality, vegetables

INTRODUCTION

Despite foods being an important source of nutrients to consumers, it serves as an excellent medium for microbial growth, some of which are pathogenic. Food safety is a global issue, affecting food production and processing.

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Food-borne illnesses are reported to be a major international problem and continue to be a public health concern, especially in developing countries where food standards, regulations and safety policies are not well established or are seldom in place (De Sousa, 2008; Food Safety Programme, 2002; Mensah et al., 2002). Despite the health risk and widespread concern of food-borne infections, only a fraction of them are diagnosed and reported, or can be traced to a definite source (Lukinmaa et al., 2004).

Fruits and vegetables are an important part of a healthy and balanced diet in all societies around the globe. They are known to carry natural non-pathogenic epiphytic microbiota but can be contaminated with pathogens from human, animal or environmental sources during growth, harvest, transportation, processing and handling (Beuchat, 2002; Olsen et al., 2000). While the prevalence of food-borne pathogens on dried fruits and vegetables and their involvement in food-borne outbreaks are not well documented, fresh fruits have been implicated in a number of documented outbreaks of food-borne illnesses particularly in Europe, Japan, United States and Canada (ECSCF, 2002; Erickson and Doyle, 2012). The major causes of the illnesses in the outbreaks were bacteria, viruses and parasites.

Drying is one of the oldest and most common methods of processing and preserving food. Home food drying remains popular as an alternative to canning (Wilhelm et al., 2004) in developing countries such as Lesotho, where cooling and suitable storage facilities are scarce. Fresh fruits and vegetables are highly perishable due to their high water content of about 80% (Kaleta et al., 2013; Karam et al., 2016). Drying fruits and vegetables does not only inhibit the growth of spoilage microorganisms, but also halts the occurrence of browning and other moisture-driven deterioration reactions and thus, preserving the structure, characteristics and nutritional value of the original material (Karam et al., 2016). The drying of fruits and vegetables, more importantly, minimises losses, enhances storage stability, reduces packaging and handling requirements, and makes transportation easier and cheaper because of reduced weight and volume (Kaleta et al., 2013; Karam et al., 2016). However, traditional sun drying is often a slow process and this increases the chances of microbial contamination (Karam et al., 2016).

Foods, such as fruits and vegetables, are sliced to increase surface-area to volume ratio for the loss of moisture during drying. Slicing is often done in the open with bare hands using a knife. The pieces are then dried in the open on the sun where they can come in contact with microorganisms, dirt, soil and insects and this sub-jects the products to possible microbial contamination. The food slices are left in full sun for some days until they have lost much of the moisture. Furthermore, some of the dried food products, especially fruits, are often consumed without any further processing steps such as cooking. Over and above these factors, proper hygienic practices are often limited in most rural areas of developing countries (Vivas et al., 2010). Furthermore, the difficulty in significantly reducing microbial hazard contamination of dried foods by traditional processing methods such as heating has been reported (Beuchat et al., 2013; Finn et al., 2013). On the contrary, commercial drying of fruits is done in protected environments and often, under more hygienic conditions.

Dried fruits and vegetables form one of the seven categories of low moisture foods (LMF) that were assessed by the Food and Agriculture Organization (FAO) of the United Nations and the World Health Organization (WHO), following concerns raised on their microbial quality (FAO/WHO, 2014). The FAO/WHO (2014) define low moisture foods (LMF) as foods that are naturally low in moisture content or are produced from higher moisture foods through drying or dehydration processes. The low water activity (a_w) of these foods prolongs their shelf life and, has for many years, promoted the perception that these foods are microbiologically safe (FAO/WHO, 2014; Finn et al., 2013).

Given that drying of fruits and vegetables prohibits microbial growth, very little attention has been given to the safety of home dried foods until recently. Never-theless, notwithstanding the fact that micro-organisms cannot grow in these LMF, recent outbreaks of foodborne illnesses linked to these food products have demonstrated that pathogenic microorganisms in LMF can persist for long periods of time even under conditions of low a_w (Beuchat, 2002; Beuchat et al., 2013; FAO/WHO, 2014; Finn et al., 2013; Prescott et al., 2001). These micro-organisms have the potential to cause illness due to their low infectious dose or possible subsequent favourable temperature that allows the organism to grow (Beuchat et al., 2013; Finn et al., 2013).

Generally, growth of bacteria and fungi is confined to water activity (a_w) levels above 0.90 (Lund et al., 2000). Lund et al. (2000) observe that pathogenic bacteria grow best at a_w levels of at least 0.98, which is the range in which fresh fruits and vegetables are found. Unlike bacteria that cannot grow below 0.85 a_w, fungi are more tolerant to reduced a_w and can grow even below 0.80 (Lund et al., 2000; Sagar and Suresh, 2010). While, the lowest a_w at which growth of bacteria has been reported was 0.75, fungal growth has been recorded at a_w levels as low as 0.61 (Lund et al., 2000).

Leistner (1992) report that moisture content and water activity (a_w) of artificially dried fruits and vegetables range from 15 to 50% and 0.60 to 0.89, respectively. These conditions can only cause dormancy in bacteria and fungi until a favourable environment is attained (Prescott et al., 2001; Sagar and Suresh, 2010). Microbial counts and pathogens higher than international stipulated limits (10^3 $CFUg^{-1}$ for fungi and 10^1 $CFUg^{-1}$ for bacteria) in both commercially and conventionally produced fresh, and

Table 1. Type and number of samples collected for commercially fruits and home dried fruits and vegetables.

Commercially dried	Number of samples	Home dried	Number of samples
Peeled peaches	20	Peeled peaches	20
Mangoes	20	Unpeeled peaches	20
Prunes	20	Apples	20
Pears	20	Spinach	15
Apricots	20	Traditional vegetable (*Wahlenbergia androsacea)*	15
Raisings	23	Pumpkin leaves	15
Dates	20	-	–
Guavas	21	-	–
Total	**164**		**105**

dried fruits and vegetables have been reported (Barkai-Golan and Paster, 2011; Johannessen et al., 2002; Mensah et al., 2002; Witthuhn et al., 2005). Pathogenic strains of *Salmonella* and *Escherichia coli* have been isolated from home dried food products (Beuchat et al., 2013; Finn et al., 2013). Studies in South Africa on the microbiological content of high moisture dried fruits showed the presence of pathogenic species such as *Salmonella*, *Staphylococcus*, *Clostridium* and high levels of fungi (Unicomb et al., 2005; Witthuhn et al., 2005).

Extensive scientific progress and technological developments in food processing has been achieved in the past few decades. Nonetheless, food safety problems remain a global challenge and may actually increase in future due to increase in food demand and the associated production. In response to the increasing number of food-borne illnesses, governments all over the world are intensifying their efforts to improve food safety (Thyagarajan and Ashok, 2014).

While the consumption of dried food products and their risk to human health increased globally, concerns have been raised on the microbial quality of the products (FAO/WHO, 2014). There has also been global recognition of the need to consider and manage the microbiological hazards associated with these products. A review on the prevalence of pathogenic microbial contamination (>10^3 CFU g^{-1}) in dried fruits and vegetables reported a global prevalence of about 4.84% (FAO/WHO, 2014). Subsequently, several regulatory authorities worldwide have developed recommendations and guidelines to prevent and manage potential risks of this product contamination from microbial hazards.

Conventional home drying of fruits and vegetables is practised in Lesotho where home drying processing of food is done under poor hygienic practices due to lack of education and awareness and, to a greater extent, lack of food safety polices and legislation. Despite home drying being common for food preservation in the country, the quality of home dried food is not monitored. In addition, Lesotho has limited capacity to effectively monitor food manufacturers and suppliers to ensure the bacteriological and chemical safety of the food as well as quality assurance (WHO, 2009). This, coupled with poor hygienic practices, creates high potential risk of microbial contamination and possible transmission of pathogenic microorganisms to humans. However, Lesotho imports most, if not all of its commercially dried fruits (high moisture dried fruits) and vegetables. The speculation that the presence of pathogens in dried fruits and vegetables in the markets in the country is prevalent has not been investigated. The main aim of this study was to assess the microbial quality and safety of commercially dried fruits and home dried fruits and vegetables sold in supermarkets and informal markets around the city of Maseru, Lesotho.

MATERIALS AND METHODS

The study was conducted in the city of Maseru. Commercially dried fruits (within the best-before-date) were bought from supermarkets and home dried fruits and vegetables were bought from the informal market around the city. Random sampling was used to collect samples for four weeks in February 2014. Samples were collected under normal purchase conditions and immediately packaged in sterile polythene bags that were sealed and transported to the laboratory in cooler boxes containing ice packs where they were analysed immediately or stored at 4°C until analysis. Table 1 presents the type of fruits and vegetables, and number of samples collected for each type of product. All analyses were done within 48 h of sample collection.

Moisture content (%) and pH of the samples were determined using a HANNA, HI 8314 membrane pH meter according to methods described in 923.03 of AOAC International (AOAC, 2000). Water activity (a_w) for the samples was measured using a labMaster - a_w instrument (USA) at 25ºC ± 2ºC as described by Witthuhn et al. (2005). Microorganisms were enumerated and isolated using nine different selective media that are presented in Table 2. The sample (20 g) was suspended in 80 ml of sterile 0.1% peptone water (Oxoid CM009) and homogenized for 2 min using a stomacher blender (lab-blander 400) in a sterile stomacher bag. Further serial dilutions (10^{-1} to 10^{-5}) were prepared and plated in triplicate onto each specific medium. Aliquots, 0.1 ml and 1 ml, of each dilution were used for spread plating and pour plating respectively, into the various media (APHA, 2001). Furthermore, the methods used by Ntuli et al. (2013) and Witthuhn et al. (2005) were adopted and followed for the enumeration and isolation of bacteria and fungi in this study.

Table 2. Methods for the enumeration and isolation of microorganisms in fruits and vegetables.

Media	Organism	Temperature and time of incubation
Potato dextrose agar (PDA)	Fungi	25°C for five days
Plate count agar (PCA)	Total aerobic counts	37°C for 24 h
Violet red bile agar (VRBA)	Total coliform	37°C for 24 h
Faecal coliform agar (FCA)	Faecal coliforms	44°C for 48 h
Eosin methylene blue agar (EMBA)	*E. coli*	37°C for 24 h
Reinforced clostridial agar (RCA)	*Clostridium* spp.	37°C for 24 h
Bacillus cereus agar (BCA)	*Bacillus* spp.	37°C for 48 h
Brilliant green agar (BGA)	*Salmonella* spp.	37°C for 24 h
Salmonella –Shigella agar (SSA)	*Salmonella* and *Shigella* spp.	37°C for 24 h

All media used were from Merck South Africa. Method was adopted from Witthuhn et al. (13).

Table 3. Physico-chemical parameters (mean ± SE) of commercially dried and home dried fruits and vegetables (n=280). SE=standard error.

Parameters	Sample	pH	Moisture (%)	A_w
Commercially dried	Peeled peaches	3.81±0.11	17.41±0.21	0.81±0.07
	Mango	4.00±0.01	29.90±5.90	0.82±0.04
	Prunes	3.82±0.02	24.64±0.70	0.83±0.03
	Pears	3.41±0.02	23.41±0.28	0.82±0.11
	Apricot	3.37±0.68	21.85±0.14	0.79±0.04
	Raisings	3.85±0.04	15.78±0.21	0.78±0.05
	Dates	4.63±0.02	21.25±0.70	0.82±0.15
	Guavas	4.37±0.04	12.95±0.84	0.83±0.03
Home dried	Peeled peaches	4.53±0.02	19.0±0.02	0.82±0.13
	Unpeeled peaches	4.12±0.01	20.0±0.01	0.84±0.01
	Apples	4.03±0.10	19.85±0.10	0.83±0.02
	Spinach	6.69±0.04	8.75±0.04	0.78±0.03
	Traditional vegetable	6.74±0.01	7.35±0.01	0.79±0.02
	Pumpkin leaves	8.27±0.40	8.05±0.040	0.71±0.01

Triplicate determinations were carried out and standard errors were calculated for all microbial counts. All data collected were analyzed using one way analysis of variance (ANOVA) to determine significant differences ($p < 0.05$) among the means. All statistical tests were carried out using the SPSS for Windows Version 16.0 package by SPSS Inc., USA.

RESULTS

The physico-chemical characteristics of commercially dried fruits and home dried fruits and vegetables are presented in Table 3. Moisture content (%) and pH differed significantly ($P<0.05$) among the samples. However, there was no significant difference in the water activity of the samples.

The moisture content (%), pH and a_w ranged from 12.95±0.84 to 29.90±5.90, 3.37±0.68 to 4.63±0.02 and 0.78±0.05 to 0.83±0.03, respectively for the commercially dried samples and from 7.35±0.01 to 20.0±0.01, 4.03±0.10 to 8.27±0.40 and 0.71±0.01 to 0.84±0.01, respectively for the home dried samples. Albeit home dried vegetables recording low moisture content, they had high pH values with the highest, 8.27±0.40, recorded in pumpkin leaves. Unpeeled peaches recorded the highest a_w value (0.84±0.01).

Table 4 presents the microbial content (mean ± SE) of the commercially dried fruits and home dried fruits and vegetables. Commercially dried fruits contained fungi and viable aerobic bacteria only. Fungi counts for commercially dried fruits ranged between $2.0x10^2$± 32 and $6.3x10^3$±90 CFU g^{-1}, while total viable (PCA) counts ranged from $5.4x10^1$± 0.6 to $1.0x10^3$ ±100 CFU g^{-1}. Commercially dried guavas recorded the highest fungi counts (6.3×10^3±90 CFU g^{-1}) while mangoes had the highest total viable counts (PCA) of $1.0x10^3$±100 CFU g^{-1}. Similarly, Table 4 indicates that *Bacillus spp.* was present only in home dried peeled peaches ($2.0x10^1$ CFU g^{-1}) and apples ($4.5x10^1$ CFU g^{-1}). The home dried peeled

Table 4. Microbial content (mean ± SE) (CFU g^{-1}) of different commercially dried fruits and home dried fruits and vegetables.

	Sample	PDA	PCA	VRBA	FCA	EMBA	RCA	BCA	BGA	SSA
Commercially dried	Peeled peaches	$2.6\times10^2 \pm 10$	$2.4 \times 10^2 \pm 15$	0	0	0	0	0	0	0
	Mangoes	$4.2 \times 10^3 \pm 69$	$1.0 \times 10^3 \pm 100$	0	0	0	0	0	0	0
	Prunes	$3.8 \times 10^3 \pm 89$	54 ± 6	0	0	0	0	0	0	0
	Pears	$2.0 \times 10^2 \pm 32$	$2.0 \times 10^2 \pm 85$	0	0	0	0	0	0	0
	Apricots	$7.2 \times 10^2 \pm 88$	59 ± 4	0	0	0	0	0	0	0
	Raisins	$1.0 \times 10^3 \pm 20$	$2.6 \times 10^2 \pm 7$	0	0	0	0	0	0	0
	Dates	$4.6 \times 10^3 \pm 10$	79 ± 0.89	0	0	0	0	0	0	0
	Guavas	$6.3 \times 10^3 \pm 90$	$1.3 \times 10^2 \pm 5$	0	0	0	0	0	0	0
Home dried	Peeled peaches	$4.0 \times 10^2 \pm 18$	$3.0 \times 10^2 \pm 17$	0	0	0	0	$2.0 \times 10^1 \pm 1$	0	0
	Unpeeled peaches	$3.1 \times 10^3 \pm 104$	$1.0 \times 10^3 \pm 60$	0	0	0	0	0	0	0
	Apples	$3.9 \times 10^2 \pm 8$	$2.0 \times 10^2 \pm 10$	0	0	0	0	$4.5 \times 10^1 \pm 2$	0	0
	Spinach	$6.5 \times 10^5 \pm 1000$	$9.2 \times 10^5 \pm 896$	$5.6 \times 10^6 \pm 100$	$8.9\times10^1 \pm 3$	$9.0 \times 10^1 \pm 6$	0	$6.1 \times 10^1 \pm 3$	0	0
	W. androsacea	$9.4 \times 10^5 \pm 667$	$5.4 \times 10^6 \pm 1100$	$3.5 \times 10^6 \pm 350$	6.5×10^1	3.2×10^2	0	$5.6 \times 10^1 \pm 2$	10 ± 1	0
	Pumpkin leaves	$8.7 \times 10^5 \pm 200$	$6.7 \times 10^6 \pm 790$	$7.7 \times 10^7 \pm 2980$	$4.0 \times 10^4 \pm 500$	$2.9 \times 10^2 \pm 40$	0	$5.7 \times 10^1 \pm 2.5$	$3.0\times10^1 \pm 2$	6 ± 0.1

Traditional vegetable = *Wahlenbergia androsacea*.

peaches recorded higher counts for fungi ($4.0\times10^2 \pm 10$ CFU g^{-1}) than commercially dried peeled peaches ($2.6\times10^2 \pm 10$ CFU g^{-1}).

There was no *E. coli* and coliforms in home dried fruits. However, very high levels of coliforms ($5.6\times10^6 \pm 100$, $3.5\times10^6 \pm 350$, $7.7\times10^7 \pm 2980$) and faecal coliforms ($8.9\times10^1 \pm 3$, 6.5×10^1, $4.0\times10^4 \pm 500$) were found in dried spinach, traditional vegetable (*Wahlenbergia androsacea*) and pumpkin leaves, respectively. More than 45% and 38% of commercially and home dried samples were above the World Health Organisation (Food Safety Programme, 2002) recommended limits in terms of fungi counts and total aerobic counts, respectively.

Possible pathogens of the genus *Salmonella*, *Shigella*, *Bacillus* and other Enterobacteriaceae were isolated from home dried samples. However, the pathogenicity of the organisms was not confirmed in this study. Faecal coliforms were detected in 55% of the home dried fruits and vegetables. *Bacillus spp.* were isolated in home dried peeled peaches, apples, spinach, traditional vegetable and pumpkin leaves and the counts were 2.0×10^1, 4.5×10^1, 6.1×10^1, 5.6×10^1 and 5.7×10^1 CFU g^{-1}, respectively.

DISCUSSION

Water activity levels and pH values for dried raisins, peaches, apricots and prunes were comparable to those recorded by Witthuhn et al. (2005) in South Africa. However, the current study recorded much lower levels of moisture than those found in South Africa. Moisture content and water activity of dried fruits and vegetables met the WHO guidelines. Microorganisms present in dried fruits and vegetables are directly related to the water used and the hygienic conditions practised during their cultivation, harvesting, post-harvest handling, processing and distribution of the produce (Beuchat, 1996; Halablab et al., 2011). Consequently, these microorganisms on fruits and vegetables may act as a reservoir which may be responsible for further post-harvest contamination, if not reduced or eliminated (Barth et al., 2010; Joint FAO/WHO Codex Alimentarius Commission, 1994).

Results presented in Table 4 are in line with the guidelines by Gilbert et al. (2000). According to Gilbert et al. (2000), total aerobic count (TAC) of $<10^2$ CFU g^{-1}, total coliform (TC) $<10^4$ CFU g^{-1} and faecal coliform (FC) counts of $<10^2$ CFU g^{-1} are acceptable. Accordingly, the bacteriological quality (CFU g^{-1}) based on total aerobic count (TAC), TC and FC for all commercially dried selected produce

were within acceptable levels. For commercially dried fruits, TAC values were ranging from $5.4x10^1$ to $1x10^3$ CFU g^{-1} and no TC and FC counts were detected on VRBA and FCA. More importantly, TAC and FC are real indicator organisms (that is, for hygiene and sanitary conditions) and for this reason their presence in high numbers in dried fruits and vegetables, as observed in this study, implies poor hygiene and sanitary conditions during processing (Oranusi and Braide, 2012).

Absence of FC and TC in commercially dried fruits (Table 4) may signify good hygienic and handling practices. Generally, this is an indication of minimum adherence to Good Health Practices (GHP) and Good Manufacturing Practices (GMP) applied to commercially dried fruits and vegetable products as stipulated by the Joint FAO/WHO Codex Alimentarius Commission (1994). Furthermore, the low pH (3.41 - 4.63) of the food products (Table 3) may also explain the absence. Absence of the possible microbial pathogens can also be attributed to the fact that, unlike home dried foods, many commercially dried food products undergo specific pathogen reduction treatments to reduce potential hazards for consumers (FAO/WHO, 2014). In contrast, a study (Witthuhn et al., 2005) in South Africa, from which most if not all commercially dried fruits are imported, and others in other parts of the region (Boyacioglu and Gönül, 1990; Olsen et al., 2000) revealed presence of pathogenic microorganisms and their toxins in these products. These studies identified species such as *Salmonella, E. coli, Staphylococcus, Clostridium,* and high levels of fungi.

The commercially produced food products analysed were mostly fruits such as peaches, mangoes, prunes, pears, apricots, raisins, dates and guavas. Typically, fruits have low pH (Uzeh et al., 2009) since fruits increase in acidity as they ripen and this may not favour growth of pathogenic microorganisms (see Table 4 under commercially dried products), although some moulds and yeasts can endure such high acidity. Thus, the considerable counts of TAC (5.4×10^1 - 1.0×10^3 CFU g^{-1}) and fungi (2.0×10^2 - 6.3×10^3 CFU g^{-1}) in these fruits could be due to the high a_w (0.78 - 0.83). Witthuhn et al. (2005) report that fungi are the main causes of the spoilage of dried high-moisture fruits. Additionally, Sagar and Suresh, (2010) observe that fungi are more tolerant to reduced water activity than bacteria and grow even below 0.80. Generally, food products with high a_w have substantial amount of unbound water molecules that support growth and survival of microorganisms favourably (El-Halouat et al., 1998; Ferrati et al., 2005).
Unhygienic conditions, combined with poor sanitary environments could account for the presence of pathogenic microorganisms in home dried fruits and vegetables. For instance, slicing of the fruits and vegetables for drying is often done in the open and with bare hands and this subjects the products to possible microbial contamination. The pieces are also dried in the open in full sun for some days, which further exposes the products to possible contamination. However, results for unpeeled peaches and apples, in which FC and TC were not detected, were in line with Gilbert et al. (2000) guidelines. Equally, TAC values for these fruits were within the acceptable levels recommended by Gilbert et al. (2000). Within these produce, TAC ranged from $2.0x10^2$ to $1x10^3$ CFU g^{-1}. In contrast, leafy vegetables such as spinach, traditional vegetable and pumpkin leaves were highly contaminated with TAC, followed by TC and FC. While TAC ranged from $9.2x10^5$ to $6.7x10^6$ CFU g^{-1}, TC ranged from $3.5x10^6$ to $7.7x10^7$ CFU g^{-1} and FC from $6.5x10^1$ to $4.0x10^4$ CFU g^{-1}. Verma and Joshi (2000) highlight that dried vegetables tend to record higher levels of contamination than dried fruits. The low a_w, coupled with the high sugar and/or fat content in these vegetables, can enhance the survival and heat resistance of microorganisms in these foods (Beuchat et al., 2013; Finn et al., 2013).

These results concur with the findings by Amoah et al. (2006), who report heavy faecal coliform contamination in vegetables (ranging from 4.0×10^3 to 9.3×10^8 MPN/g) in Ghana. Halablab et al. (2011) and Mensah et al. (2002) ascribe microbial contamination in vegetables to sources such as soil, manure, water and poor post-harvest handling and storage. Significantly, leafy vegetables such as spinach, traditional vegetable and pumpkin leaves grow closer to the ground, hence are more easily contaminated from soil microorganisms as compared to apples and peaches which are high up the tree bushes, far away from soil microbial contaminants (Bello et al., 2014). Generally, if the soil has been treated with poor quality animal manure as fertilizer or irrigated with contaminated water, vegetables are also likely to be affected.

According to Beuchat (2006), vegetables are among the food groups implicated with greater frequency in recent years, in causing enteric diseases through use of treated or fully composited animal manure (Johannessen et al., 2002). Irrigation of vegetables through use of drip rather than spray/overhead and flood have been noted to reduce this effect (Aycicek et al., 2006). On the other hand, higher pH in leafy vegetables (6.69 - 8.27) in this study may also promote growth of these microorganisms unlike the acidic (4.03 - 4.53) nature of most fruits which tend to inhibit pathogenic growth (Bello et al., 2014).

Dried leafy vegetables produced at home (spinach, traditional vegetable and pumpkin leaves) were highly contaminated with fungi (ranging from 6.5×10^5 to 9.4×10^5 CFU g^{-1}) followed by unpeeled dried peaches (3.1×10^3 CFU g^{-1}). Commercially dried fruits such as guavas, dates, prunes and mangoes also recorded high fungal contamination levels, ranging from $3.8x10^3$ to 6.3×10^3 CFU g^{-1}. According to the recommended guidelines for dried fruits and vegetables, the yeast and fungi counts should not exceed 1.0×10^3 CFU g^{-1} (Witthuhn et al., 2005). Among the commercially produced dried fruits, the

lowest fungi counts were detected in pears (2.0×10^2 CFU g^{-1}) followed by peeled peaches (2.6×10^2 CFU g^{-1}) and then in apricots (7.2×10^2 CFU g^{-1}). While fungi and total aerobic counts in the current study were recorded in less than 50% of the samples, Witthuhn et al. (2005) reports their presence in almost all products examined in South Africa. Nonetheless, as presented in Table 4, low counts of fungi were also recorded in home dried fruits such as apples (3.9×10^2 CFU g^{-1}) and peeled peaches (4.0×10^2 CFU g^{-1}). Beuchat and Mann (2014) recommend that dried fruits should be subjected to a lethal process and further contamination prevented before they are consumed.

Notably, possible pathogenic microorganisms, except *Clostridium spp*, were detected in all home dried leafy vegetables (Table 4). *E. coli* (9.0×10^1 - 3.2×10^2 CFU g^{-1}) and *Bacillus* spp (5.6×10^1 - 6.1×10^1 CFU g^{-1}) were detected in spinach, traditional vegetable and pumpkin leaves. While *Salmonella* spp was absent in spinach, it was detected in traditional vegetable (10 CFU g^{-1} detected on BGA). In pumpkin leaves, *Salmonella* spp and *Shigella* spp were detected both on BGA (3.0×10^1 CFU g^{-1}) and SSA (6 CFU g^{-1}).

The most contaminated home dried vegetable were pumpkin leaves, followed by traditional vegetable and then spinach. Correspondingly, several studies also report the presence of these pathogens in semi processed vegetables (Abadias et al., 2008; Beuchat, 1996; Beuchat, 2002; National Advisory Committee on Microbiological Criteria for Foods, 1999) though they significantly vary. According to European Commission Scientific Committee on Food (2002), while pathogenic *E. coli* is more frequently isolated as compared to *Salmonella*, the prevalence of *Salmonella* is generally high in vegetables, ranging between 4 and 8%. Likewise, in these leafy vegetables, *Salmonella* prevalence ranged between 33 and 67% while *E. coli* was present in all the vegetables (Table 4). *Salmonella* has been reported to survive on dried food products for up to eight months (Beuchat and Mann, 2014). It is acknowledged that, while traditional processing interventions such as drying are effective when applied to high moisture foods, they often fail to reduce microbial hazard contamination of LMF significantly and to non-detectable levels (Beuchat et al., 2013; FAO/WHO, 2014; Finn et al., 2013). Because home drying temperature is usually not high enough to kill contaminating microorganisms, blanching of fruits and vegetables before drying is recommended in order to reduce the microbial load and also to prevent colour changes.

More than 60% of the samples did not meet the international standards; thus not recommended for human consumption. The high microbial counts in home dried fruits and vegetables sold on the market in Maseru makes them unsuitable for human consumption. A global review on the prevalence of pathogenic microbial contamination (>10^3 CFU g^{-1}) in dried fruits and vegetables reported a prevalence of about 4.84% (FAO/WHO, 2014). To limit introduction of pathogenic microorganisms in dried vegetable products, especially in leafy vegetables, their respective pre and post-harvest sources of contamination that include soil, faeces, irrigation and rinse water, dust, insects, inadequately composted manure, wild and domestic animals, and unhygienic processing equipment and human handling should be minimised (Beuchat, 1996; Burnett and Beuchat, 2001). *E. coli* and *Salmonella* can be found in animal faeces or from contaminated sources of water used for both irrigation and further processing. According to Solomon et al. (2002) and National Advisory Committee on Microbiological Criteria for Foods (1999), spreading of *E. coli* from manure-contaminated soil and irrigation water to subsequent processed vegetables has been reported.

Importantly, during processing, stringent quality control measures need to be taken and enforced in order to reduce pathogenic prevalence in dried fruits and vegetables. To enable this, government has to play a pivotal role in coming up with appropriate legislation that should be incorporated into existing food safety regulations and implemented. In addition, government needs to draw a food safety control policy and develop stringent quality control measures for such products at the market place and stores. Once the policy is in place it should act as a guideline to regulatory authorities such as local municipal authorities, analytical labs and food standard authorities who should then enforce the policy. Thereafter, appropriate food safety training, including Hazard Analysis and Critical Control Points (HACCP), Good Manufacturing Practices (GMP) and Good Hygienic Practices (GHP) should be initiated since they can significantly reduce pathogenic hazards in foods (Blumenthal et al., 2000; Ijabadeniyi and Buys, 2012). Lastly, the Joint FAO/WHO Codex Alimentarius Commission (1994) guidelines on processing dried vegetables and fruits should be followed stringently to reduce the risk of post-harvest contamination. One major limitation of this study is that potential microbial pathogens were identified only to the genus level and not to species level, which would confirm pathogenicity and improve the results of the study.

Conclusions

In this study, more than half of the samples analysed were disqualified from human consumption due to poor microbial quality, which fell short of meeting international standards. The presence of possible pathogenic species on food products poses a potential public health hazard to consumers. Therefore, the findings from this study highlight the importance and need for adequate processing of home dried fruits and vegetables.

Responsible authorities have to embrace measures

and policies focused on food safety and hygienic practices through practical education and re-training programs for food business operators at all steps of food production (from farm to folk), especially the home based producers. Also, once these measures are in place they need monitoring and enforcement through properly gazetted food laws and regulations. The findings from this study can be valuable for further risk assessment of the impact on human health of consuming, especially, home dried fruits and vegetables.

Conflict of interests

The authors have not declared any conflict of interest.

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Full Length Research Paper

Enhancement of the expression of defense genes in tomato against *Ralstonia solanacearum* by N-octanoyl-L-homoserine lactone

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Many Gram-negative bacteria use N-acyl-homoserine lactone (AHLs) as quorum sensing (QS) signalling molecules to monitor their population density and to regulate gene expression in a density dependent manner. Recently, it has been shown that AHLs are detected by the plants and they trigger plant defense responses. In this study, N-octanoyl-L-homoserine lactone (C8-HSL) has been used as resistant inducer against bacterial wilt disease of tomato caused by *Ralstonia solanacearum*. The present investigation focused on the role of defense related enzymes (phenylalanine ammonia lyase, peroxidase, polyphenol oxidase and lipoxygenase) in imparting resistance in tomato against *R. solanacearum*. Activities of these defense enzymes, increased in C8-HSL treated tomato plants, which were challenged with *R. solanacearum*. The transcripts accumulation studies for these enzymes were carried out using semi-quantitative reverse transcription PCR, with maximum mRNA accumulation in resistant cultivar upon treatment with C8-HSL. Quantitative real time-PCR (qRT-PCR) confirmed the maximum induction of all these four genes in C8-HSL treated plants. However, the expression of defense genes was higher in C8-HSL treated resistant cultivar than that of susceptible cultivar. Therefore, the results support the view that C8-HSL molecule enhances disease protection against *R. solanacearum* infection in tomato through the activation of defense genes.

Key words: N-octanoyl-L-homoserine lactone, *Ralstonia solanacearum*, tomato, defense genes, quantitative real time-polymerase chain reaction (PCR).

INTRODUCTION

Many bacteria use small signalling molecule to communicate with each other and to co-ordinate their growth activities, this process is commonly referred to as quorum sensing (QS). The most common signalling molecules in Gram negative bacteria are N-acyl-L-homoserine-lactones (AHLs). The AHLs are composed of a conserved homoserine lactone moiety and a fatty acid side chain that can vary in length (4 to 18 carbon atoms)

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(Decho et al., 2011). To date, approximately 50 types of AHL signalling molecules are found in bacteria (Jin et al., 2012). Recent reports indicate that bacteria commonly associated with plants are capable of producing a variety of AHLs (Cha et al., 1998). However, only little is known about the molecular ways of plants reacting with these bacterial QS signals. Bacteria like *Xanthomonas oryzae* pv. *oryzae*, *Ralstonia solanacearum*, *Pseudomonas syringae* and *Dickeya didantii* cause disease on plants. In recent years, evidence has accumulated that AHL molecules are able to function as priming agents. AHL molecules induce resistance against a broad spectrum of plant pathogens in different plant species (Schikora et al., 2016). Schenk and Schikora (2015) showed that AHL primed plants, upon a challenge with pathogens accumulate callose and phenolic compounds. Similar to AHL molecules produced by bacteria, commercially available pure AHL molecules also induce priming. The transport of AHLs within plants has been studied initially in Barley and *Arabidopsis* (Gotz et al., 2007; von Rad et al., 2008) by using radioactive labelled AHLs. Mathesius et al. (2003) observed that the legume plant, *Medicago truncatula* are able to respond to nanomolar concentrations of synthetic and purified AHLs and these compounds elicit major changes in protein expression. These changes suggest that bacterial QS signalling molecules might regulate the functions of these proteins, which include roles in defense responses of host plants, primary metabolism, plant hormonal response, transcriptional gene regulation, protein processing and activities of the cytoskeleton (Mathesius et al., 2003). Schuhegger et al. (2006) showed that treatment of roots with synthetic N-hexanoyl-L-homoserine lactone (AHLs (C6-HSL)) enhanced the expression of salicylic acid and ethylene dependent defense genes in tomato against the fungal leaf pathogen, *Alternaria alternata*. A transcriptomic approach by von Rad et al. (2008) in *Arabidopsis* showed gene expression changes for several hundred genes in shoots and roots in response to 10 µM concentration of C6-HSL. However, if plant can detect low concentration of AHLs, they might be able to respond before pathogen concentration build up (Teplitski et al., 2010). The long chain AHL, oxo-C14-HSL, activates resistance towards different obligate bio-trophic pathogens such as *Golovinomyces orontii* and *Blumeria graminis* in *Arabidopsis* and barley, respectively (Schenk et al., 2014). All these studies demonstrate that AHLs can induce resistance in plant by activating the defense mechanism.

Tomato (*Solanum lycopersicum* Mill.) is one of the important vegetable grown and consumed worldwide. Tomato is prone to a number of bacterial diseases among which bacterial wilt caused by *R. solanacearum* (Smith) is a very destructive harmful disease resulting in complete loss of the crop (Vanitha and Umesha, 2008; Prakasha et al., 2016). Control of bacterial wilt has been difficult due to the high variability of the pathogen, high ability to survive in diverse environments and its extremely wide host range. Using chemicals to control plant diseases is hazardous to the environment and living beings, so using biological control can overcome this problem. Schuhegger et al. (2006) results suggest that AHL molecule play an important role in the biocontrol activity of *Serratia liquefaciens* and other rhizobacteria in tomato, act as mediators of communication between prokaryotes and eukaryotes. AHLs may therefore be considered as potential candidates for a new group of general elicitors of plant defense as they induce expression of typical defense related proteins resulting in increased resistance against pathogen (Venturi and Fuqua, 2013).

Plants possess a range of active defense responses that contribute to resistance against a variety of pathogens. They respond to bacterial pathogen attack by activating various defense responses that are associated with the accumulation of several factors like defense related enzymes and inhibitors that serve to prevent pathogen infection. The interaction between the pathogen and host plant induces some changes in cell metabolism, primarily in the enzyme activities, including that of phenylalanine ammonia lyase (PAL), peroxidase (POX), polyphenol oxidase (PPO), lipoxygenase (LOX), superoxide dismutase and β-1,3-glucanase (Kavitha and Umesha, 2008). These enzymes play a crucial role with respect to the degree of host resistance, by increasing anti microbial activity, bio synthetic processes related to wall development such as phenol, lignification, polymerization of hydroxyproline-rich glycoproteins, regulation of cell wall elongation and wound healing (Belkhadir et al., 2004).

The aim of this study was to investigate whether C8-HSL induce resistance in tomato and also its efficacy in controlling bacterial wilt disease through differential expression of defense genes (PAL, POX, PPO and LOX). The changes in the gene expression patterns were also studied using specific primers for these enzymes. Defense genes were assessed to determine possible relationships between the activation of these enzymes and the protection of plants following treatment with C8-HSL and its mRNA accumulation was measured by quantitative real time-PCR upon challenge inoculation with the pathogen.

MATERIALS AND METHODS

Plant material and growth conditions

Seeds of tomato cultivar, resistant (R) (cv. Golden) and susceptible (S) (cv. Rasi) to bacterial wilt were procured from private seed agencies in Mysore, Karnataka, India. From earlier studies in our laboratory, these two cultivars of tomato were selected on the basis of their response to bacterial wilt disease caused by *R. solanacearum* inoculation (Vanitha and Umesha, 2008). All seed samples were surface sterilized with 3% (v/v) sodium hypochlorite solution for 5 min and washed with distilled water three times.

Table 1. List of primers.

Gene product	Forward primer (5'-3')	Reverse primer (5'-3')
Phenylalanine ammonia lyase	GTGACTAACCACGTCCAAAG	CAAAGCGCCACGAGATAG
Peroxidase	GTTGCCTTGGTTGCTAGAG	GACGTCTGGAGACTGGAA
Polyphenol oxidase	ACTACGGAGGTGCCATAC	CGGCTAATCGCCAGATTG
Lipoxygenase	GGACATGGCGACAAGAAA	GTAGGGCGATTAGGGAGATA
18S rRNA	GTGCATGGCCGTTCTTAGTTG	CAGGCTGAGGTCTCGTTCGT

Tomato plants were raised in plastic trays filled with mixture of sterilized soil, sand and farm yard manure (2:1:1). Tray were maintained in the green house conditions and watered as and when required.

Treatment with C8-HSL and challenge with *R. solanacearum*

Four-week-old plants grown in sterilized soil were treated with 10 µM C8-HSL (Cayman, USA) (Schuhegger et al., 2006). A100 x stock in dimethyl sulfoxide (DMSO) was diluted in 5 ml sterile distilled water and pipetted on the soil to avoid contact with shoots and leaves. Control plants received equal amounts of DMSO in water.

The wilt causing *R. solanacearum* (strain: RS-lpxC-DOB-2) inoculum was prepared by growing bacteria on 2,3,5-triphenyltetrazolium chloride (TZC) agar medium for 48 h at 30°C (Kumar et al., 2016). The bacterial cells were collected in sterile distilled water and pelleted by centrifugation at 12,000 rpm for 10 min. The pellet was resuspended in distilled water and bacterial concentration was adjusted to 1 x 10^8 cfu/ml at absorbance 610 nm using UV-visible spectrophotometer (Hitachi U-2000, Japan) according to Ran et al. (2005). 15 ml of bacterial suspension was poured onto the soil near the roots of tomato plants.

The four-week-old tomato plants inoculated and uninoculated were harvested at 0, 3, 6, 9, up to 72 h post inoculation (hpi) and stored at -80°C for subsequent analysis.

Enzyme studies

Phenylalanine ammonia lyase (PAL) activity was performed according to Lisker et al. (1983). One gram of tomato seedling, fresh mass was homogenized to fine paste in a pre-chilled mortar with 25 mM Tris-HCl buffer (pH 8.8). The homogenate was centrifuged at 10,000 rpm for 12 min at 4°C and the supernatant was directly used as enzyme source. The enzyme activity was determined by measuring the production of trans-cinnamic acid from L-phenylalanine using spectrophotometer (Hitachi U-2000, Japan). The reaction mixture contained 1 ml enzyme extract, 0.5 ml 50 mM L-phenylalanine and 0.4 ml 25 mM Tris-HCl buffer (pH 8.8). After incubation for 2 h at 40°C, the activity was stopped by the addition of 60 µl 5 M HCl and the absorbance was read at 290 nm against the same volume of reaction mixture without L-phenylalanine which served as blank.

Peroxidase (POX) assay was carried out as described by Hammerschmidt et al. (1982). One gram of fresh mass of plants was homogenized in 1 ml of 10 mM phosphate buffer (pH 6.0) and centrifuged at 10,000 rpm for 12 min at 4°C and the supernatant served as enzyme source. The reaction mixture consisted of 0.25% (v/v) guaiacol in 10 mM potassium phosphate buffer (pH 6.0) containing 10 mM H_2O_2. Addition of 0.1 ml of crude enzyme extract initiated the reaction and absorbance at 470 nm was measured for 1 min.

Polyphenol oxidase (PPO) activity was determined according to Mayer et al. (1966). The reaction mixture consisted of 1.5 ml of 0.1 M sodium phosphate buffer (pH 6.5) and 0.2 ml of the enzyme extract. The reaction was started with the addition of 0.2 ml of 10 mM catechol. The increase in absorbance was measured at 420 nm for 1 min.

Lipoxygenase (LOX) activity was estimated according to Borthakur et al. (1987). The activity was determined spectrophotometrically by monitoring the appearance of conjugated diene hydroperoxide, absorbing at 234 nm. The reaction mixture contained 2.7 mL of 0.2 M sodium phosphate buffer (pH 6.5), 0.3 ml of 10 mM linoleic acid in Tween 20 and 0.05 ml of the enzyme extract. Protein contents of the extracts were determined according to standard procedure of Bradford (1976) using BSA (Sigma, USA) as standard.

Analysis of defense genes by semi-quantitative reverse transcription PCR

The total RNA from four-week-old plants of resistant and susceptible tomato cultivars based on enzyme assays (PAL, POX, PPO and LOX) were extracted for analysis. RNA isolation was done using RNeasy Plant Mini Kit (QIAGEN, Germany) according to the manufacturer's instruction.

The complementary DNA was synthesised using 2 µg of RNA and first strand cDNA synthesis kit (Thermo Scientific, India). The reverse transcribed RNA was used as PCR template with gene specific primers for all the four genes (PAL, POX, PPO and LOX). 18S rRNA gene primer specific to tomato was used as a constitutive control in all gene expression studies (Chandrashekar and Umesha, 2014). All the primer sequences were reconfirmed by BLAST analysis. The primers used are shown in Table 1. Semiquantitative RT-PCR assay conditions were, initial 3 min denaturation at 94°C, followed by 94°C for 30 s, 60°C for 40 s, and 72°C for 1 min and a final extension for 10 min at 72°C. The number of cycles was 35.

Analysis of defense gene expression by quantitative real time PCR

Each qPCR reaction (20 µl) consisted of 1 x SYBR Green (Thermo Scientific, India) PCR master mix, 3 pmol of each primer and 20 ng each of cDNA by using StepOnePlus™ Real Time PCR machine (Applied Biosystems, USA). qPCR steps were: denaturation at 95°C for 10 min, 40 cycles of 15 s at 95°C, 60 s at 60°C. For calculating the fold change in expression of genes in plants, the transcripts in both the control and treated were normalized to 18S rRNA and the difference in the 18S rRNA normalized cycle threshold value ($\Delta\Delta$CT) was used to obtain fold change (Livak and Schmittgen, 2001), with standard error being calculated from three replicated derived from each independent experiment.

Statistical analysis

All enzyme assay experiments were carried out in triplicates. Further, the experimental results were subjected to Duncan's multiple range tests at a significance level of $P < 0.05$. All statistical tests were performed using SPSS software.

RESULTS

Enzyme studies

Plants have endogenous defence mechanisms that can be induced in response to attack by pathogens. Inducing the plants own defense mechanisms by prior application of AHL (biological inducer) is thought to be a novel plant protection strategy. The temporal changes of all four enzyme activities of 10 µM C8-HSL primed followed by challenge inoculated with the pathogen along with their respective uninoculated controls were assayed. The temporal pattern studies of enzymes were undertaken to estimate the PAL, POX, PPO and LOX highest activities at regular intervals from 0 to 72 h.

In the resistant tomato cultivar, the temporal pattern of PAL enzyme revealed the maximum activity at 9 hpi (140 units) (Figure 1a), while in susceptible tomato cultivar, PAL enzyme revealed the maximum activity at 18 hpi (85 units) (Figure 1b). In the resistant tomato cultivar, the temporal pattern of POX enzyme revealed the maximum activity at 6 hpi (65 units) (Figure 1c), while in susceptible tomato cultivar POX enzyme revealed the maximum activity at 15 hpi (28 units) (Figure 1d). In the resistant tomato cultivar, the temporal pattern of PPO enzyme revealed the maximum activity at 12 hpi (72 units) (Figure 1e), while in susceptible tomato cultivar, PPO enzyme revealed the maximum activity at 24 hpi (35 units) (Figure 1f). In the resistant tomato cultivar, the temporal pattern of LOX enzyme revealed the maximum activity at 6 hpi (40 units) (Figure 1g), while in susceptible tomato cultivar, LOX enzyme revealed the maximum activity at 12 hpi (23 units) (Figure 1h). Therefore, gene expression studies were concentrated on only that particular time interval.

Semi-quantitative reverse transcription PCR

The gene expression pattern was altered when the plants were treated with C8-HSL and challenged with *R. solanacearum* in both resistant and susceptible tomato cultivars. The PAL, POX, PPO and LOX gene expressions was higher in resistant cultivar when the plants were treated with C8-HSL. The respective controls also expressed the accumulation of gene expression but they were not significant. The housekeeping gene, 18S rRNA expression was found to be unaltered in both resistant and susceptible cultivar in all the treatments (Figure 2).

Gene expression analysis by quantitative real-time PCR

Both resistant and susceptible tomato cultivars were raised and the four-week-old plants were treated with C8-HSL and inoculated with *R. solanacearum* (concentration of 1×10^8 cfu/ml). The gene expression studies were carried out based on the temporal pattern studies of enzymes. Based on the temporal activity of PAL (Figure 1a and b) and followed by semi quantitative RT-PCR (Figure 2); the authors selected 9 and 18 hpi for resistant and susceptible tomato cultivars, respectively. Based on the temporal activity of POX (Figure 1c and d) and followed by semi quantitative RT-PCR (Figure 2); 6 and 15 hpi were selected for resistant and susceptible tomato cultivars, respectively. Based on the temporal activity of PPO (Figure 1e and f) and followed by semi quantitative RT-PCR (Figure 2); 12 and 24 hpi were selected for resistant and susceptible tomato cultivars, respectively. Based on the temporal activity of LOX (Figure 1g and h) and followed by semi quantitative RT-PCR (Figure 2); 6 and 12 hpi were selected for resistant and susceptible tomato cultivars, respectively. For qRT-PCR analysis, the authors have selected the time interval which showed highest activity in both temporal as well RT-PCR analysis for PAL, POX, PPO and LOX gene and the total RNA was isolated and converted into cDNA (Thermo Scientific, India) as per manufacturer's instructions.

In the resistant tomato cultivar, the relative gene expression of PAL was up-regulated to 20 fold upon 10 µM C8-HSL treatment as compared to the control. Whereas pathogen inoculated resistant tomato cultivar up-regulates PAL activity to 16 fold which was significantly increased to 30 fold upon C8-HSL treatment and challenge inoculation with pathogen (Figure 3a). In the case of susceptible tomato cultivar, the relative gene expression of PAL was up-regulated to 12 fold upon 10 µM C8-HSL treatment as compared to the control. Whereas pathogen inoculated susceptible tomato cultivar down-regulates PAL activity to 4 fold which was significantly increased to 20 fold upon C8-HSL treatment and challenge inoculation with pathogen (Figure 3b).

In the resistant tomato cultivar, the relative gene expression of POX was up-regulated to 10 fold upon 10 µM C8-HSL treatment as compared to the control. Whereas, pathogen inoculated resistant tomato cultivar up-regulates POX activity to 5 fold which was significantly increased to 20 fold upon C8-HSL treatment and challenge inoculation with pathogen (Figure 3c). In the case of susceptible tomato cultivar, the relative gene expression of POX was up-regulated to 7 fold upon 10 µM C8-HSL treatment as compared to the control. Whereas pathogen inoculated susceptible tomato cultivar down-regulates POX activity to 2 fold which was significantly increased to 12 fold upon C8-HSL treatment and challenge inoculation with pathogen (Figure 3d).

In the resistant tomato cultivar, the relative gene

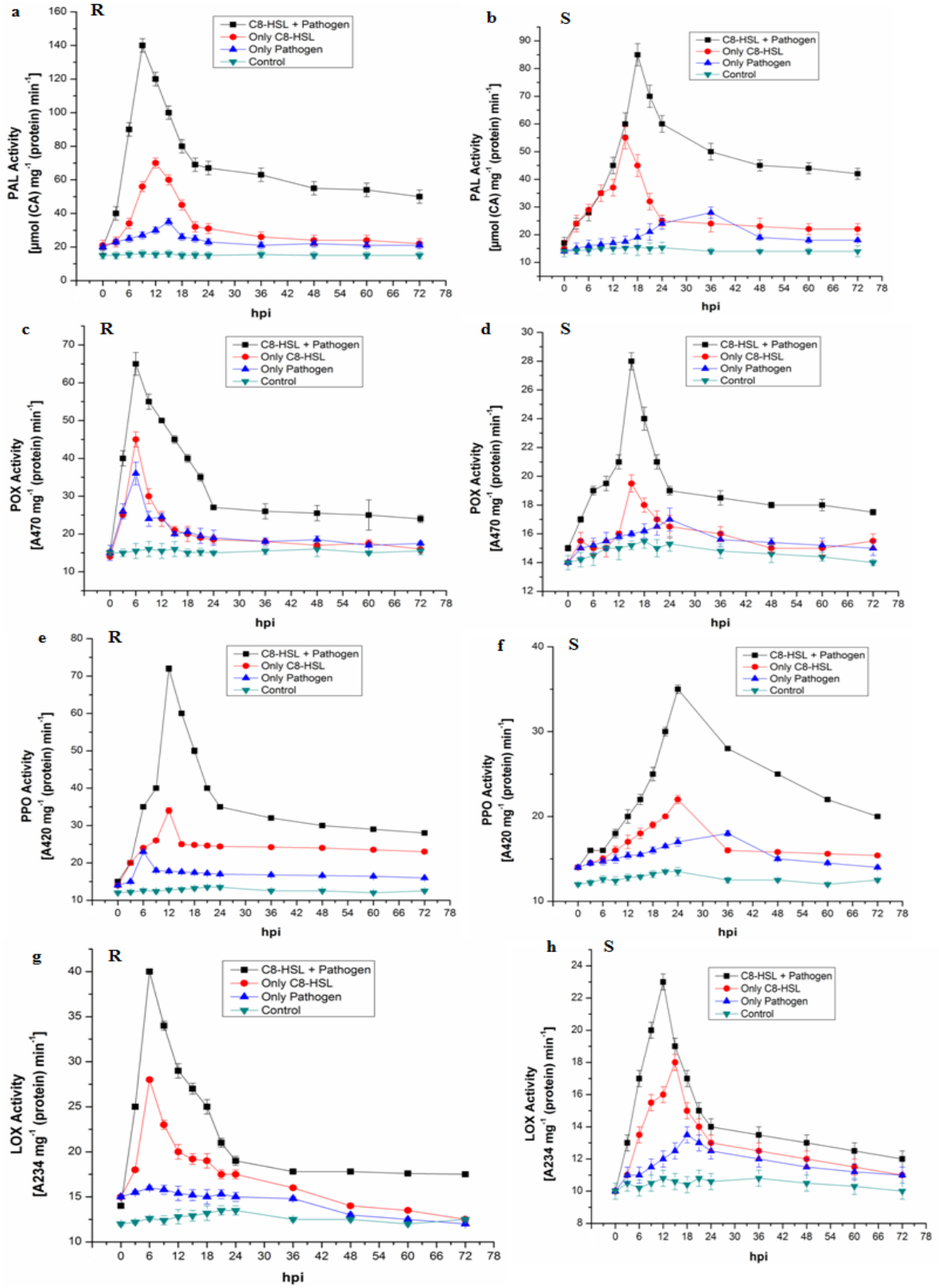

Figure 1. Temporal pattern study of PAL, POX, PPO and LOX activity in resistant (R) and susceptible (S) tomato cultivars. Four-week-old plants were treated with C8-HSL, followed by challenged with pathogen. Both treated and control plants were harvested at different hours after pathogen inoculation, and subjected to enzyme estimation. The data are expressed as the average of three independent experiments with three replicates each. Bars indicate standard errors.

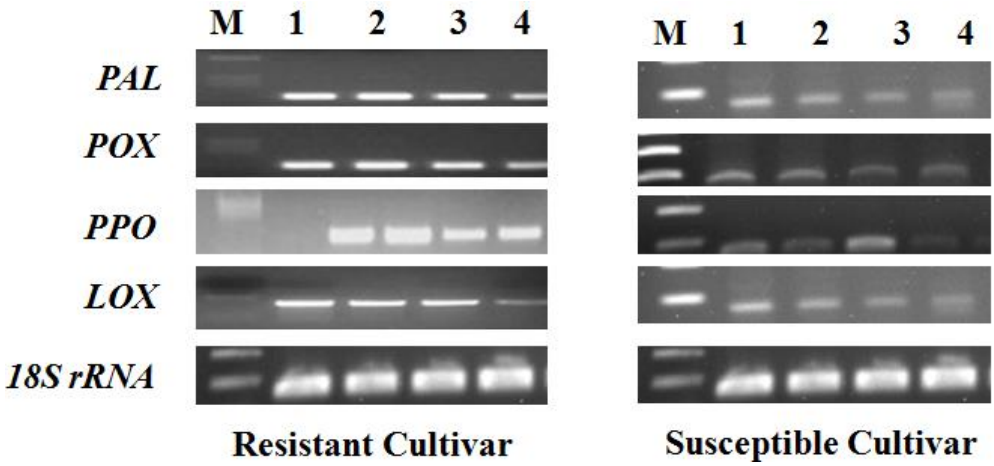

Figure 2. The PAL, POX, PPO and LOX gene expression was assessed by semi-quantitative PCR. After the C8-HSL (10 µM) treatment, the PCR product was assayed by electrophoresis (2% agarose gels) stained with ethidium bromide. Lane M: DNA ladder; Lane 1: pathogen + C8-HSL; Lane 2: C8-HSL; Lane 3: pathogen only; Lane 4: control.

expression of PPO was up-regulated to 12 fold upon 10 µM C8-HSL treatment as compared to the control. Whereas, pathogen inoculated resistant tomato cultivar up-regulates PPO activity to 7 fold which was significantly increased to 25 fold upon C8-HSL treatment and challenge inoculation with pathogen (Figure 3e). In the case of susceptible tomato cultivar, the relative gene expression of PPO was up-regulated to 9 fold upon 10 µM C8-HSL treatment as compared to the control. Whereas, pathogen inoculated susceptible tomato cultivar down-regulates PPO activity to 1 fold which was significantly increased to 15 fold upon C8-HSL treatment and challenge inoculation with pathogen (Figure 3f).

In the resistant tomato cultivar, the relative gene expression of LOX was up-regulated to 5 fold upon 10 µM C8-HSL treatment as compared to the control. Whereas pathogen inoculated resistant tomato cultivar up-regulates LOX activity to 4.5 fold which was significantly increased to 12 fold upon C8-HSL treatment and challenge inoculation with pathogen (Figure 3g). In the case of susceptible tomato cultivar, the relative gene expression of LOX was up-regulated to 4 fold upon 10 µM C8-HSL treatment as compared to the control. Whereas pathogen inoculated susceptible tomato cultivar down-regulates LOX activity to 1.5 fold which was significantly increased to 9 fold upon C8-HSL treatment and challenge inoculation with pathogen (Figure 3h).

DISCUSSION

Resistance in plants is a highly regulated phenomenon depending on several signalling pathways, each activated by a set of different biotic and abiotic stimuli (Schuhegger et al., 2006). Recently, it has become evident that plants can sense and respond appropriately to bacterial AHLs. It is reasonable that cross-kingdom signalling exits between plants and bacteria because plants and bacteria co-habited the earth for millions of years during which they might have evolved complex signalling networks consisting of different signalling molecules (Jin et al., 2012). In addition, plants seem to be able to detect various AHLs at quite low concentration (Mthesius et al., 2003). Schuhegger et al. (2006) in tomato reported that C6-HSL were able to induce resistance to the fungal leaf pathogen, *Alternaria alternata*. Mathesuis et al. (2003) found that over 150 proteins of approximately 2000 resolved protein spots were significantly altered in their accumulation in *M. truncatula* roots after the treatment with low concentration of 3OC12-HSL and 3OC16-HSL. In addition, von Rad et al. (2008) showed that the contact of *Arabidopsis* roots with C6-HSL resulted in distinct transcriptional change in the roots. Miao et al. (2012) found significant changes in protein accumulation for approximately 6.5% proteins of the total resolved proteins on 2-DE gels after the interaction of *Arabidopsis* roots with 3OC8-HSL, indicating that the responses of plants to AHLs are quite extensive. However, it is becoming increasingly evident that AHLs plays a positive role in activation of defense gene expression and pathogen defense. These data suggested that AHL play an important role in plant bacterial communication and a possible role in pathogen defense, and the authors decided to analyze the effect of C8-HSL on tomato plant along with *R. solanacearum* challenge inoculation.

Early and elevated levels of expressions of various defense enzymes are important features of plant resistance to pathogens. This is the first report where the role of defense related enzymes such as PAL, POX, PPO and LOX during the C8-HSL mediated elicitation of

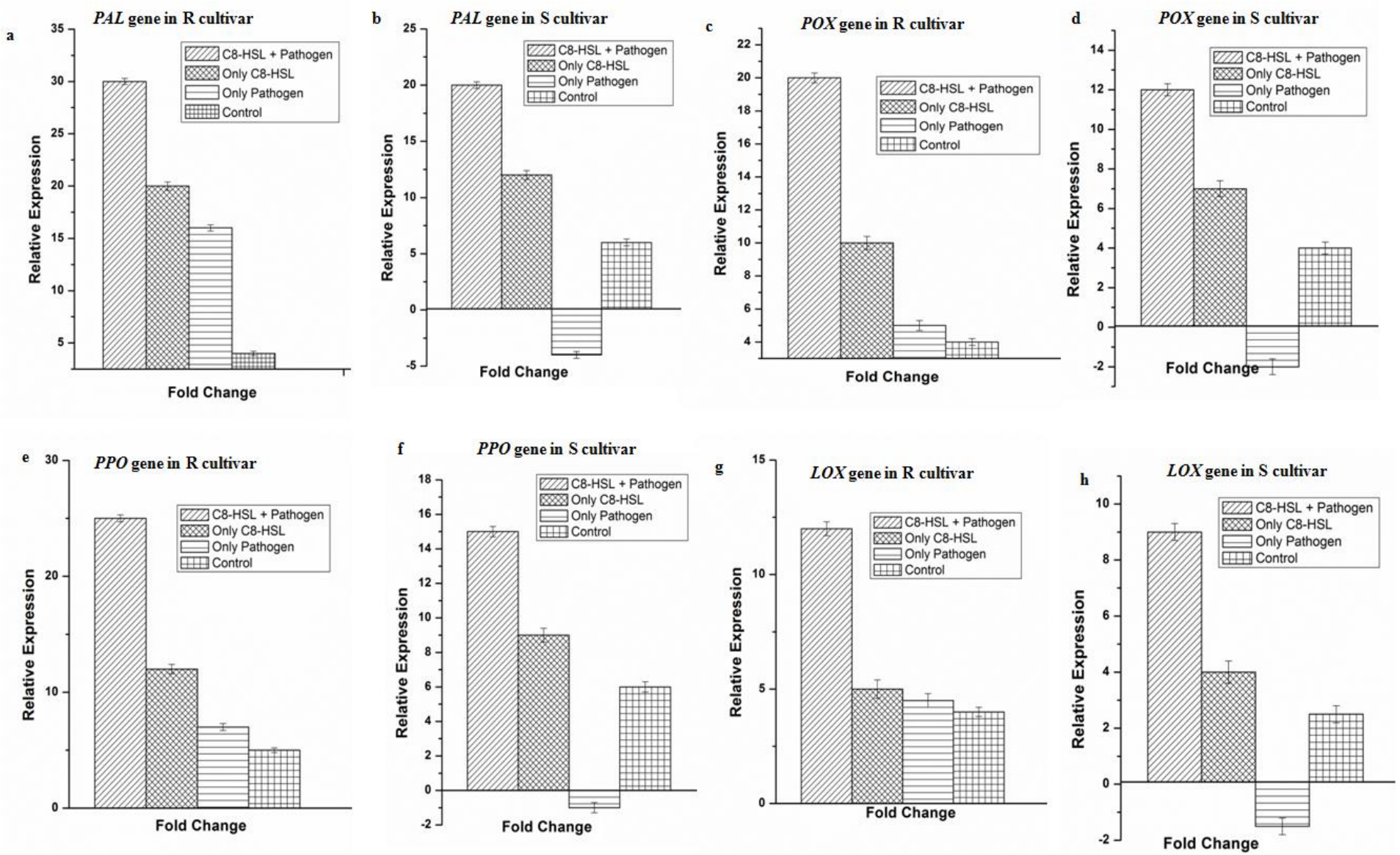

Figure 3. Relative expression levels of PAL, POX, PPO and LOX genes in four-week-old plants of both R and S tomato cultivars upon C8-HSL (10 µM) treatment and challenged with the pathogen. Total RNA (2 µg) was used to synthesis the cDNA of which 20 ng of individual cDNA was used to check the fold change of the PAL, POX, PPO and LOX genes which were carried out in three replicates. The gene expression levels were measured by qRT-PCR and normalized to the constitutive 18S rRNA gene. Each bar represents the mean of three independent experiments with standard error. R: resistant (cv. Golden); S: susceptible (cv. Rasi).

resistance in tomato against *R*. *solanacearum* was studied. Expression of these defense related enzymes (PAL, POX, PPO and LOX) are known to play a major role in determining the host resistance against various phytopathogens. These enzymes are either directly or indirectly involved in hypersensitive reaction (HR) development (Rusterucci et al., 1999), biosynthesis of cell wall strengthening material (lignin and suberin) and anti-microbial compounds (phytoalexins, furanocoumarin, quinines and pterocarpan) (Daayf et al., 1997), as also signalling molecules (salicylic acid and jasmonic acid) (Creelman and Mullet, 1997; Hammerschmidt, 1999).

Early induction of PAL is more important because it is the first key regulatory enzyme in the phenyl propanoid pathway leading to the production of phytoalexins and phenolic substances (Wang et al., 2004). In this study, maximum PAL activity was 9 h after inoculation (hpi). PAL activity increased in C8-HSL treated tomato plants challenged with the *R*. *solanacearum*, while tomato plants inoculated with the *R*. *solanacearum* alone had lower PAL activity. The role of PAL in imparting resistance to tomato against bacterial canker disease has been discussed by Umesha (2006). In contrast with the results from the present study, the PAL activity in roots of pepper plants from a resistant cultivar was high than for a susceptible cultivar after inoculation with *Phytophthora capsici* (Zhang et al., 2013). Iqbal et al. (2005) showed that during the infection of *F*. *solani* f. sp. *glycines* on the roots of soybean plants from a resistant cultivar, the PAL enzyme was up-regulated, and this was not observed in the susceptible cultivar.

POX is a key enzyme in the biosynthesis of lignin, in addition to its antimicrobial activity (Torres et al., 2006). Increased activity of cell wall bound peroxidises has been elicited in different plants due to pathogen infection. In this study, POX activity up-regulated after inoculation and reached its maximum at 6 hpi. Similar to PAL activity, tomato plants inoculated with the *R*. *solanacearum* alone recorded lower POX activity than C8-HSL treated plants. Leite et al. (2014) reported that POX activity was higher in the plants of a resistance genotype of common bean in response to *Sclerotinia sclerotiorum* infection than for a susceptible genotype.

PPO catalyses the oxidation of phenolic compounds to highly toxic quinines which play an important role in plant disease resistance. In this study, the activity of PPO reached maximum at 12 hpi in tomato plants. The PPO activity in plants treated with C8-HSL alone did not reach the level of activity seen in the plants treated with C8-HSL and inoculated with the *R*. *solanacearum*. PPO also plays a critical role in tomato's disease resistance to *Pseudomonas syringe* pv. tomato (Thipyapong et al., 2004).

The lipoxygenese enzyme initiates a metabolic route leading to the synthesis of various antimicrobial compounds involved in plant defense. In this study, the LOX activity was maximum at 6 hpi in plants treated with C8-HSL and challenged with *R*. *solanacearum*. Similar to the above mentioned enzymes, LOX activity were lower in other treatments. However, high LOX activity may constitute in plants resistance to pathogens but with an addition increase upon infection (Devi et al., 2000).

The plants treated with C8-HSL followed by pathogen inoculation accumulated increased amounts of defense enzymes (PAL, POX, PPO and LOX) when compared with untreated control. Similar results were reported when the activities of PAL, POX, PPO, LOX were increased in tomato plants pre-treated with *Pseudomonas fluorescens* and challenged with *R*. *solanacearum* (Vanitha and Umesha, 2011). The RT-PCR studies were carried out to investigate the gene expression pattern of defense related enzymes (PAL, POX, PPO and LOX). The genes were compared with the internal control being 18S rRNA. The 18S rRNA was expressed in both cultivars. The expression of the defense genes was higher in resistant cultivar.

qRT-PCR was performed to evaluate the mRNA accumulation of differentially expressed defense genes in both resistant cultivar and susceptible cultivar. In the resistant tomato cultivar, the relative gene expression of defense genes (PAL, POX, PPO and LOX) was up-regulated upon C8-HSL treatment as compared to the control, and significantly increased upon *R*. *solanacearum* inoculation. Whereas, in susceptible cultivar, the defense genes (PAL, POX, PPO and LOX) were down-regulated upon *R*. *solanacearum* inoculation as compared to the control, and interestingly up-regulated upon C8-HSL treatment. Thus, the results show that C8-HSL can induce significant defense gene (PAL, POX, PPO and LOX) in both the tomato cultivar (Figure 3). Our findings were in accordance with Lata et al. (2010) who have showed the relationship of PEG-induced dehydration stress in tolerant and sensitive millet, plants where transcripts showed a differential expression pattern in both cultivars at different time points of stress treatment as analyzed by qRT-PCR. Song et al. (2011) results showed the treatment with abscisic acid (ABA) on tomato against *Alternaria solani*, effectively reduced disease severity in tomato plants, as enzyme activities were maintained at higher levels in ABA pre-treated and *A*. *solani* challenged tomato plants. Tomato defense genes were rapidly and significantly up-regulated by ABA treatment which is well correlated with the present study.

In conclusion, the results of the present study confirm that C8-HSL trigger the defense mechanism in tomato plants by activating the defense enzymes and protect itself from the pathogen attack. Thus, this investigation shows that C8-HSL can be considered as potential candidates for elicitors for tomato plants against wilt disease, as they induce expression of typical defense related enzymes resulting in increased resistance against *R*. *solanacearum*. To the best of the authors' knowledge, this is the first report on the interaction between the C8-

HSL – tomato plants and its defense enzymes against *R. solanacearum* under greenhouse conditions.

Conflict of interest

The authors have not declared any conflict of interest.

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Microbiological quality of raw vegetables and ready to eat products sold in Abidjan (Côte d'Ivoire) markets

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Vegetables are usually consumed raw. This implied best hygienic conditions from the harvest to the processing because of the gastro-enteritis that they could provoke. This study was conducted with the aim to appreciate microbiological quality of raw tomatoes, endives and ready-to-eat products sold in markets. Samples were taken randomly in two markets of Abidjan. A microbiological analysis was done in order to identify and enumerate faecal coliforms, *Escherichia coli*, *Enterococcus* and *Pseudomonas*. A decontamination treatment based on washing samples with running water and sodium hypochlorite solution I° chlorymetric was also applied to tomatoes and endives. The results indicated that, for tomatoes and endives, the average load was $1.5.10^4$ CFU/g of *Enterococcus,* $1.3.10^3$ CFU/g of *Pseudomonas* and $1.7.10^2$ CFU/g of faecal coliforms. In ready-to-eat products, the load was $9.3.10^1$ CFU/g for *Enterococcus*, $l.03.10^1$ CFU/g for *Pseudomonas* and $9.9.10^1$ CFU/g for faecal coliforms. The disinfection with a sodium hypochlorite solution I° chlorymetric reduced *Enterococcus* and faecal coliforms load to 98% and *Pseudomonas* load to 97% as compared to the washing with running water in which *Enterococcus* was only reduced to 80%, faecal coliforms to78% and *Pseudomonas* to 73%. *Escherichia coli* were isolated in 28 samples as follow: 15 stumps from endives (54%), 10 stumps from tomatoes (36%) and 3 stumps from ready-to-eat products (10%). Results showed that before consumption, vegetables need to be washed, cleaned and disinfected. This will avoid sanitary hazard.

Key words: Hygienic quality, raw vegetables, ready-to-eat products, disinfection, germs.

INTRODUCTION

Vegetables have beneficial effects on health and diseases prevention (Remesy et al., 1998). Their consumption is encouraged in many countries by governmental agencies to protect against a range of illnesses such as cancers and cardiovascular diseases. They are important components of a healthy and balanced diet because of their nutrients content such as vitamins, minerals and dietary fibber (Koffi-Nevry et al., 2012).

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Vegetables are consumed in the raw or cooked form. In the raw state, they can be sold entirely or after minimal processing which consist of peeling, cutting and slicing the fresh product in order to increase their functionality (Siddiqui et al., 2011). This second presentation constitutes the ready-to-eat products. However, vegetables must be treated cautiously while processing because of microbial flora and pathogen germs contamination which can represent a serious health risk (Cardamone et al., 2015).

In Côte d'Ivoire, these vegetables are not always cultivated under the best hygienic conditions. Sallou (2001) showed that farmers often used partially decomposed organic manure and polluted water for their cultures. Moreover, according to Sackou et al. (2006), 75% of lettuce sold in Abidjan markets did not meet the microbiological criteria set up for vegetables and, most of the time, the immediate surroundings of sales points of vegetables are real sources of contamination (Koffi-Névry et al., 2012).

In Côte d'Ivoire, several consumers (44%) did not disinfect vegetables before eaten (Sackou et al., 2006) and unfortunately, ready-to-eat products are sometimes handled in such conditions by sellers or kitchen personnel. Seow et al. (2012) have showed that among several vegetables and fruits, raw and ready-to eat lettuce are more contaminated with high level of mesophilic bacteria and coliforms. Vegetables need to be well washed and disinfected before been processed. According to Alvaro et al. (2009), the most common method to reduce the microbial activity of fruits and vegetables is the disinfection of washing water by chlorination. Lorougnon (1996) and Sorianoa et al. (2000) have also demonstrated that disinfection by chlorination reduced microbial load around 99%.

This study was led firstly, with the aim to evaluate the microbiological quality of tomatoes and endives, two vegetables highly consumed by Ivorian population, and ready-to-eat salads. Secondly, to appreciate the effect of decontamination treatment by simple washing with clean water or washing with sodium hypochlorite l° chlorymetric on *Escherichia coli*, *Enterococcus, Pseudomonas* and coliforms load in these vegetables.

MATERIALS AND METHODS

Sampling sites

Samples have been collected in the principal market of Adjamé, the most famous market of Abidjan city. This market received food and vegetables coming from different part of the country. The second market chosen is the market of Cocody. The neighbourhood of Cocody is considered to be the rich part of the city. So, one market is used by all kind of social categories of populations and another one is supposed to be used by the wealthy persons.

Sampling procedure

Vegetables were sampled as follow: 50 raw tomatoes (*Lipopersicum esculentum* var *Lima*), 50 raw endives (*Cichorium endivia* var *latifolia*), 50 ready-to-eat product consisted of onions, tomatoes and endives, all cut up and seasoned with oil and salt.

In this study, sampling of the different products was made by the same method. Two sellers were randomly chosen by market and by vegetables. So, 2 women were chosen for the tomatoes, 2 for ready to eat products and 2 others for endives. They participated in the study. Sampling was done 3 times per week during 4 weeks. One sample by kind of vegetable was taken randomly each time and packed in plastic bag by the seller. Before going to the laboratory for the analysis, samples were placed in an isothermal box equipped with ice packs.

Microbiological analysis

For the microbial analyses, *Escherichia coli* was only researched (presence or absence) on, while the others (faecal coliforms, *Enterococcus* and *Pseudomonas*) were enumerated. Coliforms, *E. coli* and *Enterococcus* are faecal contamination indicator germs, while *Pseudomonas* is vegetables deterioration indicator germ. A portion of vegetable (10.0 g) was taken into plastic sterile bags with 90 ml of plugged water. The mixture was blended in a Stomacher. Decimal dilutions were made by adding 1 ml of the first solution to 9 ml of a solution of salt trypton. *Enterococcus* was counted on Bile Esculin Agar (BEA) (BIORAD®) after incubation 24 h at 37°C (NF T 90-416, 1985). *Pseudomonas* grows on Cetrimid agar (BIOMERIEUX®) and was counted after incubation for 48 h at 30°C (NF V 04-504, 1995). For species identification, two check stumps were used: *Enterococcus faecalis* ATCC 29212 and *Pseudomonas aeruginosa* ATCC 27853. Faecal Coliforms were counted on violet red bile lactose agar (VRBL) (BIORAD®) after incubation for 24 h at 44°C (V 08-010, 1982; Le Minor et al, 1989). *E. coli* were detected by selecting five characteristic colonies of faecal coliforms (AFNOR, 1999; Le Minor et al., 1989). Isolation was made on methylene blue eosin agar (EMB) (BIOMERIEUX®). The check stump of *E. coli* gave by Pasteur Institute of Côte d'Ivoire was identified and confirmed by API 20^E gallery.

Microbiological criteria

The bacteriological criteria used for faecal coliforms in vegetables and ready-to-eat products was m = 10^2 CFU/g, M = 10^3CFU/g. The bacteriological criteria for *Enterococcus*, *Pseudomonas* were determined from the numeration criteria and was m = 1.5. 10^3 CFU/g, M = l.5. 10^3 CFU/g for *Enterococcus* and m = 30 CFU/g, M = 3.10^2 CFU/g for *Pseudomonas*. Concerning *E. coli*, the criteria was absence or presence after research on characteristic colonies of faecal coliforms (Table 1). "m" is the value below which the product is considered to have a very good and satisfactory quality, so it could be used. "M" is the value above which the quality of the product is considered to be bad, and it have to be rejected. The values between "m" and "M" characterize products qualities as acceptable.

Treatment method

Only tomatoes and endives were subjected to decontamination treatment because they were sold entirely and not cut up like the ready-to-eat products. Three different treatments were used and consisted of: no washing samples, washing with running water by soaking samples during 10 min and washing with a sodium hypochlorite solution 1° chlorymetric by soaking also the samples during 10 min.

Table 1. Microbiological criteria for vegetable and ready to eat.

Microorganisms	**Microbiological criteria**	**Microbiological quality**
Enterococcus	$<1.5\times10^2$ cfu/g	Satisfactory
	$1.5x10^2$ to 1.5×10^3 cfu/g	Acceptable
	$>1.5\times10^3$ cfu/g	Unsatisfactory
Pseudomonas	<30 cfu/g	Satisfactory
	30 to 3×10^2 cfu/g	Acceptable
	$>3\times10^2$ cfu/g	Unsatisfactory
Faecal Coliform	$<10^2$ cfu/g	Satisfactory
	10^2 to 10^3 cfu/g	Acceptable
	$>10^3$ cfu/g	Unsatisfactory
Escherichia Coli	Absence	Satisfactory
	Presence	Acceptable

Table 2. Total repartition of isolated species in the samples from 2 markets of Abidjan.

Species	**Tomatoes**		**Endive**		**Ready to eat products**		**Total**	
Enterococcus faecalis	7	47	30	50	10	34	47	131
Enterococcus (Others species)	40		20		24		84	
Pseudomonas aeruginosa	4	11	14	50	8	24	26	85
Pseudomonas (Others species)	7		36		16		59	
Faecal coliforms	34		42		8		84	
Escherichia coli	10		15		3		28	

Statistical analysis

A statistical study was made using STATISTICA 7.1. A Friedman Anova and Khi-2 tests were made (α = 5%) to discriminate the treatments.

RESULTS

Microbiological repartition of germs in tomatoes, endives and ready-to-eat products

In all analysed samples (tomatoes, endives and ready-to-eat products), 131 (87%) revealed the presence of *Enterococcus*, 85 (57 %) this of *Pseudomonas*, 84 (56 %) that of faecal coliforms and *E. coli* was isolated in 28 samples (19 %) (Table 2).

The percentage of *Enterococcus* is higher than that of *Pseudomonas* and faecal coliforms. The most important loads of microorganisms were in endives. In this product, *Enterococcus* and *Pseudomonas* were isolated in all samples, faecal coliforms in 42 samples and *E. coli* in 15 samples. In tomatoes, *Enterococcus* was isolated in 47 samples while only 11 samples contain *Pseudomonas*. In ready-to-eat products, the prevalence of germs is lower than that of tomatoes and endives. *Enterococcus* was counted in 34 samples, *Pseudomonas* in 24 samples, faecal coliforms in 8 samples and *E. coli* in 15 samples.

At the species level, *E. faecalis* was isolated in 7 samples of tomatoes, 30 endives and 10 ready-to-eat products. *P. aeruginosa* was isolated in 4 samples of tomatoes, 14 endives and 8 ready-to-eat products. The repartition of *E. coli* stumps in the samples was as follow: 15 stumps from endives (54%), 10 stumps from tomatoes (36%) and 3 stumps from ready-to-eat products (10%) (Figure 1). Others species of *Enterococcus* and *Pseudomonas* were present on the samples (Table 2).

Microbiological quality of samples

The numeration revealed that the load of *Enterococcus* is higher than that of others microorganisms. There was 1.4. 10^3 CFU/g of *Pseudomonas* in endives and faecal coliforms were in lower number (Table 3).

According to microbiological criteria presented in the Table 1, considering *Enterococcus*, 50% of tomatoes, 90% of endives and 18% of ready-to-eat products have an unsatisfactory microbiological quality. For

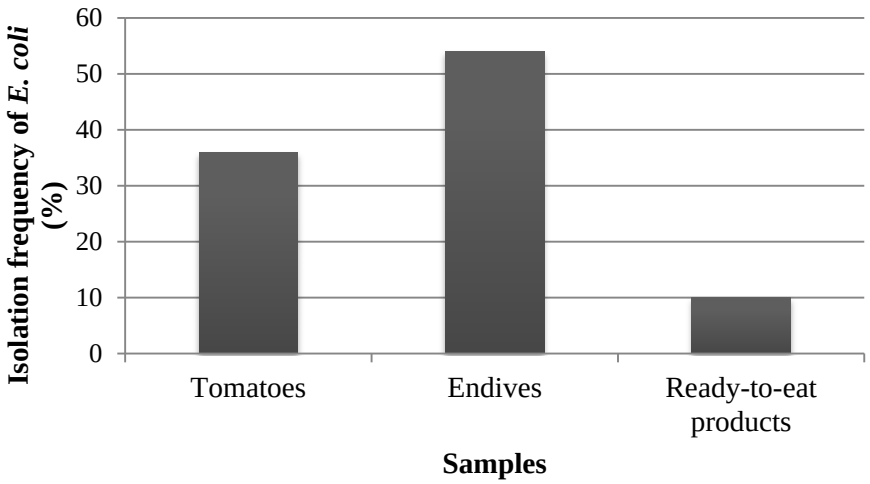

Figure 1. Repartition of *E. coli* in vegetables.

Table 3. Average load of germs in samples of 2 markets of Abidjan.

	***Enterococcus* (CFU/g)**	***Pseudomonas* (CFU/g)**	**Faecal coliforms (CFU/g)**
Tomatoes	$1.82x10^4 \pm 3.68x10^4$	$6.63x10^1 \pm 2.48x10^2$	$1.05x10^2 \pm 1.75x10^2$
Endives	$1.33x10^4 \pm 1.58x10^4$	$1.63x10^3 \pm 2.93x10^3$	$2.12x10^2 \pm 2.96x10^2$
Ready-to-eat products	$6.29x10^2 \pm 3.68x10^4$	$4.99x10^1 \pm 8.53x10^1$	$1.58x10^1 \pm 4.99x10^1$

Table 4. Microbiological quality of vegetables and ready-to-eat products of 2 markets of Abidjan.

Microorganisms	**Microbiological quality**	**Tomatoes (%)**	**Endives (%)**	**Ready-to-eat products (%)**
Enterococcus	Satisfactory	22	0	50
	Acceptable	28	10	32
	Unsatisfactory	50	90	18
Pseudomonas	Satisfactory	84	6	66
	Acceptable	10	34	30
	Unsatisfactory	6	60	4
Faecal coliforms	Satisfactory	74	56	94
	Acceptable	26	44	6
	Unsatisfactory	0	0	0
E. coli	Satisfactory	80	70	94
	Acceptable	20	30	6

Pseudomonas, 6% of tomatoes, 60% of endives and 4% of ready-to-eat products have an unsatisfactory microbiological quality. The ready to eat products had in percentage the most samples with satisfactory microbiological quality. This good quality is observed in 50% of samples for *Enterococcus*, 66% for *Pseudomonas*, 94% for faecal coliforms and *E. coli*. Samples of endives have an unsatisfactory microbiological quality (Table 4).

Effect of decontamination treatment

Washing methods applied on tomatoes and endives have reduced significantly, the rate of germs when compared

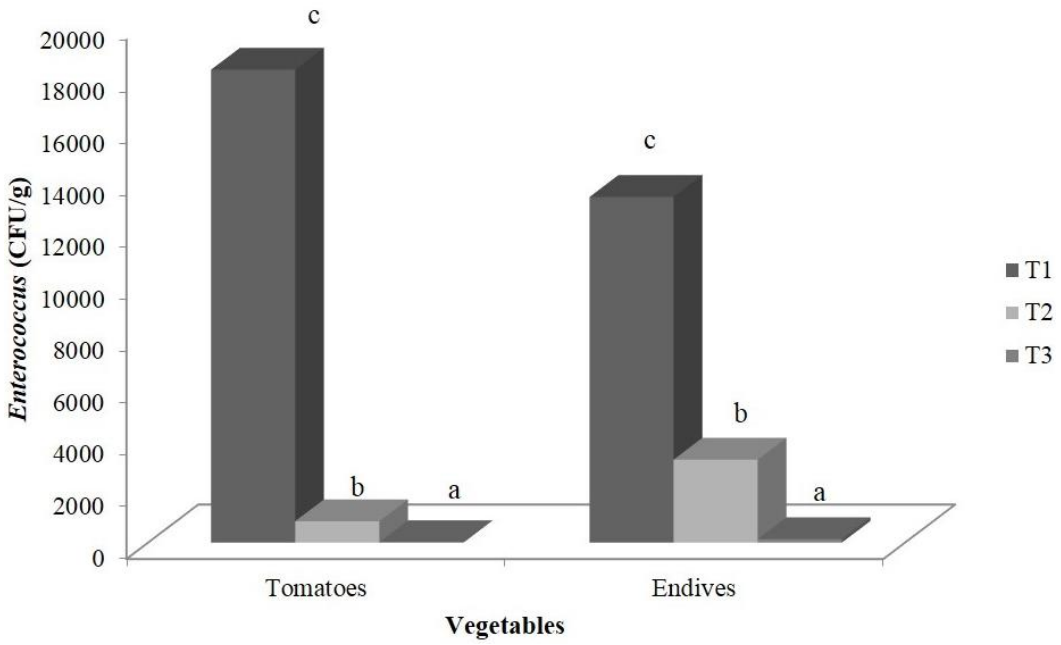

Figure 2. Variation of *Enterococcus* load according to treatments. T1: No washing; T2: Washing with running water by soaking sample during 10 min. T3 Washing with a 1° Chl sodium hypochlorite solution by soaking samples during 10 min. Letters indicate significant difference between the treatments for each vegetable (χ^2 test, $p \leq 0.05$).

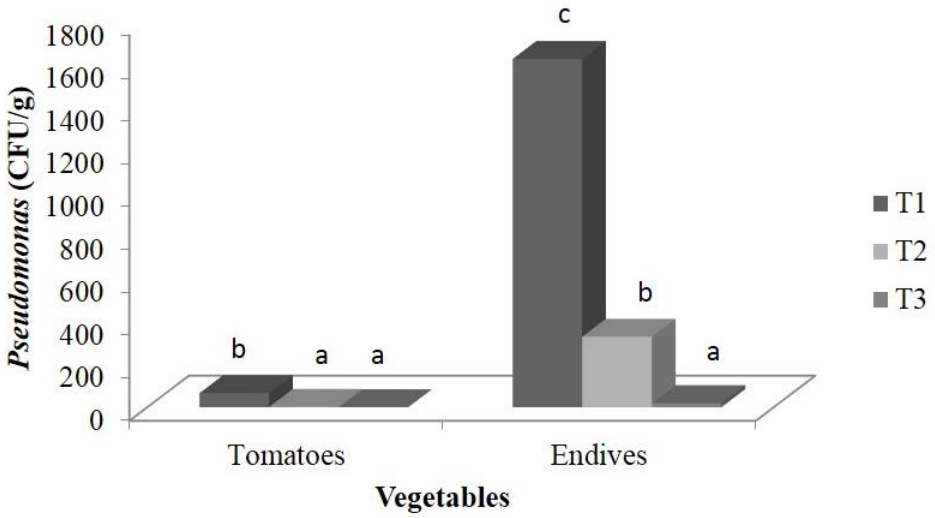

Figure 3. Variation of *Pseudomonas* load according to treatments. T1: No washing. T2: Washing with running water by soaking sample during 10 min. T3: Washing with a 1° Chl sodium hypochlorite solution by soaking samples during 10 min. Letters indicate significant difference between the treatments for each vegetable (χ^2 test, $p \leq 0.05$).

with that of no washing samples (Figures 2, 3 and 4).

All treatments differed significantly ($p<0.05$) for each microorganism except in tomatoes where there is any statistical difference between washing with running water and washing with sodium hypochlorite water 1° chlorimetric for *Pseudomonas* load. The percentage of reduction was 80% for *Enterococcus*, 78% for faecal coliforms and 73% for *Pseudomonas* with the washing with running water and 98% for *Enterococcus* and faecal coliforms and 97% for *Pseudomonas* with the sodium hypochlorite water 1°

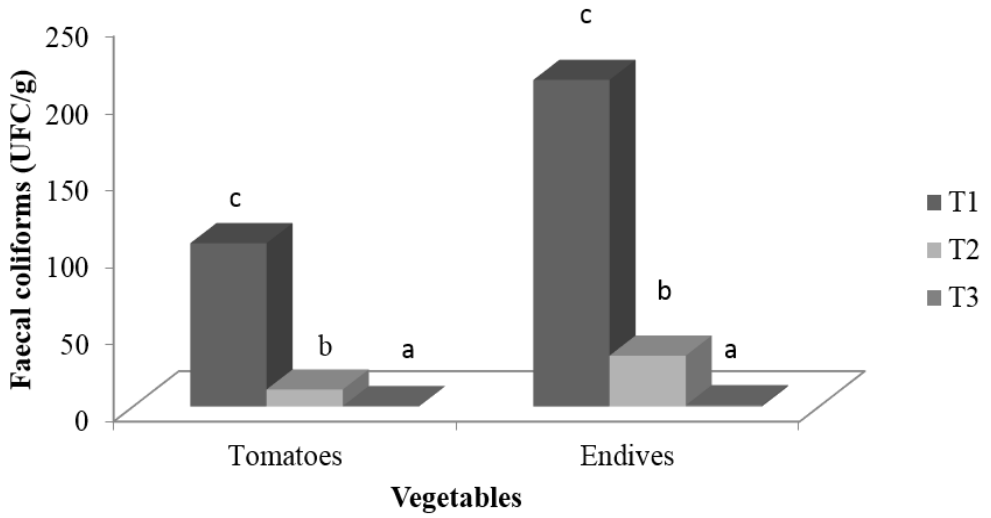

Figure 4. Variation of faecal coliforms load according to treatments. T1: No washing. T2: Washing with running water by soaking sample during 10 min. T3: Washing with a 1° Chl sodium hypochlorite solution by soaking samples during 10 min. Letters indicate significant difference between the treatments for each vegetable (χ^2 test, $p \leq 0.05$).

Table 5. Percentage of microrganisms reduction in all samples.

Germs	Washing with running water	Washing with a sodium hypochlorite solution 1° chl
Enterococcus	80	98
Pseudomonas	73	97
Faecal Coliforms	78	98

chlorimetric (Table 5).

DISCUSSION

The percentage of *Enterococcus* is more important than that of *Pseudomonas* and faecal coliforms. This could be due to the large range of temperature (10 and 45°C) and pH (4 to 9) in which *Enterococcus* is able to grow. It seems to be an advantage for this microorganism (Rollins and Joseph, 2000; Nar et al., 1991). They may be on the vegetables before the harvest but they can also be brought by seller's manipulations. *Pseudomonas* is present in all the samples of endives which constitute a good habitat for microorganism. Indeed, leafy vegetables (like endives) have some large and rough surfaces which are in contact with the ground and the irrigation water facilitating the accumulation of dirt and the adhesion of bacteria (Abadias et al., 2008). Certain species such as *Pseudomonas cichorii* and *Pseudomonas cepacia* can impair the commercial quality of leafy vegetables by inducing some brown or black blemish (Chaux and Foury, 1994). *E. faecalis* is responsible of 90% of infections induced by the *Enterococci* and it was an important life-threatening nosocomial infection (Rollins and Joseph, 2000). *E. coli* used to be more isolated in endives than in tomatoes and ready-to-eat products. This is in phase with Bohaychuk et al. (2009) who have revealed a load of 0.48 log CFU/g in minimally processed vegetables and a load of 2 log CFU/g in raw vegetables.

P. aeruginosa is an opportunistic pathogen of humans responsible of most of the nosocomial infections (Todar, 2002). According to Kominos et al. (1972), a patient consuming an average portion of tomato salad might ingest as many 5.10^3 CFU of *P. aeruginosa*. The detection of faecal coliforms is low in ready-to-eat products and can be explained by the fact that vegetables are thoroughly washed. This result is confirmed by Pingulkar et al. (2001). In their study, Faecal coliforms were absent in ready-to-eat salad samples and *E. coli* was detected in relatively low numbers in batches of some prepared salad vegetables (Brocklehurst et al., 1987).

Tomatoes and endives sold in the markets did not fit to the bacteriological criteria. Most of them have an acceptable or an unsatisfactory microbiological quality. The first cause is the use of partially decomposed organic manure and polluted water on the crops. So, environment

germs are seen on vegetables. A second cause is the bad conditions of harvesting, transportation, sale in market and processing (Sallou, 2001; Adjrah et al., 2011). In fact, markets have an unhealthy environment and sellers usually wash and soak the roots of endives in water in which germs could increase. In ready-to-eat products the contamination was made from the cooking to the selling. They were prepared at markets and although they were thoroughly washed, vegetables could be contaminated by the hands and the utensils which are not disinfected. So, besides the disinfection of food, the kitchen environment must be clean because it can also serve as a reservoir of large numbers of micro-organisms (Kagan et al., 2002). Sodium hypochlorite water 1° chlorimetric has an efficient action on germs. The efficient action was also mentioned by Lorougnon (1996) who revealed 100% of reduction.

Conclusion

The microbiological analysis of raw tomatoes, endives and ready-to-eat products sold in the market revealed a high level of contamination by *Enterococcus*, *Pseudomonas* and faecal coliforms. Most of samples have an unsatisfactory microbiological quality. The treatment applied on vegetables reduced the microorganisms. The best one was the decontamination with sodium hypochlorite solution I° chlorimetric because it reduces the presence of germs to 97% at least. The hygienic quality of ready-to-eat vegetables is satisfying because *E. coli* were detected only in 3 samples. However, all sanitary risk due to the consumption of these vegetables must be avoided. So vegetables need to be cleaned, washed and disinfected.

Conflict of interest

The authors declare that there is no conflict of interest.

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