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Full Length Research Paper

Degradation kinetics of 2,4-dichlorophenoxyacetic and atrazine by *Trametes versicolor* (L.:Fr.) Pilát

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The degradation kinetics of different sources of 2,4-dichlorophenoxyacetic (2,4-D) and atrazine by the natural strain Mo008 of the basidiomycete fungus *Trametes versicolor* (L.:Fr.) Pilát was studied, knowing that in this process the strain used produces an enzyme complex composed of manganese peroxidase (MnP), lignin peroxidase (LiP) and laccase, and the response on the global consumption rate and yield is dependent on the 2,4-D source, being more efficient with an analytical source and mixture with atrazine than amine salt; however, it was more efficient to degrade atrazine. The strain studied (Mo008) consumed 1000 ppm of 2,4-D analytical, 2,4-D amine, 2,4-D plus atrazine and atrazine in 750, 850, 650 and 550 h, respectively.

Key words: *Trametes versicolor*, degradation kinetics, biodegradation, 2,4-dichlorophenoxyacetic (2,4-D), atrazine.

INTRODUCTION

Lignin is one of the most abundant polymers in nature and the fungi that cause white rot of wood are responsible for initiating its depolymerization (Buswell and Odier, 1987; Gold et al., 1989; Kirk and Farrell, 1987; Szklarz et al., 1989). Ligninolytic fungi such as *Trametes versicolor, Phanerochaette chrysosporium* and *Pleurotus ostreatus*, have been extensively studied in the recovery of effluents from various industries due to the production of an enzymatic system capable of degrading lignin and phenolic compounds (Yateen et al., 1998; *Tortella* et al., 2008), which is composed of the manganese peroxidase (MnP), lignin peroxidase (LiP) and laccase (Rothschild et al., 1999; Guo et al., 2000; Ullah et al., 2000; Gómez et al., 2005).

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Diverse species of basidiomicetes have been studied in recent years, due to their ability to degrade lignin and phenolic compounds (Córdoba et al., 2012; Betancur et al., 2013; Stamatiu et al., 2015). Studies in biotechnology and industrial applications of lignolytic fungi are focused on the species that cause white rot of the wood as a group in general, although this is taxonomically and physiologically heterogeneous, which has overestimated the importance of the best studied fungus, *P. chrysosporium* and has left aside other species with better qualities for biotechnological applications (Peralta et al., 1998; Nyanhongo et al., 2007).

T. versicolor is a basidiomycete that has the ability to degrade compounds with varying kinetics, which is attributed to the production of enzymes that degrade lignin, particularly laccase and MnP, which are excreted in amounts that increase during lignolytic activity (Hobbs, 2004; Lin et al., 2008; Lau et al., 2004; Sedarati et al., 2003). In this sense, it is reported that at an initial concentration of 50 mg L^{-1}, *T. versicolor* showed a complete removal of benzene after 14 h of exposure and by increasing it to 300 mg L^{-1} the removal took 42 h. Concentrations of 50 ml L^{-1} of toluene were removed in 4 and 36 h when increased to 300 ml L^{-1} (Demir, 2004). On the other hand, Megan et al. (2010) reported that this species degrades trifluralin and dieldrin in maximum amounts of 24.6 and 115 ml L^{-1} as well as the mixture of both in a concentration of 32.3 ml L^{-1} in a time of 480 h. Within this degradation process, *T. versicolor* excretes an enzyme that acts as a kind of Mn(II)-dependent peroxidase (Johansson and Nyman, 1987). MnP is able to catalyze the oxidation of phenanthrene, fluorene and other phenolic compounds (Collins and Dobson, 1996) suggesting that this enzyme is capable of degrading a large number of phenolic compounds as well as the LiP; however, the laccase enzyme, which is also present in this fungus, does not degrade phenanthrene and fluorene, but does have activity over a wide range of polyphenols (Collins and Dobson, 1996; Majcherczyk et al., 1998). Obtained data by Pozdnyakova et al. (2018) support the hypothesis that, the degree of degradation of the phenolic compounds can depend of the composition of the extracelular ligninolytic complex of strain used. In this regard, for the correct selection of fungal strains for remediation, it is necessary to study the activity of the basic ligninolytic enzymes. This will allow the development of a technological process to avoid the accumulation of toxic substances in the treated objects. On the basis of their degradative properties and the composition of the ligninolytic enzyme system, *T. versicolor* can be employed for detailed study and for the development of technologies of remediation of contaminated environments. The present research was carried out to determine the degradation kinetics of *T. versicolor* natural strain Mo008 as well as to know the enzimatic complex involved in the degradation process over acid 2,4-dichlorophenoxyacetic (2,4-D) and atrazine.

MATERIALS AND METHODS

Strain

In the present investigation, the native strain of *T. versicolor* (L.:Fr.) Pilát isolated from *Crescentia alata* in Jojutla, Mor., México was used (Mo008).

Detection of 2,4-D in liquid medium

To extract 2,4-D from a solution, a methodology proposed by Anonimo (1995) was used, the residues were collected from a steam rod at 50°C and 50 rpm and 50 mL of ethyl alcohol (95%) was added to concentrate the 2,4-D residues.

Calibration curve of 2,4-D, 2,4-D amine and atrazine

This was obtained using the methodology of Bhoi (2011), for which a standard solution of ethylic alcohol (95%) was prepared with 100 mg L^{-1} of 2,4-D analytical (SIGMA®) and 2,4-D amine (Hierbamina®). A solution in distilled water of 10 mg L^{-1} of atrazine was prepared too and from each standard solutions aliquot was taken to prepare a solution of 0, 2, 5, 10, 15 and 20 mg L^{-1} from both sources of 2,4-D and 0, 0.5, 1, 2, 3, 4, 5 and 10 mg L^{-1} of atrazine. The absorbance was measured in a spectrophotometer (Genesys 10uv®) at 287 nm for 2,4-D and 220 nm for atrazine.

Bioreactors for biodegradation of 2,4-D, 2,4-D amine and atrazine

PVC containers with 15 L^{-1} solution with 1000 ppm of 2,4-D (SIGMA®), 2,4-D amine (Herbamina®) and the mixture of 2,4-D amine plus atrazine (Atrazine 90®) at pH 5.0 and 25°C were used as bioreactors. Filters with steril polyurethane fiber support and 10 g of mycelium of *T. versicolor* strain Mo008 and previously developed for 15 days in malta-agar culture medium were constructed and adapted to each biorreactor. The strain was exposed for 1000 h at continuous flow. Every 50 h, 3 samples of 100 ml were collected from each reactor in amber glass bottles and stored at 0°C.

Degradation kinetics

The degradation kinetics was obtained by estimating yield of biomass-substrate and consumption global velocity (CGV) based on the methodology presented by Rubio (2005) and Marron et al. (2006). The yield represents the amount of mycelium produced by the fungus per each ppm consumed (Equation 1) and the CGV like decrease of the concentration of the substrate in a given time (Equation 2).

$$Y = \frac{(Xf - Xi)}{Si - Sf} \quad (1)$$

Where, Y is the yield; Xf is the final biomass; Xi is the initial biomass; Sf is the final concentration; Si is the initial concentration.

$$CGV = \frac{(Si - Sf)}{Tt} \quad (2)$$

Where, CGV is the consumption global velocity; Is is the initial substrate; Sf is the final substrate; Tt is the total time.

Enzymatic analysis

Preparation of enzyme expression fluid

An enzyme expression fluid was prepared according to the methodology of Penninckx and Jiménez (1996) and Jiménez et al. (1999) which contained 1% of glucose, 0.02% of ammonium tartrate, 0.05% of $MgSO_4.7H_2O$, 0.01% of $CaCl_2.12H_2O$, 0.05% of Tween 80, 0.1 mg of thiamine chloride, veratryl alcohol 2.5 mM, 70 ml L^{-1} of trace elements (which contains per liter: 1.5 g of nitriloacetic acid, 3 g of $MgSO^4.5H_2O$, 1 g of NaCl, 0.1 g of $FeSO_4.7H_2O$, 0.1 g of $CaCl_2.2H_2O$, 0.1 g of $ZnSO_4.7H_2O$, 0.01 g of $CuSO_4.5H_2O$, 0.01 g of ALK $(SO_4).12H_2O$, 0.01 g of HBO_3 and 0.01 g of $Na_2MoO_4.2H_2O$) and brought to pH 4.5 with sodium tartrate buffer. In 500 ml Erlenmeyer flasks, 70 ml of expression fluid was added and sterilized at 120°C for 15 min. A gram of mycelium of the Mo008 strain was added with 15 days of development (Jiménez et al., 1997) and it was placed on a shaker at 150 rpm at room temperature. At 48 h, 40 ppm of Mn^{+2} was added and held for an additional 48 h on the shaker.

Preparation of extracts

Mycelium (1 g^{-1}) developed in the enzyme expression fluid was centrifuged (Hermle®) at 5000 rpm for 5 min (Pennickx and Jimenez, 1996). The supernatant was stored at -15°C in total darkness for further use. This same methodology was used to obtain extracts of the mycelium developed in the filters of the bioreactors with the solution of 1000 ppm of 2,4-D amine after 1000 h of exposure in continuous flow.

Conditioning of extracts

The temperature was elevated to 20°C to each extracts, as each one of them was used for the detection of the enzymatic activity.

Calibration curves for the substrates used

To measure the enzymatic activity of LiP, a calibration curve was performed for concentrations from 0 to 5 mM of veratril alcohol in a sodium tartrate buffer 50 mM pH 4.5 and 25°C. For the case of Manganese Peroxidasse (MnP), the curve was estimated for concentrations from 0 to 0.5 mM of phenol red [0.01%] in sodium succinate buffer 0.1 M and for laccase, concentrations from 0 to 9 mM of ABTS 5 mM in 0.1 M sodium acetate buffer pH 5.0. The absorbance was recorded at 310, 610 and 420 nm for each substrate, respectively. From the absorbance data recorded for each of the enzymes studied, the concentration of the substrate (mM) was estimated from the equation obtained from the calibration curve, the extinction coefficient and the enzymatic activity (mM/min/ml). From these results, the Michaelis-Menten kinetic model was obtained as well as the Lineweaver-Burk representation to obtain the maximum reaction velocity (Vm) and the concentration of the substrate for which the reaction speed is half the speed maximum (km)

Determination of enzymatic activity

The activity of LiP was measured using the method proposed by Tien and Kirk (1983), which records the increase in absorbance at 310 nm due to the oxidation of veratril alcohol to veratryl aldehyde. The reaction employs 2.2 ml of sodium tartrate buffer (50 mM, pH 4.5 at 25°C), 40 µl veratryl alcohol (2 mM) and 240 µl of the culture extract. The reaction is initiated by adding 20 µl H_2O_2 (0.2 mM) and the absorbance is measured at 310 nm. The activity of MnP was recorded following the methodology described by Glenn and Gold (1985). This method is based on the oxidation of Mn (II) to Mn (III) and uses as a substrate 2.5 ml of red phenol (0.01%) and $MnSO_4$ (0.1 mM) in sodium succinate buffer (0.1 M). The reaction mixture contains 2.5 ml of substrate and 200 µl of the culture extract. The reaction is started by adding 20 µl of H_2O_2 (0.1 mM), after incubation for 2 min at 30°C and the absorbance WAS measured at 610 nm. On the other hand, laccase activity was recorded using the method described by Bourbonnais et al., (1995) which records the oxidation of 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS substrate, 5 mM). After dissolving the substrate in 2.5 ml of sodium acetate buffer (0.1 M, pH 5.0), 100 µl of the extract was added, the mixture was incubated for 2 min at 30°C and after that time the absorbance was recorded at 420 nm. For all cases, the absorbance was measured at intervals of 30 s for 5 min.

All analysis had three replicates and the variance analysis and Tukey test (α=0.01) was made with SAS® Stadistical Software.

RESULTS AND DISCUSSION

Calibration curve for detection of analytical 2,4-D, 2,4-D amine and atrazine

The obtained curves are represented by a linear model with a correlation coefficient that allows to affirm that the detected absorbance is a direct function of concentration in each one of the samples (Figure 1).

Degradation kinetics of *T. versicolor*

Specifically, 2,4-D is one of the herbiides most used to control boradleaf weeds; the active ingredient has chronic health effects, and lethal on the soil because it has a residual effect. It has been shown that there are microorganisms that have the capacity to degrade these phenolic compounds, which is called bioremediation (Akintui et al., 2015). The degradation kinetics in bioreactors was determined according to the yield of the biomass-substrate, which expresses the increase of biomass of *T. versicolor* for each ppm of consumed substrate (Rubio, 2005), with a response differentiated by the source 2,4 -D (Table 1) as well as the mixture of this with atrazine (Figure 2), confirming a differentiation in the yield for each substrate (Figure 3A), which coincides with Field et al. (1992), Bhalerao and Puranik (2007) and Siddique et al. (2003) who attribute the development of filamentous fungi on different substrates which directly influence respiration and biomass as the only carbon source. Stamatiu et al. (2015) found that some strains exposed to chlorpyrifos and endosulfan outgrow the mycelial development of their respective controls after a period of inhibition (3 to 16 days). *Phanerochaete chrysosporium, P. ostreatus, Bjerkandera adusta* and *T. versicolor* are the most commonly used for the degradation of such compounds owing to their production of ligninolytic enzymes such as LiP, MnP and laccase (Pozdnyakova et al., 2018).

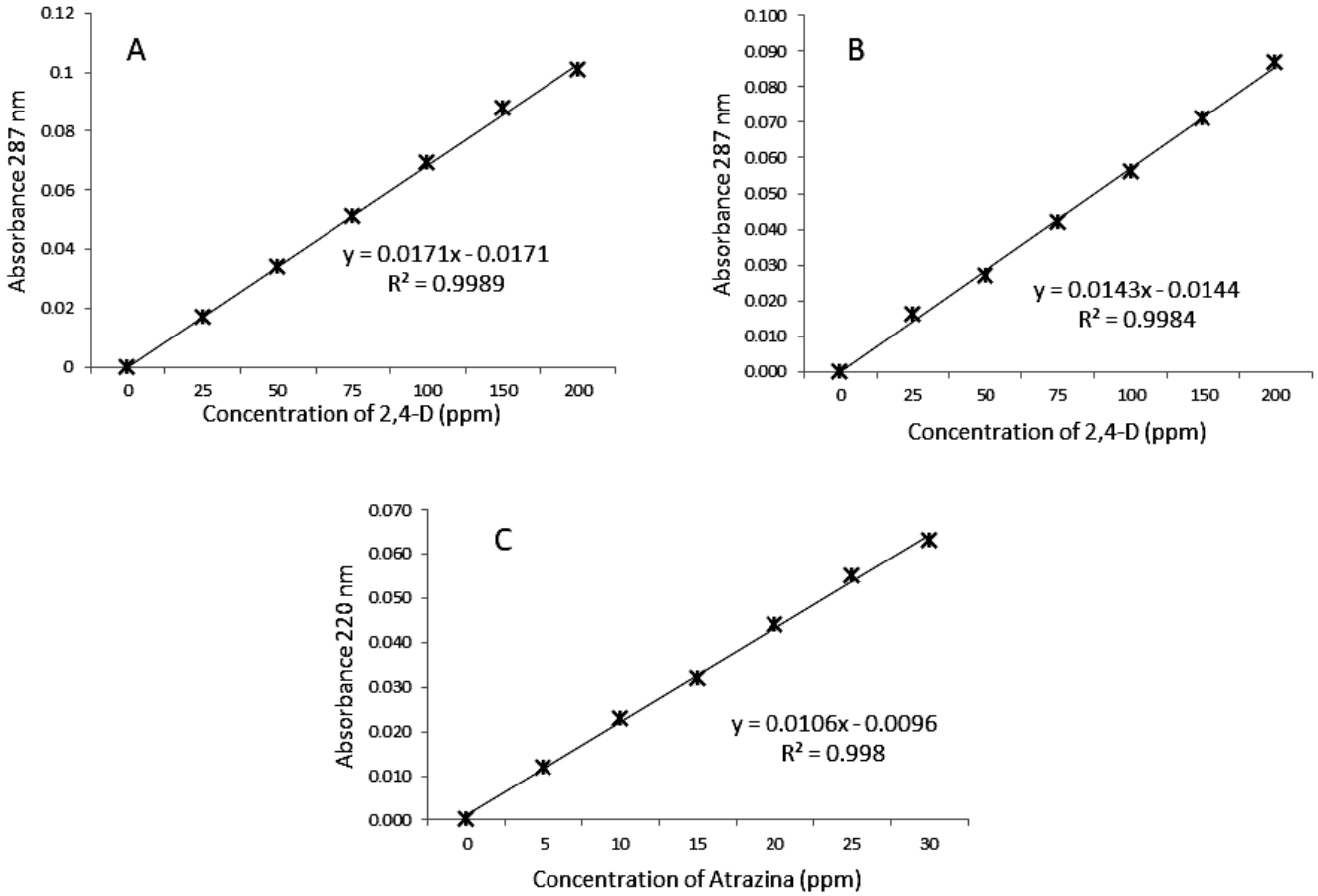

Figure 1. Calibration curves for the detection of analytical 2,4-D (A); 2,4-D-amine (B); and atrazine (C).

Table 1. Bioreactor degradation kinetics of *Trametes versicolor* (L.:Fr.) Pilát strain Mo008.

Parameter	Yield (g ppm^{-1})	GCR* (ppm h^{-1})	Filter biomass (g)	Consumption time (h)
Analytical 2,4-D	0.0121	1.333	12.113	750
2,4-D amine	0.0085	1.176	8.480	850
2,4-D amine + atrazine	0.0117	1.538	11.747	650
Atrazine	-	1.818	-	550

*Global consumption rate.

On the other hand, Dutta et al. (2010) reported a stimulus in basal respiration and in microbial biomass when chlorpyrifos like substrate is used in comparison with the control, whereas Das and Mukherjee (2000) and Eisenhauer et al. (2009) mentioned that soil micro-organisms treated with organophosphorus and organochlorine pesticides increase their population. The global consumption rate (GCR) that expresses the amount of ppm degraded by *T. versicolor* per hour indicates that when 2,4-D amine is mixed with atrazine, it tends to be higher (Figure 3B) than when only analytical 2,4-D and amine salt are used, and the studied strain is more efficient to degrade atrazine than 2,4-D; the 1000 ppm added to the bioreactor was degraded in a time of 550 h with a GCR of 1,818. These results indicate that *T. versicolor* (Mo008) can be used as a biodegradation tool for this type of compounds in high concentrations (1000 ppm) as compared to what has been reported by Demir (2004), Wu and Yu (2006), Srivastava et al. (2008), Kumar et al. (2009), Megan et al. (2010) and Pozdnyakova (2018).

Chlorophenols are the most common organic compounds which are widely used in agricultural industry and public health. The most important pollution sources

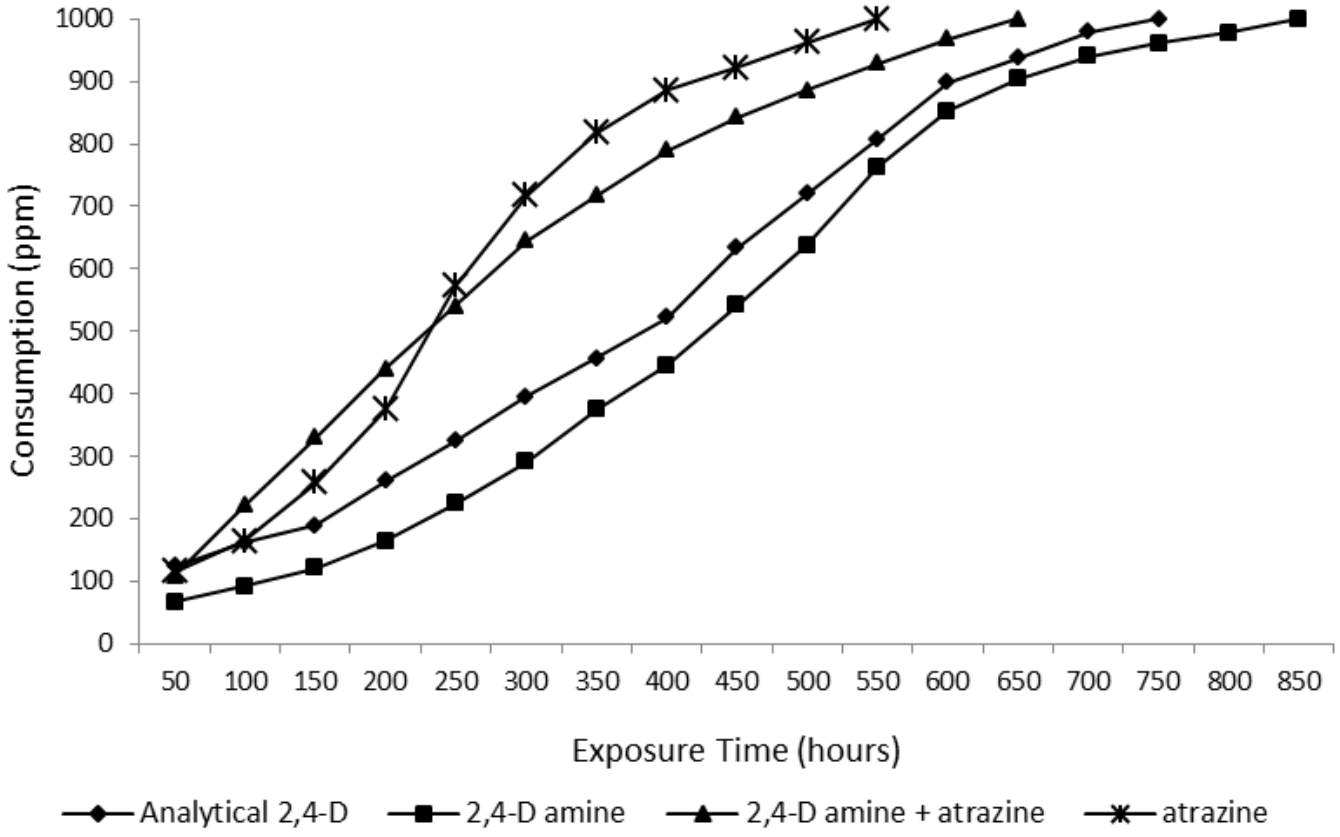

Figure 2. Bioreactor consumption by *Trametes versicolor* (L.:Fr.) Pilát strain Mo008 of each of the substrates evaluated.

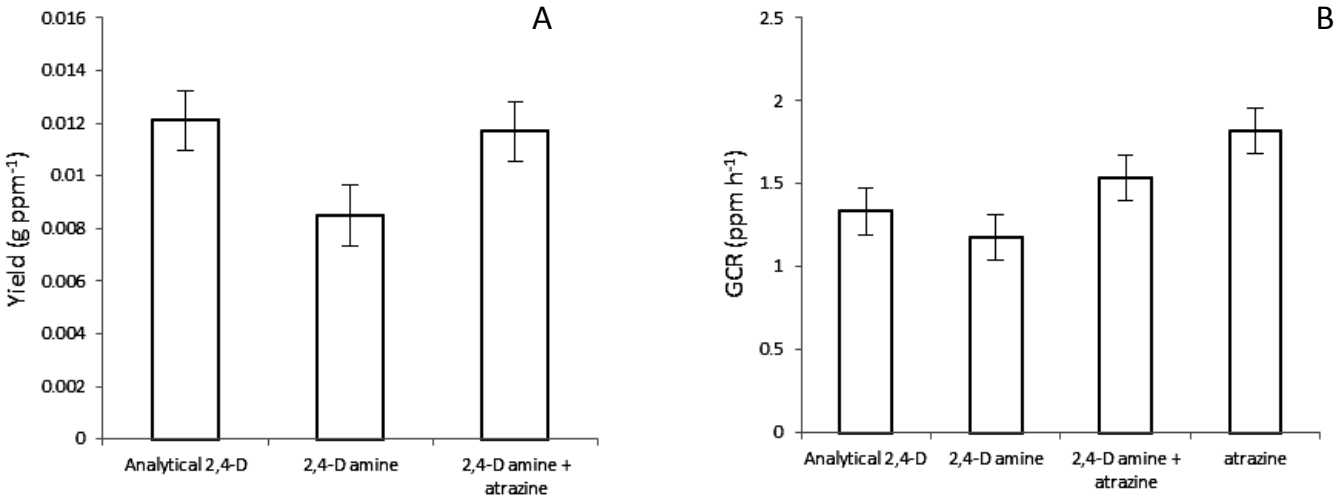

Figure 3. Bioreactor degradation kinetics of *Trametes versicolor* (L.:Fr.) Pilát strain Mo008. **A,** Yield biomass/substrate (g ppm^{-1}); B, global consumption rate (GCR) in ppm h^{-1}.

of chlorophenols are the waste waters from pesticide, paint, solvent, pharmaceutics, wood-preserving chemicals, paper and pulp industries and water disinfecting processes. Because these types of products are toxic, resistant to microbial degradation and can accumulate in the food chain, many countries have restricted or

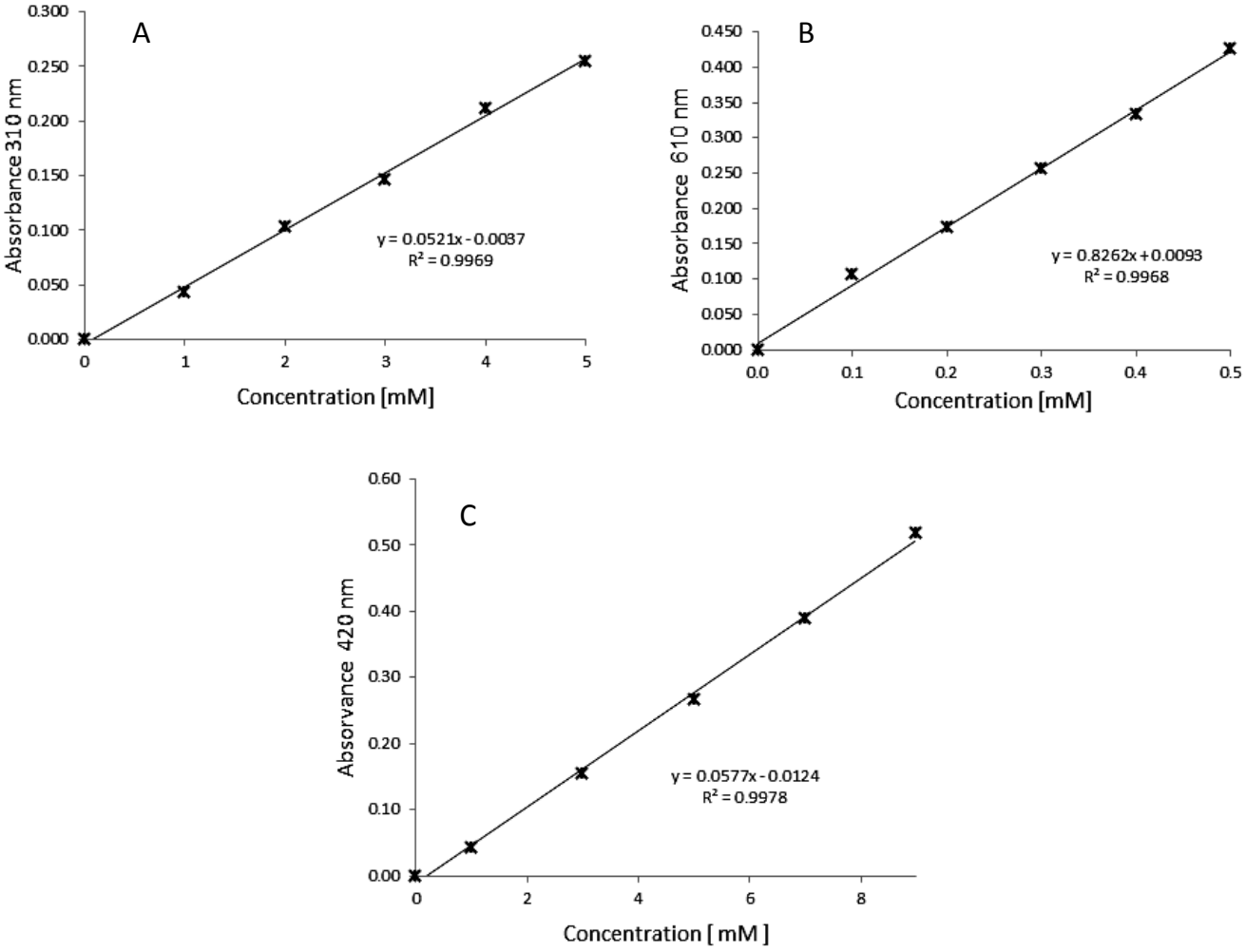

Figure 4. Calibration curve for each substrates to detection of enzymatic complex involved in degradation of 2,4-D by *Trametes versicolor* (L.:Fr.) Pilát strain Mo008. **A,** Lignine peroxidase; B, manganese peroxidase; **C,** lacase.

banned their production or use and have designated them as priority pollutants in their own list of hazardous wastes (Ruiying and Jianlong, 2007). *T. versicolor* has the ability to produce different enzymes that can degrade recalcitrant compounds, for this reason, it is used in biotechnology for bioremediation studies, likewise it can be use to remove Cu^{2+}, Pb^{2+}, Cd^{2+}, Ni^{2+} and Zn^{2+} from organic textile dyes (Congeevaram et al., 2007; Fu and Viraraghavan, 2001; Baldrian, 2003; Bayramoğlu et al., 2003; Solis et al., 2015).

Calibration curves for the measurement of enzymatic activity in degradation of 2,4-D amine and the mixture of 2,4-D amine and atrazine

The calibration curves for each extracts used to detect enzymatic activity are represented by linear models with correlation coefficients above 0.99 as shown in Figure 4.

Enzymatic activity in the degradation of 2,4-D amine and 2,4-D amine plus atrazine

The complex enzymatic involved in the degradation of phenolic compounds (LiP and MnP) was detected in the micelium extracts exposed to 2,4-D amine as well as to the mixture of this with atrazine in continuous flow bioreactor and phenol oxidases or polyphenolic oxidases as laccase. For each enzyme detected, the mycelial extract developed in the enzymatic expression fluid (Control) showed a higher affinity than the mycelial extract developed in the bioreactor (Table 2); however, the development of *T. versicolor* in 2,4-D amine as well as in the mixture with atrazine indicate that this fungus produces the enzymatic complex involved for the degradation of both compounds (Karam and Nicell, 1997; Duran, 1997; Duran and Esposito, 2000) and that the degradation process was greater when the 2,4-D amine was mixed with atrazine than when it was exposed

Table 2. Enzymatic activity of *Trametes versicolor* (L.:Fr.) Pilát detected in strain Mo008.

Km expressed in mM						
	Lignin Peroxidase		Manganese Persoxidase		Laccase	
	2,4-D[a]	2,4-D[b] + Atrazine	2,4-D[a]	2,4-D[b] + Atrazine	2,4-D[a]	2,4-D[b] + Atrazine
Control	0.1377	0.0878	1.7202	0.4092	0.9056	0.9393
Extract	0.1684	0.1217	1.0412	0.5337	0.9047	4.8181
a: amine; b: analytical						
Vm expressed in mM·min^{-1}						
	Lignin Peroxidase		Manganese Persoxidase		Laccase	
	2,4-D[a]	2,4-D[b] + Atrazine	2,4-D[a]	2,4-D[b] + Atrazine	2,4-D[a]	2,4-D[b] + Atrazine
Control	51.0204	67.5674	3.5958	0.5510	188.6792	303.0303
Extract	52.6315	64.1025	0.5605	0.4450	158.7301	416.6666
a: amine; b: analytical						

individually due to the values of speed maximum detected in the last process (Figure 5). Oxidative enzymes play an important role in the decontamination of effluents and soil and *T. versicolor* possesses a complex mechanism involving enzymes that attack lignin directly, like LiP, MnP and laccase (Córdoba et al., 2012); these enzymes can be used in the management of environmental pollutants such as textile effluents, pulp effluents, organochloride agrochemicals and crude oil residues (Kantharaj, 2017) which were detected in the present study. The genus *Trametes*, which belongs to the White-rot fungi, is assumed to be one of the main producers of laccases. *T. versicolor* produces laccase and MnP as major lignolytic enzymes; however, and in a particular case, the role of these enzymes in decolorization of azo dyes is not yet clear. Laccase and/or MnP activities in culture fíltrate of *T. versicolor* were not able to decolorize azo dyes, thus indicating a role of other enzymes or cell-bound components in azo dye degradation (Swamy and Ramsay, 1999). The use of enzymes for the treatment of contaminants has been proposed by numerous researchers, however, most of these investigations are focused on demonstrating the decrease of several contaminantsby biological organisms that produce the enzyme complex studied as a basis for future remediation engineering projects, a fundamental step for its implementation (Gianfreda et al., 1999; Heitzer, 1993; Aitken, 1993; Heitzer, 1998). This enzyme complex is mainly composed of the so-called lignolytic enzymes that include laccasa, MnP and LiP. These enzymes catalyze the oxidation of lignin, but its non-specific nature allows the degradation of xenobiotic compounds with a chemical structure similar to lignin (Dominguez et al., 2010). *T. versicolor* (L.:Fr.) Pilát is a basidiomycete that produces extracellular enzymes that participate in the degradation of lignin in a nonspecific way (García and Torres, 2003) and has the possibility of using it in a broad spectrum of recalcitrant substances that show structural similarities with lignin. However, Katharaj (2017) reports that MnP degrades the lignin mainly by attacking phenolic lignin component. In the presence of H_2O_2, this enzyme oxidizes the phenolic structures by converting Mn^{2+} to Mn^{3+}. Oxalato and malonate are the mediators that produce carbon centered radicals, peroxyl radicals and superoxide radicals which improve the effective lignin-degradings system.

MnP is an essential component to certain basidiomycetes and some wood decaying white-rot fungi, which secrete MnP in several forms into their environment. Laccases are the copper containing polyphenol oxidases which enable degradation of phenolic compounds and also reduce molecular oxygen to wáter (Arora et al., 2010; Divya et al., 2013). Laccases oxidize the phenolic units in lignin to phenoxy radicals, which can lead to aryl-C cleavage (Kawai et al., 1988). Laccase can also oxidize non-phenolic substrates in the presence of certain auxiliary substrates (Call and Muncke, 1997; Kantharaj et al., 2017). Compounds such as chloro-phenols, polychlorinated biphenyls (PCBs), DDTs, dioxins, polycyclic aromatic hydrocarbons (PAH's), alkyl halides, nitrotoluenes, azo dyes and polymers can be modified or degraded to varying extents (Linn et al., 1993; García, 2001; Raimbault, 1998; Gold and Allic, 1993; Karam and Nicell, 1997; Kuhad et al., 1997; Majcherezyk et al., 1998); in addition, 2,4-D amine and atrazine can be efficiently degraded by this species as shown in this investigation.

The enzymes produced by the fungi *T. versicolor* was also employed for the detoxification of aromatic pollutants like agrochemicals and industrial effuents (Kantharaj et al., 2017). In recent years, the presence of micropollutants such as pharmaceuticals, industrial chemicals, personal care products and many other chemical compounds in the aquatic enviroment have become a significant problem worldwide (Doruk et al., 2018). Sahadevan et al. (2016) reported that lignin-degrading enzymes, LiP, MnP and laccase can be used like appropriate biological substitute to treat highly alkaline effluents like pulp, paper industry and waste water and various non-steroidal, anti-

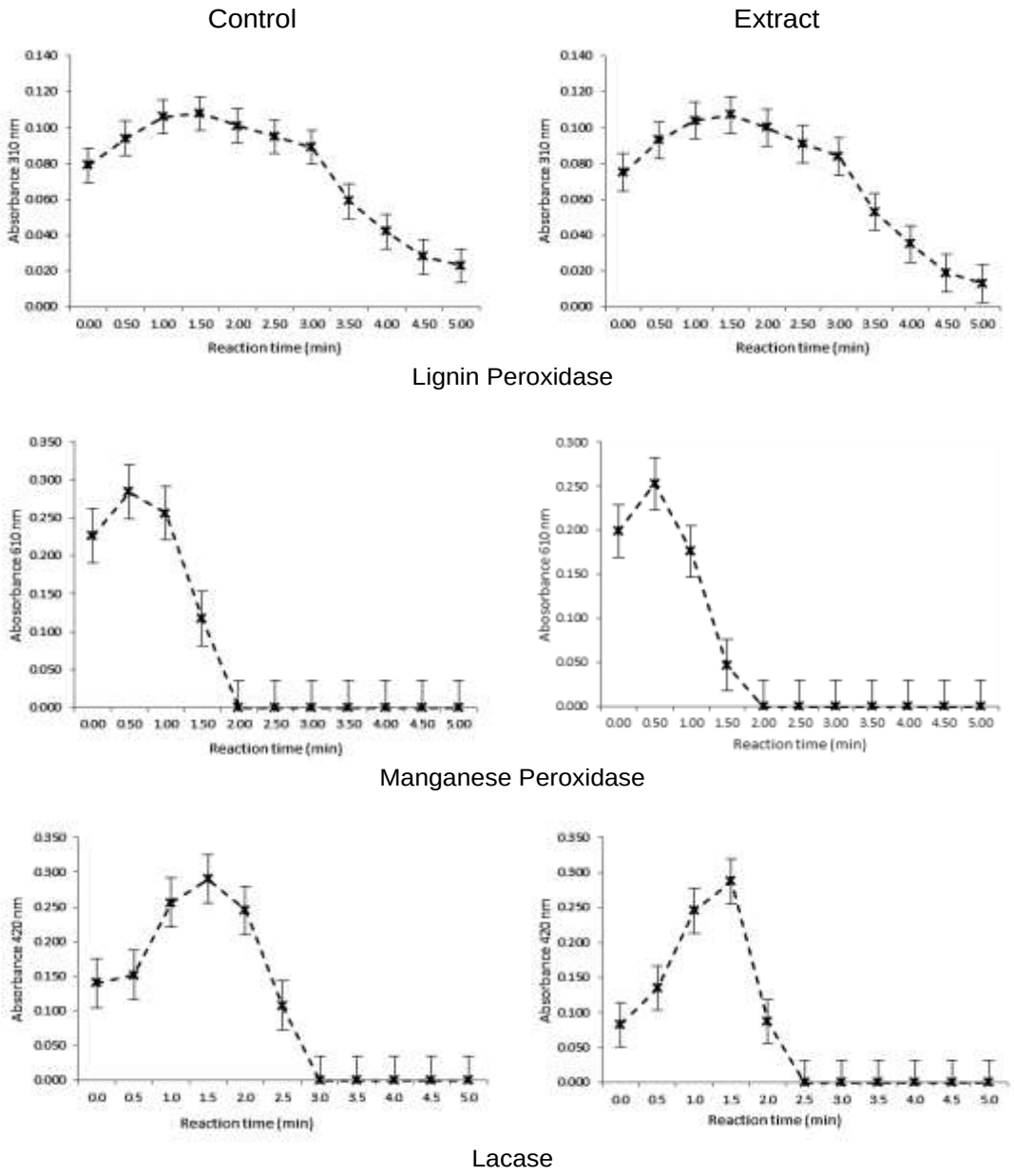

Figure 5. Average absorbance for each of the enzymes detected in the *Trametes versicolor* (L.:Fr.) Pilát strain Mo008 extract developed in the bioreactor filters exposed to analytical 2,4-D, 2,4-D amine, 2,4-D amine plus atrazine and atrazine.

inflammatory drugs such as naproxen, ketoprofen and ibuprofen (Marco et al., 2009; Marco et al., 2010a, 2010b).

Conclusions

T. versicolor (Natural strain Mo008) efficiently degraded atrazine and 2,4-D, being more efficient in the degradation of 1000 ppm of atrazine (550 h) than of 2,4-D (850 h); however, the mixture of both herbicides was consumed in a time of 650 h. Likewise, in the biodegradation of both, alone and as a mixture, the studied strain presented the activity of the enzymatic complex which was composed of laccase, LiP and MnP which have an important role in the degradation of phenolic compounds and other recalcitrants wastes due to their similiar structure to lignin. *T. versicolor* can be employed as a bioremediation tool for water contaminated with acid 2,4-D and atrazine and others phenolics compounds as well as pharmaceuticals wastes.

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CONFLICT OF INTERESTS

The authors have not declared any conflict of interests in this research and its publication.

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Full Length Research Paper

Isolation and characterization of *Ralstonia solanacearum* strains causing bacterial wilt of potato in Nakuru County of Kenya

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In Africa, cultivation of potato (*Solanum tuberosum*) represents an important food source and income generation. However, its productivity is constrained by biotic and abiotic stresses. Bacterial wilt caused by *Ralstonia solanacearum* is an important constraint to the world potato industry. In Kenya, the disease affects 77% of potato farms causing yield losses of up to 100%. Control methods are limited mainly due to the broad diversity and wide spread of its pathogen. Understanding the population structure and geographical distribution of this pathogen is an important starting point in the development of effective control strategies. In this study, *R. solanacearum* strains affecting potato cultivation in Nakuru county were successfully isolated and characterized. Kelman's triphenyl tetrazolium chloride media were used to isolate the pathogen and 20 isolates were selected based on their virulence for further characterization and confirmation of their status at the molecular level through polymerase chain reaction using 759/760 primers and sequencing of partial endoglucanase (*egl*) gene. The phylogenetic assay was done using specific primers and it was found that the phylogenetic diversity was highly heterogeneous, since all the four phylotypes of *R. solanacearum* were identified. Phylotype I was the most prevalent phylotype and represented 50% of the collection. Based on their ability to utilize sugars and alcohols, all the isolates were grouped as biovar III except 2 (Rs18 and Rs49). The aggressiveness of isolated bacteria was then evaluated using a hypersensitive reaction test on tobacco and their virulence was further confirmed on a susceptible potato variety Shangi under greenhouse conditions. All isolates elicited a reaction in tobacco with different grades. They also showed varying levels of virulence with Rs6 isolate being the most virulent. Taken together, these findings provide baseline information for improvement programs targeting host-based resistance to multiple strains causing bacterial wilt of potato in this region.

Keywords: Bacterial wilt, Biovar, hypersensitive reaction, pathogenicity, phylotype, potato, *Ralstonia solanacearum*.

INTRODUCTION

Potato (*Solanum tuberosum*), a starchy edible tuber belonging to the Solanaceae family is one of the most important food crops in the world (FAO, 2010). The nutritional value of this crop is attributed to its ability to supply high quality proteins, essential amino acids, vitamins, minerals and trace elements to the human diet

(Abong et al., 2009; Ahuja et al., 2013). The plant is ranked the fourth staple food crop after rice, wheat and corn with its average global production estimated at 380 million tonnes under production on 20 million hectares of land in 2016 (FAOSTAT, 2018). In Kenya, potato is the second most cultivated crop after maize with an annual production of about 3 million tonnes which is valued at USD 500 million (GIZ-PSDA, 2011; MoALF, 2016). This represents 8 to 15 t/ha in terms of average production relative to cultivated area which is low compared to the expected 30 to 40 t/ha under normal field conditions (Muthoni et al., 2013; Gitari et al., 2018). The low yield is mainly attributed to various challenges ranging from biotic to abiotic stresses. This includes lack of nutrients, pests and high incidences of diseases particularly, bacterial wilt disease caused by *Ralstonia solanacearum* which is still the most devastating disease of potato and the Solanaceae family as whole (Kaguongo et al., 2010). The disease has been found to occur in all the potato growing areas of the country affecting 77% of potato farms and causing yield losses of 50 to 100% (Kaguongo et al., 2010; Muthoni et al., 2012).

Several management practices have been proposed and implemented to control the disease and these include phytosanitary, cultural and chemical methods as well as breeding for resistance. However, these strategies have not been 100% effective in controlling the disease, although in locations where the pathogen is established, a combination of diverse methods have shown some promising results (Champoiseau et al., 2009). The diversity of *R. solanacearum* species, couple with its wide host-range as well as its persistence in the soil are the most significant impediments to the existing control methods. Diversity is key not only in understanding the phyto-pathological interactions between the host and pathogens but also in developing control measures. Virulence and pathogenicity (Hayward, 1991; Kinyua, 2014), phylotype (Fegan and Prior, 2005; Sagar et al., 2014) and biovar determination based on biochemical properties (Fegan and Prior, 2005) are among strategies employed in identification and characterization of *R. solanacearum* strains. Recently, phylotype have been improved to sequevar level based on the similarity of a 750-bp fragment of the endoglucanases (*egl*) gene and so far, 55 sequevars have been identified (Li et al., 2016; Liu et al., 2017).

The current study sought to isolate, characterize and define the population structure of *R. solanacearum* strains affecting potato cultivation in Nakuru County-Kenya. Here, successful recovery and identification of the pathogen from infected potato plants with further classification of the isolates into respective biovars and phylotypes were reported. It is envisaged that this information will contribute towards an integrated management approach for better control of bacterial wilt disease, resulting in reduced losses and poverty.

MATERIALS AND METHODS

Survey site, sample collection and identification of *R. solanacearum*

Samples entailing plants presenting typical bacterial wilt symptoms (CIP, 2017) were randomly collected from ten farms in three different sub-counties in Nakuru county located within the Great Rift Valley. A total of 10 farms were surveyed for wilting plants in Kuresoi North, Njoro and Mau Narok. Five samples per farm were packaged in collection bags and transported to the Plant Transformation Laboratory at Kenyatta University for isolation and further characterization of pathogen. Prior to isolation, infected samples were first watched with tap water for 5 min, surface sterilized using 2% sodium hypochlorite (NaOCl) and rinsed three times with sterile distilled water. The pathogen isolation was done by plating 0.5 cm of surface the sterile plant tissue on Kelman's triphenyl tetrazolium chloride (TZC or TTC) medium (Kelman, 1954). The plated tissues were incubated at 28°C in an incubator and monitored until the bacteria colonies were formed. Individual distinct colony from each sample was streaked onto new TZC medium to obtain pure culture. The resulting isolates were given codes based on the collection areas and preserved in 25% (v/v) glycerol solution at -20°C to be used in subsequent experiments. Morphological characteristics were used to classify the isolated bacteria into virulent (based on milky, flat, irregular, fluidal colonies with pink or red color center and whitish margin) and avirulent strains (smaller, off-white and non-fluidal or less fluidal colonies). This was done on TTC medium containing 0.005% TTC according to Kelman (1954). The virulent isolates were selected for further analysis.

Molecular characterization of *R. solanacearum*

The isolated *R. solanacearum* were characterized using polymerase chain reaction (PCR) to validate their species, determine their phylotype and infer their evolutionary relationships by sequence analyses of the partial endoglucanase (*egl)* gene. To extract genomic DNA, bacterial cells retrieved from the glycerol stock cultures were grown on TZC agar medium and a single colony was transferred into nutrient broth. The cultures were then incubated overnight at 28°C in a shaking incubator. DNA was extracted using a DNA extraction kit (Qiagen, USA) according to the manufacturer's instructions. The DNA quality was checked through gel electrophoresis and quantified using a nanodrop spectrophotometer (Maestrogen, Taiwan). The *R. solanacearum* species was determined by using universal primer of *R. solanacearum* species: 759/760 which produces 281 bp amplicons of the species genome, a common region among *R. solanacearum* (Fegan and Prior, 2005). Identification of the phylotypes was done using a multiplex PCR based on phylotype-specific primers shown in Table 1 as described by Fegan and Prior (2005) and Sagar et al. (2014). A DNA sample was randomly selected from each resulting phylotype, to carry out a PCR targeting the endoglucanase gene

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Table 1. Primers used for species validation, phylotypes determination and endoglucanase gene sequencing.

Primer name	Sequences	Amplicon size (bp)	Tm (°C)	Remark
759R	5'-GTCGCCGTCAACTCACTTTCC-3'	280	55	Species validation
760F	5'-GTCGCCGTCAGCAATGCGGAATCG-3'			
Nmult:21:1F	5'-CGTTGATGAGGCGCGCAATTT-3'	144	55	Phylotype I
Nmult:21:2F	5'-AAGTTATGGACGGTGGAAGTC-3'	372	55	Phylotype II
Nmult:23:AF	5'-ATTACGAGAGCAATCGAAAGATT-3'	91	55	Phylotype III
Nmult:22:InF	5'-ATTGCCAAGACGAGAGAAGTA-3'	213	55	Phylotype IV
Nmult:22:RR	5'-TCGCTTGACCCTATAACGAGTA-3'	-	-	-
Endo-F	5′-ATGCATGCCGCTGGTCGCCGC-3′	~750	62	*egl* gene
Endo-R	5′-GCGTTGCCCGGCACGAACACC-3′			

Tm, Melting temperature.

shown in Table 1. All PCR amplifications were carried out in a 25 µl reaction mix containing: One Taq 2X Master mix with standard buffer (New England, Biolabs), 0.2 µM of each primer and 2 µl of the 5 µg/µl DNA template. Amplifications were done in an automated thermocycler (Eppendorf AG, 22331 Hamburg, Germany) using the following conditions: initial denaturation at 94°C for 3 min, followed by 35 cycles comprising denaturation at 94°C for 30 s, the annealing temperatures specific to each primer for 30 s and extension at 72°C for 30 s. Final extension was done at 72°C for 5 min. The phylotype of each isolate was determined according to the size of the amplified product (Table 1) after separation on 1.5% agarose gel stained with SYBR® Green and visualized under UV light transilluminator (UVIdoc HD2, Cambridge). The PCR products for sequencing were cleaned using a PCR purification kit (Qiagen, USA) then sequenced using the forward and reverse primers at Inqaba Biotech (Inqaba, South Africa).

Sequence analysis and phylogeny

The sequences obtained from Sanger sequencing platform were retrieved from Inqaba's server. The primer sequences were removed using Vector NTI Advance (Invitrogen, USA). Resulting sequences were then used to query nucleotide databases at National Center for Biotechnology Information (NCBI, USA) using nucleotide Basic Local Alignment Search Tool (BLASTn). Four best matching results per query sequence were selected based on the highest percentage of identity and low E-values. The selected matches were then retrieved and aligned using default parameters in ClustalW's tool in MEGA7 software. Phylogenetic tree was constructed using Maximum Likelihood (ML) algorithm based on Jukes-Cantor model with 1000 bootstrap resampling of the data to test the tree topologies (Kumar et al., 2016).

Biovar determination

Isolated *R. solanacearum* were differentiated into biovars based on their ability to oxidize three disaccharides (maltose, lactose and cellobiose) and three hexose alcohols (mannitol, sorbitol and dulcitol) as previously described by Hayward (1954). Standard biovar test medium (basal medium) was prepared by adding 1.0 g $NH_4H_2PO_4$, 0.2 g KCl, 0.2 g $MgSO_4.7H_2O$, 1.0 g bactopeptone, 3.0 g agar and 80 mg Bromothymol blue into a final volume of 1 L of distilled water (Denny and Hayward, 2001). The pH was adjusted to 7.0 then 10% solutions of cellobiose, lactose, maltose, dulcitol, mannitol and sorbitol were prepared separately and 1 part of each carbon solution was mixed with 9 parts of the basal medium to obtain a final concentration of 1% of the carbohydrate. The medium was autoclaved and allowed to cool before 150 µl of each preparation was dispensed into the 96 wells micro-titration plates. All isolates were inoculated into individual wells with 50 µl of bacterial suspensions adjusted to OD_{600} = 0.1 (~10^8 cfu/mL), with two replicates per isolate. The plates were incubated at 28°C and monitored daily for change in pH by a color change (Schaad et al., 2001). The experiment was repeated 3 times.

Determination of *R. solanacearum* aggressiveness using hypersensitivity reaction test

A total of 20 *R. solanacearum* isolates were determined as virulent based on Kelman (1954) method. To further analyze the aggressiveness of the 20 isolates, a hypersensitive reaction (HR) test was conducted using tobacco model plant through bacterial infiltration. Briefly, tobacco plants (*Nicotiana tabacum*) were first grown on autoclaved soil and maintained in a glasshouse at 18 to 22°C with regular watering. The inoculum was prepared by growing bacterial overnight in liquid nutrient broth medium as earlier described then pelleted by centrifugation. The concentration of cell suspension was adjusted to OD_{600} = 0.1 (~10^8 cfu/mL) using a spectrophotometer (JENWAY 6300, Dunmow, UK). Fully expanded leaves from 54 days old plant were infiltrated with a suspension of each *R. solanacearum* isolate using a sterile syringe following the injection technique described by Klement (1963). Sterile distilled water was used as a negative control while an isolate provided by CIP was also included as a positive control. Two leaves per plant from a total of 4 plants were infiltrated with each isolate and HR described as necrotic or yellowing areas in the region surrounding an infection point monitored daily for 2 weeks post infiltration. This was scored as described by Shahbaz et al. 2015). The experiment was repeated 3 times and the data were presented in a graph using graphpad prism version 6 to show the most frequent reaction induced by the identified isolates.

Pathogenicity test of *R. solanacearum* on potato

To confirm the virulence of the *R. solanacearum* isolates, pathogenicity test was performed on potato seedlings of variety Shangi by root irrigation method according to Rado et al. (2015).

This variety was chosen based on its agronomic traits and farmers preferences as it the most common potato cultivar cultivated in Nakuru County (Mwaniki et al., 2016; Gitari et al., 2018). Certified potato seeds of this cultivar were purchased from the Agricultural Development Corporation (ADC) in Molo-Kenya and sown in autoclaved potted soil. One tuber was sown per pot and emerging seedlings thinned to leave one plant in each pot. The plants were maintained in a glasshouse with natural light conditions (12 h of light and 12 h of darkness), temperatures of 24 to 28°C and a humidity of 60%. The seedlings with 4 to 6 expanded leaves were then infected with the *R. solanacearum* isolates. To prepare the inoculum, one *R. solanacearum* isolate was randomly selected from each for each surveyed areas and grown on TZC plates for 48 h at 28°C. A single colony showing virulence (fluidal, irregular and creamy white with pink at the center) was selected from each culture and transferred into a 10 mL tube containing modified Kelman media (MKM) (French and Elphinstone, 1995) then incubated at 28°C for 24 h on a shaker (180 rpm). The cultures were then pelleted by centrifugation, the bacterial cells suspended in sterile distilled water and their concentration normalized to OD_{600} = 0.1 (~10^8 cfu/mL). Plant infection was done using the root irrigation method described by Rado et al. (2015). According to the protocol, the soil layer around the stem was scooped to the side of the plant, the main root wounded by gently scratching with a sterile 1 mL tip and 10 mL of the prepared bacterial suspension poured around the base of the injured plant. The scooped soils were returned to cover the injured area and the plants maintained under the same conditions. Ten plants were then inoculated with each of the four selected isolates. Sterile distilled water was also included as a mock infection to act as a normal control and the experiment was repeated 3 times. Virulence of the isolates was assessed by analyzing bacterial wilt symptoms (CIP, 2017) on infected plants. From the first day when the wilt symptom appeared, scoring was carried out weekly for a month following Timila and Manandhar (2016) method. Plants with visible symptoms (wilted leaves) were recorded as diseased plants (Park et al., 2007). The disease incidence (DI) was calculated following the method by Xue et al. (2009) as DI (%) = [∑ (number of diseased plants in this index × disease index) / (total number of plants investigated × the highest disease index)] × 100%. The data were presented as mean of disease incidence (DI) percentage in a disease progression curve using graphpad prism version 6. The pathogen was re-isolated from diseased plants for confirmation.

RESULTS

Isolation, species validation and virulence of the pathogen

Cultural methods and media used in the current study allowed isolation of 54 isolates from the 3 sub-counties Kuresoi North, Mau Narok and Njoro sampled in Nakuru county. These isolates, suspected to be *R. solanacearum,* were subjected to various confirmatory biochemical and molecular tests to ascertain their identity. The Kelman's TZC agar differentiation test gave pink or light red color colonies with characteristic red center and whitish margin for the virulent isolates while the avirulent isolates produced smaller, off-white and non-fluidal or dry colonies on TZC medium after 48 h of incubation (Figure 1). Twenty isolates from those producing fine pink or light red color colonies were randomly selected from the sample areas for further characterization. A PCR analysis on the 20 isolates from those producing fine pink or light red color with whitish merges colonies using 759/760 primers returned a 281 bp amplicons confirming the isolates to belong to *R. solanacearum* species as shown in the representative gel in Figure 2.

Phylotype analyses

The *R. solanacearum* strains of potato from Nakuru showed different levels of genetic diversity. The prevalence and distribution of the 4 phylotypes varied throughout the 3 major potato-growing areas in the County. The greatest diversity was found in Kuresoi North. The second highest diversity was found in Njoro, with 3 different phylotypes (I, III and IV) while all strains from Mau Narok were only identified as phylotype I. In addition, it was found that the most prevalent phylotype was phylotype I, which represented 50% of the collection (Table 2).

Characterization of partial endoglucanase (*egl*) gene sequences

Sequencing of the partial endoglucanase (*egl*) gene allowed further confirmation of the cultures to belong to *R. solanacearum* species. The sequences have been deposited in the NCBI database awaiting accession numbers. Multiple sequence alignment and phylogenetic analysis resulted in generation of a phylogenetic tree indicating the evolutionary relationships of the current isolates with previous isolates deposited at NCBI (Figure 3). According to the Maximum Likelihood algorithm, the analyzed *R. solanacearum* clustered into 3 groups with the isolates from this study falling in groups I and III. Isolate Rs15 was found in group I, with close relation to other *R. solanacearum* and *Ralstonia syzygii* the causal agent of banana blood disease. The remaining three isolates were clustered alone in group III (Figure 3).

Differentiation of the identified *R. solanacearum* into biovars

All the 20 isolates of *R. solanacearum* with the exception of 2 (Rs18 and Rs49 whose biovars were not identified) were able to oxidize the disaccharides and sugar alcohols within 3 to 5 days although at different rates. Their ability to oxidize the substrates was confirmed by a change of the basal media color from blue-green to yellow upon incubation with bacteria isolates. Eighteen isolates which oxidized all substrates were therefore classified as belonging to biovar III. The 2 other isolates Rs18 and Rs49 were not identified due to their inability to oxidize certain substrates (Table 3). Rapid oxidation of mannitol and sorbitol were observed in relative to other

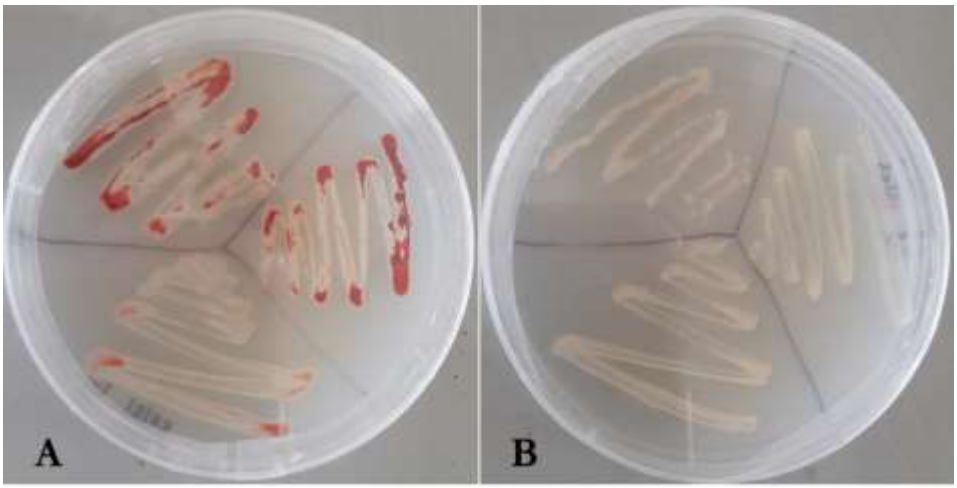

Figure 1. Appearance of *R. solanacearum* isolated on TZC medium. A, virulent colonies; B, avirulent colonies.

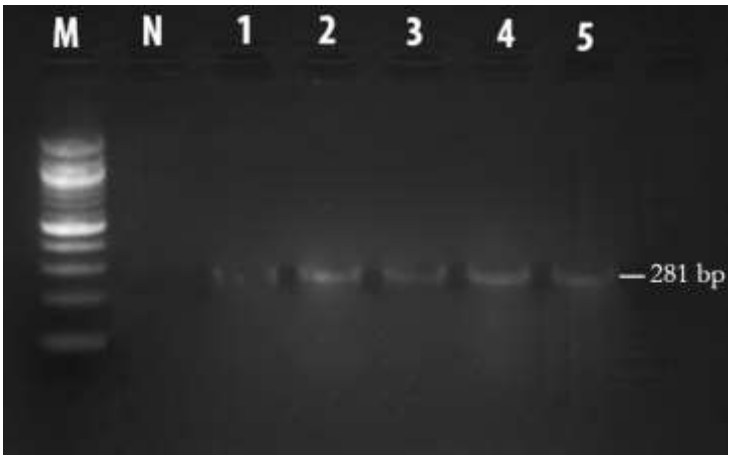

Figure 2. *R. solanacearum* species validation with 759/760 primers; M, 1kb DNA ladder; N, negative control; 1-5, bacterial isolates.

substrates. A color change in the test medium containing these substrates was observed by 2 days after culture while in dulcitol, complete color change occurred at 4 days after incubation. All the 3 disaccharides were utilized at a similar rate and resulted in complete color changes at the 4th day. Control plates with sterile distilled water did not show a color change following incubation with all bacterial isolates.

Analysis of the virulence and pathogenicity of *R. solanacearum* in plants

To assess whether there were any differences in virulence and pathogenicity of the identified *R. solanacearum* isolates, tobacco leaves were infiltrated with bacterial inoculum of the 20 isolates and monitored development of hypersensitivity reactions. It was observed that all the isolates resulted in a hypersensitivity reaction from the infiltrated plants manifested by slight localized chlorosis followed by necrosis, although at different grades (Figure 4). Tobacco leaves infiltrated with sterile water were unaffected. Isolates Rs9A and Rs37 resulted in the highest hypersensitive response in the tobacco leaves and were therefore considered the most virulent (Table 1). On the other hand, most of the isolates were able to induce only the first grade of reaction, slight localized chlorosis (Figure 5).

Four isolates representing the four phylotypes were randomly selected following the HR assay and were used to analyze their aggressiveness on the most cultivated potato cultivar Shangi. Upon infection, disease symptoms

Table 2. Characteristics of the identified *R. solanacearum* isolates.

S/N	Isolate	Origin	Virulence	HR	Phylotypes
1	Rs1A	Muthera farm1	+	+	III
2	Rs2A	Mau Narok farm2	+	+	I
3	Rs4A2	Mau Narok farm1	+	+	I
4	Rs6	Kuresoi North farm6	+	++	IV
5	Rs8	Mau Narok farm2	+	+	I
6	Rs9A	Kuresoi North farm2	+	+++	IV
7	Rs13	Muthera farm2	+	+	I
8	Rs13B	Muthera farm2	+	++	I
9	Rs15	Kuresoi North farm3	+	++	II
10	Rs18	Kuresoi North farm6	+	+	II
11	Rs19	Muthera farm1	+	++	III
12	Rs21	Kuresoi North farm5	+	+	III
13	Rs26	Mau Narok farm2	+	++	I
14	Rs34	Muthera farm1	+	+	IV
15	Rs35	Muthera farm1	+	+	I
16	Rs36	Kuresoi north farm1	+	++	I
17	Rs37	Kuresoi north farm2	+	+++	IV
18	Rs49	Mau Narok farm2	+	+	I
19	Rs56	Mau Narok farm4	+	++	I
20	Rs57	Muthera farm1	+	++	III
21	Reference	CIP, Kenya	+	++	IV

HR positive score: slight localized chlorosis (+) followed by necrosis (++) and collapse of whole leaf (+++); in virulence test: + denotes a positive reaction showing colonies with fine characteristic of virulent strains of *R. solanacearum* as described by Kinuya (2014).

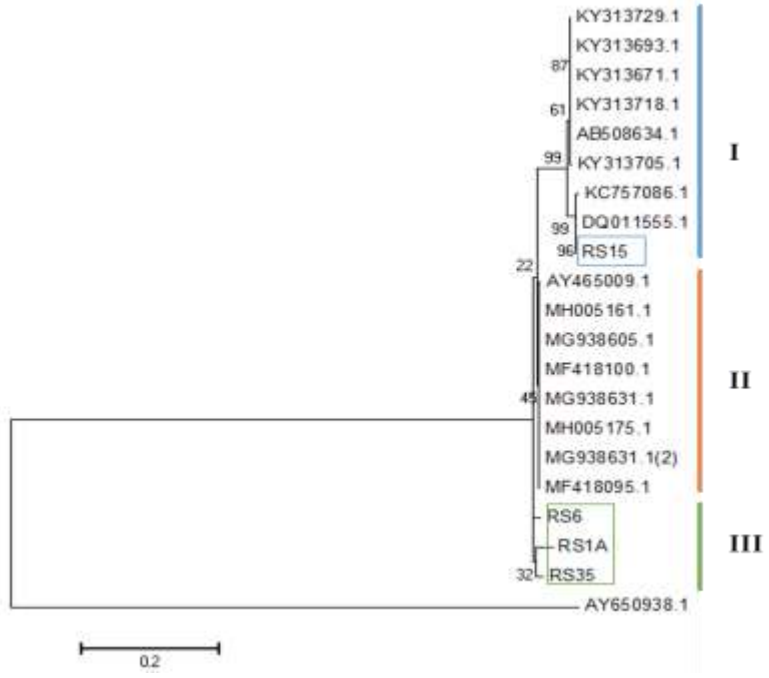

Figure 3. Evolutionary relationships between the identified *R. solanacearum* strains and other known sequences from NCBI. The phylogenetic tree was generated using the maximum Likelihood algorithm in MEGA 7 following a multiple sequence alignment.

Table 3. Biovar differentiation of the identified *R. solanacearum* isolates.

Isolate	Cello biose	Maltose	Lactose	Mannitol	Sorbitol	Dulcitol	Control	Biovar
Rs1A	+	+	+	+	+	+	-	III
Rs2A	+	+	+	+	+	+	-	III
Rs4A2	+	+	+	+	+	+	-	III
Rs6	+	+	+	+	+	+	-	III
Rs8	+	+	+	+	+	+	-	III
Rs9A	+	+	+	+	+	+	-	III
Rs13	+	+	+	+	+	+	-	III
Rs13B	+	+	+	+	+	+	-	III
Rs15	+	+	+	+	+	+	-	III
Rs18	+	+	-	+	+	-	-	*
Rs19	+	+	+	+	+	+	-	III
Rs21	+	+	+	+	+	+	-	III
Rs26	+	+	+	+	+	+	-	III
Rs34	+	+	+	+	+	+	-	III
Rs35	+	+	+	+	+	+	-	III
Rs36	+	+	+	+	+	+	-	III
Rs37	+	+	+	+	+	+	-	III
Rs49	-	-	+	+	+	-	-	*
Rs56	+	+	+	+	+	+	-	III
Rs57	+	+	+	+	+	+	-	III
Reference	+	+	+	+	+	+	-	III

*Could not be identified.

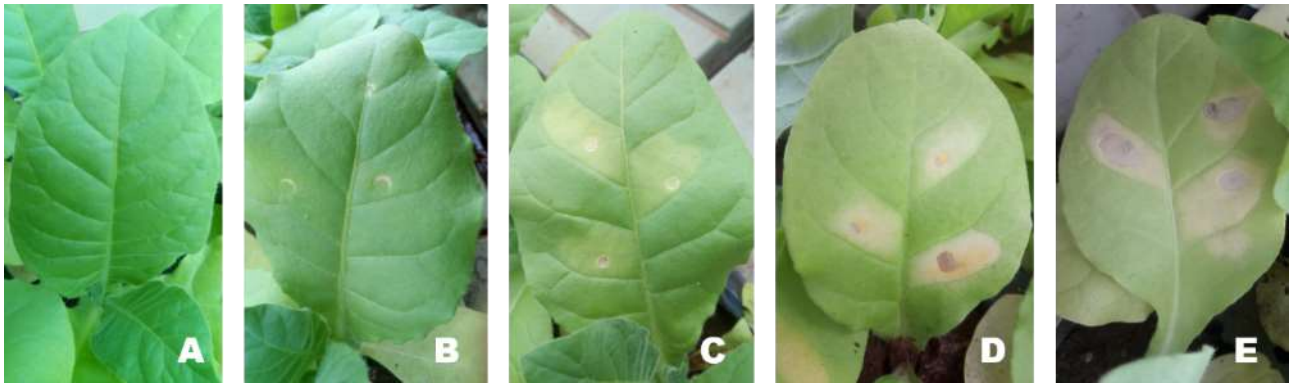

Figure 4. Hypersensitivity responses induced on tobacco plants by tested isolates. A. Original leave; B, no reaction with sterile distilled water; C, slight localized chlorosis (+); D, followed by necrosis (++); E, collapse of whole leaf (+++).

characterized by wilting, yellowing of leaves and black streak on the stem were observed from day 8 following infection (Figure 6). The disease progression was monitored weekly for one month and the observed disease incidence (DI) ranged from 0 to 40% with Rs6 isolate (from Kuresoi North) being the fastest and most virulent followed by Rs15 also from the same region. The isolates from Mau Narok and Njoro (Rs1A and Rs35) were weakly aggressive and Rs35 showed no wilt symptoms but resulted in yellowing of leaves and development of black streak on the stem, compared to the control where none of these symptoms were observed (Figure 7).

DISCUSSION

The findings of this study show successful isolation and characterization of *R. solanacearum* causing bacterial wilt of potato in Nakuru county of Kenya. Cultural, biochemical

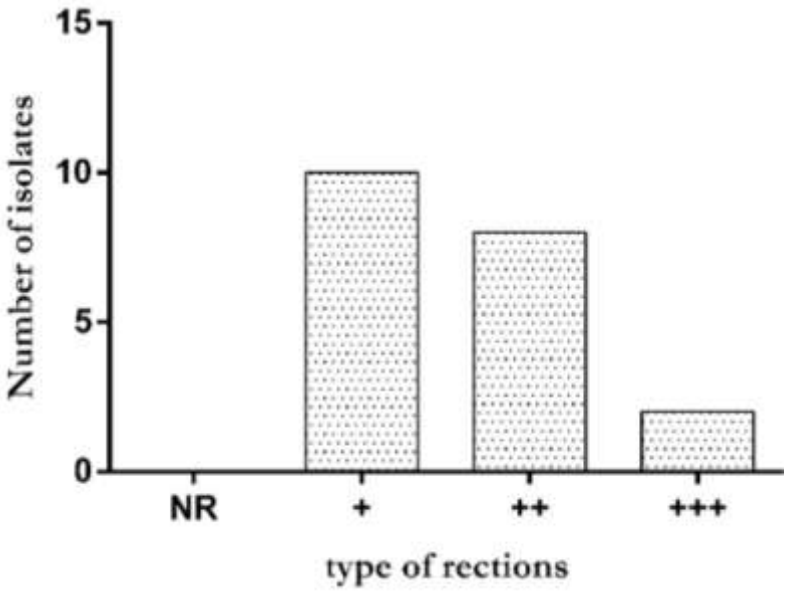

Figure 5. Different type or grade of reaction induced by the isolates and their frequency.

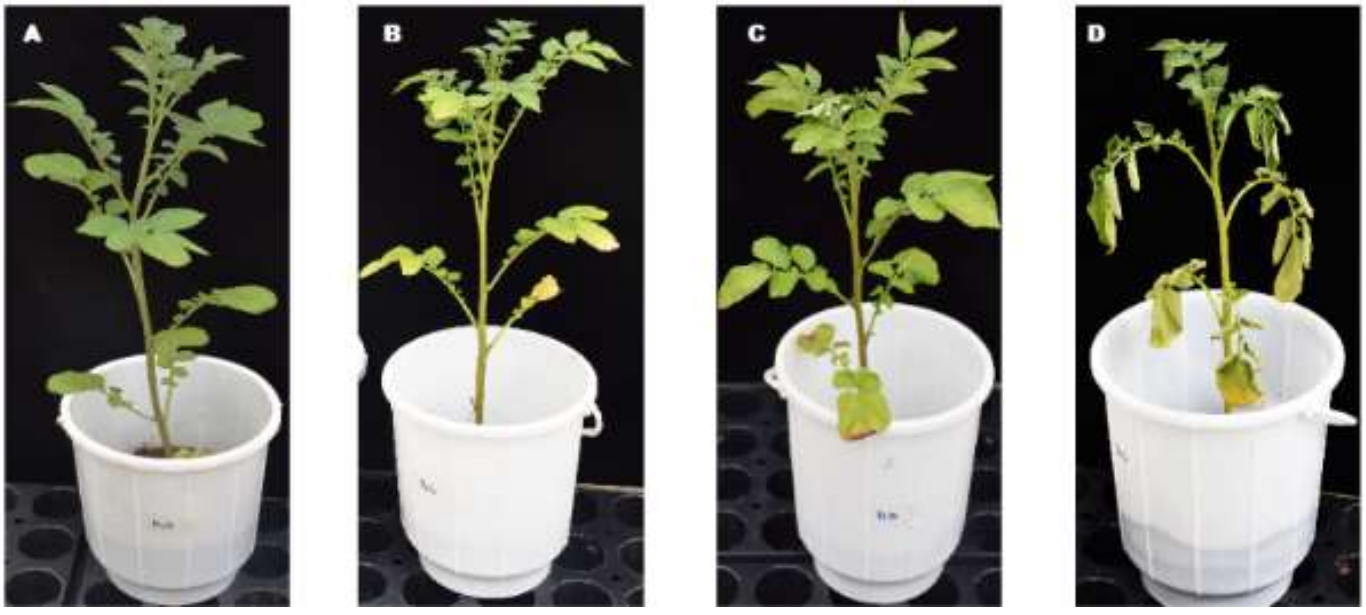

Figure 6. Different types of symptoms induced on potatoes (cv. Shangi) by the tested *R. solanacearum* strains. A. No visible symptoms (control with H_2O); B, Yellowing or chlorotic spots on the leaves; C. Black streak on the stem and wilting of the upper leaves; D, Wilting of all leaves and plant death.

and molecular methods were used to characterize the isolates into biovars, phylotypes and further determine their evolutionary relationship with other known *R. solanacearum* species all over the world. TZC medium allowed successful isolation of *R. solanacearum* as revealed by the different pigmentation. Apart from selective isolation, this medium has also been implicated in differentiating virulent from avirulent strains with Kelman (1954) reporting that virulent colonies appear white with pink or light red centers and non-virulent colonies appear as small off-white colonies. On this medium, typical virulent bacterial colonies (fluidal,

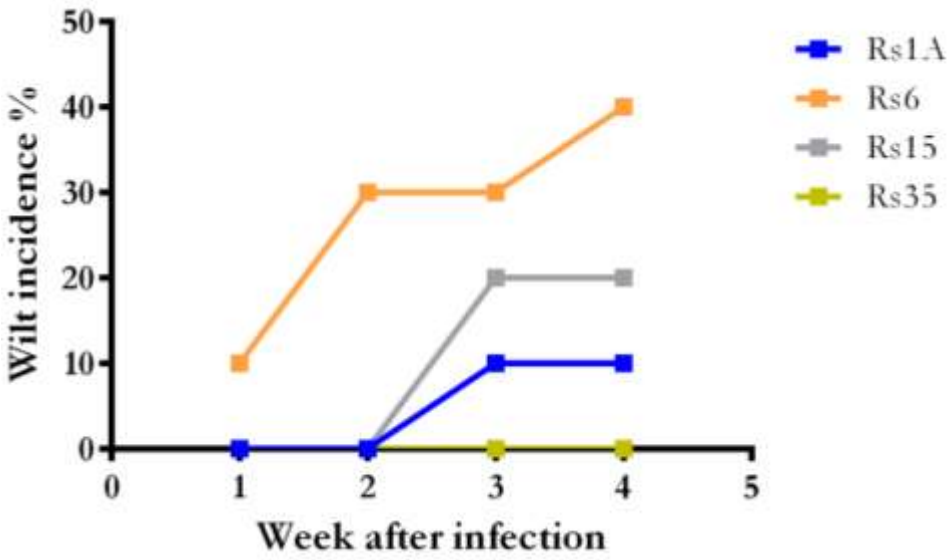

Figure 7. Tracking disease progression on potato cv. Shangi using four *R. solanacearum* isolates

irregular in shape and white with pink or light red centers) were obtained as also report by Champoiseau and Momol (2008)) and Rahman et al. (2010). The isolates were then confirmed to be *R. solanacearum* species, in line with the work by Fegan and Prior (2005) where they also reported the amplification of 281 bp of the common region of the species genome with 759/760 primers.

The isolated *R. solanacearum* was further characterized into biovars based on their ability to utilize sugars and/or alcohols. Differentiation of *R. solanacearum* into biovars based on the utilization of carbohydrates has been previously reported by Hayward (1964). It was found that all isolates from this study successfully oxidized the disaccharides and hexose alcohols and this confirmed their classification as biovar III, relative to what was established by Denny and Hayward (2001). The successful isolation of *R. solanacearum* which all belong to biovar III confirmed their ability to adapt to several conditions (Denny, 2007).

Moreover, the high virulence of biovar III strains in the region can be justified by their wide host range and compatibility with number of environmental factors favorable for disease appearance such as temperature, rainfall, soil type, inoculum potential, and other soil biological factors such as wilt complexes formed among nematodes (*Meloioigyne* species) fungi (*Fusarium* species) and *R. solanacearum* (Shahbaz et al., 2015).

Cultural and biochemical methods in bacterial identification is often supported by molecular characterization using known sequences to further affirm the categories to which each isolate belong to. In the current study, the phylotypes of each isolate were determined and it was found that all 4 phylotypes of *R. solanacearum* (I, II, III and IV) were present in the region. Even though, all phylotypes were found from the sampled regions, their distributions were not even. For instance, all the 4 phylotypes were recovered in Kuresoi North, 3 in Njoro and phylotype I was the only phylotype isolated in Mau Narok. The uneven distribution of this pathogen could be due to differences in adaptation to climatic conditions and the competitive fitness advantage of *R. solanacearum* as earlier reported by Huerta et al. (2015). Phylotype I, exhibited the highest incidence than the others phylotypes. This can be explained by its capacity to infect a wide range of hosts including herbaceous and woody plants (Hayward, 1994). Furthermore, phylotype I is known to be distributed worldwide (Hayward, 1991) and it is reported to be highly recombinogenic (Coupat et al., 2008; Wicker et al., 2012). Several studies have also reported prevalence and distribution of different phylotypes in other African countries. For instance, in Cameroon and Ivory Coast, phylotypes I, II and III have been reported (Mahbou et al., 2009; N'Guessan et al., 2012) while in Ethiopia phylotypes I and II have been identified (Lemessa and Zeller, 2007). Phylotype I was also previously reported in Madagascar and eastern African countries bordering the Indian Ocean including Kenya and South Africa (Wicker et al., 2012; Ravelomanantsoa et al., 2016; Carstensen et al., 2017). This is the first report of phylotype IV in Kenya which could have been introduced in the country through imported potato seed with latent infection (Kaguongo et al., 2010).

Sequencing of the endoglucanase gene from the isolated *R. solanacearum* confirmed the identity of these bacteria and allowed deciphering of their evolutionary relatedness with other known R. solanacearum species. This gene has previously been used for sequevar

determination (Li et al., 2016; Liu et al., 2017), evolutionary dynamics to reveal genetic relationships between *R. solanacearum* species complexes (RSSC), phylogenetic and statistical analysis of housekeeping, virulence-related and pathogenicity-related genes (Fegan and Prior, 2005; Castillo and Greenberg, 2007).

All *R. solanacearum* isolates elicited a hypersensitive response from infiltrated tobacco leaves which was a good indicator of their potential virulence. Hypersensitive reaction is a defense mechanism used by plants to prevent the spread of pathogen infection to non-infected parts. It is associated with plant resistance and characterized by rapid and programmed cell death localized in the region surrounding an infected region (Nimchuk et al., 2003). Reports have indicated that most pathogenic bacteria induce hypersensitivity in leaves of tobacco or other non-host plants and this is often used as a prescreen technique in virulence assays (Poussier et al., 2003; Yabuuchi et al., 2006). In *R. solanacearum*, the hypersensitivity reaction and pathogenicity genes or *hrp* genes have been implicated in controlling induction of both disease development and hypersensitive reactions with *hrp* mutants unable to induce symptoms in susceptible host plants (Boucher et al., 2001). The ability of all isolates under this study to induce hypersensitive reaction (HR) could be attributed to presence of the *hrp* genes in their genome as reported by Boucher et al. (2001).

The virulence of these isolates was determined through pathogenicity tests on a susceptible potato cultivar Shangi. Here, plants were infected with different isolates with a uniform concentration of the pathogen and maintained under the same conditions then evaluated for disease occurrence and severity, as previously reported by Timila and Manandhar (2016). It was observed that the isolates caused disease symptoms on potato plants albeit at different rates. The nature of these symptoms also varied from those observed under field conditions as also reported by Huerta et al. (2015). This could be due to the fact that *R. solanacearum* pathogenicity is distinctly regulated in early or late stages of infection in response to environmental conditions such as soil, humidity, temperature and texture as well as bacterial population densities (Schell, 2000; Hikichi et al., 2007). It is important to note that *R. solanacearum*-host interaction occurs through three main stages including root colonization, cortical infection and xylem penetration. These stages are affected by plant structure and metabolism; and these could have played a role in the observed phenotypes upon infection (Vasse et al., 2005).

Conclusion

R. solanacearum strains affecting potato in Nakuru county-Kenya were successfully isolated and characterized using phylogenetic and pathogenetic analyses. It was found that the pathogen is highly diverse with several phylotyes and variable distribution. Phylotype I was found as the most predominant phylotype and widely distributed in the region. Therefore, it should be considered in development of control strategies such as grafting for resistance or propagation programs. The results also demonstrated that biovar III strains have adapted themselves to the more diverse environment of Nakuru than other biovar strains showing severe reaction on tobacco and variable virulence on potato plants. These findings provide vital information on the *R. solanacearum* strains in this region as well as their associated virulence and distribution which form a basis for breeding programs for potato bacterial wilt resistance and development of control strategies with special emphasis on the improvement of pathogen-targeted and geographically-targeted management practices.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Dynamics of the concentration of the hormones triiodothyronine and thyroxine at the beginning of the reproductive season and the effect of application of iodine in Nelore heifers bred in pasture

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The aim of this study was to evaluate the dynamics of triiodothyronine (T3) and thyroxine (T4) hormones, their relation with body condition score (BCS) and reproductive stage of 248 Nelore heifers, and the effect of iodine application on the induction of cyclicity of 217 anoestrus heifers in pasture at the beginning of the reproductive season. At day zero (D0), an assessment of the BCS was performed. An examination of ovarian structures was performed by ultrasound (US) and the serum was collected for hormonal analysis. Anestrous animals received 27 mg of iodine or placebo (saline). After 40 days (D40), another US was performed, and BCS and hormonal determination evaluations were repeated. At D0 and D40, the cycling group had a BCS mean and a T3 concentration higher than the anestrous group. BCS and T3 and T4 concentrations influenced cyclicity and probability of pregnancy. The treatment did not affect cyclicity, pregnancy rate and number of days between the treatment and calving.

Key words: Bovine, reproduction, thyroid.

INTRODUCTION

The influence of thyroid hormones on reproduction has already been scientifically proven (Starling et al., 2005). Thyroid hormones are important in fertility, acting on ovarian follicle growth, cell proliferation and differentiation of most body tissues and fetal development (Kota et al., 2013). The hypothalamic-pituitary-thyroid and the hypothalamic-pituitary-ovarian axes act simultaneously on the control of folliculogenesis. Thyroid hormones may promote different effects on ovarian function, providing direct or indirect action on follicular development and maturation which results in the regulation of fertility (Saraiva et al., 2010).

Iodine is an essential trace element found in human and animal bodies in minute quantities. The only confirmed role of iodine is in the synthesis of the thyroid hormones triiodothyronine (T3) and thyroxine (T4), which

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are critical for the development of sexual and nervous system as well as the regulation of metabolism, maintaining body temperature and the energy status. Consequently, a severe iodine deficiency hinders the secretion of thyroid hormones (Samanc et al., 2010). Iodine deficiency causes reproductive failures, stillbirth, gestational age extension, abortion, delayed puberty, irregular estrus, reduced fertility and anoestrus (Blood and Henderson, 1991). Studies in pre-pubertal women report that higher concentrations of T4 are associated with precocious puberty (Wilken et al., 2016).

Thyroid hormones have been linked to the different patterns of postpartum luteal activity in dairy cows. Kafi et al., (2012) observed that the serum profile of thyroid hormones was lower in high-producing animals that showed a prolonged luteal phase, anovulation and a delayed first ovulation compared to a normal luteal activity at postpartum. Low serum levels of thyroid hormones in high production animals lead to decrease in energy metabolism; there is mobilization of body fat reserves by directing nutrients to milk production (Khatri and Bhutto, 2014).

Osorio et al., (2014) found that nutritional status is a key factor for the production of T3 and T4, and that the intake of iodine is responsible for the production of such hormones. These thyroid hormones play a crucial role in the development, growth, reproduction and production of cattle. Besides the influence of iodine, serum levels of T3 and T4 may also be influenced by a thermal stress caused by high temperatures and humidity. Júnior et al. (2003) reported that, besides causing changes to physiological and behavioral reactions, heat stress also triggers acute and chronic changes in plasma cortisol concentrations and thyroid hormones (T3 and T4). The circadian secretion rhythm of T3 and T4 is determined by TSH pulses that occur at night. T3 and T4 concentrations are variable during the day, but show significant differences at night (Teixeira et al., 2008). The concentration of iodine in the ovary has been known to be higher than in any other organ except for thyroid. The ovary iodide uptake varies according to reproductive stage. It is increased by estrogen and by a hypothyroid state, and blocked by goitrogens, substances that hinder the absorption of iodine (Slebodziński, 2005). The hypothesis of study was if the application of iodine in females without an ovarian activity is capable of causing a stimulus for the production of T3 and T4 hormones by the thyroid gland, starting cyclicity. Therefore the dynamics of the concentration of T3 and T4 was evaluated at the beginning of the reproductive stage and the effect of application of iodine on the induction of cyclicity of Nelore heifers bred in pasture.

MATERIALS AND METHODS

All procedures were approved by the Ethics Committee on Animal Use (CEUA IF Goiano) under protocol number 012/2013. The study was conducted on a farm located in the municipality of Figueirão, Mato Grosso do Sul state, Brazil (18˚36'56.51" S and 53˚35'26.80" W). Two hundred and forty-eight (n=248) Nelore heifers were selected. They were bred in a pasture, aged 20±2 months, weighing an average of 268.7±29.4 kg. They were kept in pastures with *Brachiaria brizantha*, with an adequate natural shading and free access to water. Mineral salt (Dukamp 80/S®, Monte Aprazível, SP state, Brazil) was freely provided in covered troughs with an average daily consumption of 80 g.

During the first 40 days of the experimental period (day 0 to day 40 of the study), the temperature and humidity were measured daily. A daily index of temperature and humidity (THI) was calculated using the model defined by Thom (1959) and applied by Maturana Filho et al. (2011): THI = 0.8 x T + [(RH(%)/100) x (T - 14.4)] + 46.4; where T is maximum temperature in °C, and RH is maximum relative humidity, measured by a digital thermo-hygrometer (Instrutemp®, Belenzinho, SP state, Brazil). The rainfall between days 0 and 40 was 231 mm, and the average temperature and humidity were 25.5±1.7°C and 76±10.8%, respectively. The average THI was 91.83.

On the first day (D0) of the experiment, the animals were immobilized in a restraint snare, and a gynecological examination was performed by ultrasound (DP 2200 VET), using a linear transrectal probe at 5 mHz to verify the reproductive stage. Animals were classified according to the presence or absence of corpora lutea, into cycling (n=31) and anoestrus (n=217). No signs of stress that could affect the results of the experiment were observed during the handling of the animals.

In this management, blood samples were collected through the side coccygeal vein for laboratorial analysis of T3 and T4 levels. The collection was made through the side coccygeal vein and the blood was transferred to a sterile vial without anticoagulant, using a 120 × 40 mm needle with a 5 mL volume. Blood samples were centrifuged and serum was separated and stored at -20°C pending analysis. Serum levels of T3 and T4 were determined by electrochemiluminescence; using Cobas, 411 equipment (Roche/Hitashi®) and laboratory kits (T3: Roche Diagnostics, Indianapolis, IN, USA; T4: Roche Diagnostics, Indianapolis, IN, USA). The visual analysis of heifers was performed at the exit of the chute; a time when the animals are walking, procuring body condition score. To evaluate body condition score, a scale from 1 to 5 was used; 1 is excessively thin and 5 is excessively fat. The scores were attributed to animals according to the amount of tissue stores, especially fat and muscles, associated with specific anatomic markers (Ferguson et al., 1994). Heifers diagnosed in anoestrus were divided into two groups. The treated group (TG: 110) received an application of 27 mg (3.0 mL) of iodine solution (sodium iodide at 0.6%), and the control group (CG: 107) received 3.0 mL of placebo (saline). The iodine solution was obtained from a mixture of distilled water with Sodium Iodide PA® (PM. 149.89; Labsynth, Diadema/SP state).

On the fifth day of the study, the breeding season began with the introduction of bulls selected for breeding by an andrologic examination into the batch of heifers. The proportion bull/heifer was 1/30. Forty days (D40) after the application of the iodine and the placebo, a second gynecological examination was performed to identify anoestrus, cycling and pregnant animals. New blood samples were collected to study the dynamics of the concentrations of the hormones, T3 and T4, at the early reproductive season and the effect of the application of iodine. The procedures followed the same methodology as at the first day of the experiment. Heifers were submitted to a 90-day breeding season with bulls selected through andrologic examination. 60 after days the withdrawal of the bulls, a pregnancy diagnosis was performed by means of ultrasound, to verify the final pregnancy rate in the different experimental groups. The animals remained on the same property until calving, enabling the record of the calving dates. The interval between the start of the breeding season and the calving was

Table 1. Average concentration and standard deviation of triiodothyronine (T3) and thyroxine (T4) of three body condition score (BCS) groups of cycling and anoestrus Nelore heifers raised on pasture in the first experimental day (D0) and forty days later (D40).

Day		BCS			
		≤2.0	**2.1 to 2.9**	**≥3.0**	**p-value**
D0	T3 (ng/mL)	2.71±0.08[a]	2.73±0.06[a]	2.89±0.11[a]	0.168
	T4 (ng/mL)	115.43±3.84[a]	122.73±4.02[b]	148.25±7.16[c]	≤0.001
D40	T3 (ng/mL)	2.59 ± 0.05[a]	2.50 ± 0.04[a]	2.57 ± 0.07[a]	0.905
	T4 (ng/mL)	123.41 ± 4.17[a]	131.07 ± 3.74[b]	138.63 ± 5.06[c]	0.017

*Different lowercase letters in the same line indicate statistical difference (P<0.05).

analyzed at the end of the calving season.

To perform the statistical analysis, different methodologies were used due to the nature of the study variables. For the analysis of the behavior of T3 and T4 at the two different times (D0 and D40), a Pearson's correlation was recommended to study the association between continuous variables. To evaluate differences in hormone concentrations between animals with different body condition scores as well as reproductive phases (cycling and anoestrus), an analysis of variance and a t test was used to compare means. To examine the effect of the application of iodine on cyclicity and pregnancy rate, the chi-square test was used. The comparison of means between T3 and T4 concentrations and number of days until calving of the treated and control groups was performed using analysis of variance. To study the influence of hormone levels on pregnancy rates and cyclicity, given the categorical nature of the response variables and the continuous independent variable, the logistic regression analysis using the logit function was performed. To perform the statistical analyses, the functions and packages of the statistical software R CoreTeam (2013) used a significance level of 5% probability; considering the hypothesis tests.

RESULTS AND DISCUSSION

Blood concentrations of T3 and T4 were evaluated according to different BCS, considering all animals (cycling and anoestrus) (Table 1). It is observed that average T4 concentrations in different BCS groups were statistically different at the beginning of the breeding season as well as 40 days later. Induced hypothyroidism may result in a better body weight gain and a better body condition (De Moares et al., 1998). This may explain the relative lower levels of T4 in animals with a lower BCS as a physiological mechanism to improve BCS and start cyclicity. However, results observed by Bettini et al*.,* (2006) showed that induced hypothyroidism did not affect weight variation, super-ovulatory response and activity of the corpus luteum in crossbred heifers (½ *Bos taurus* x ½ *Bos indicus*).

Cycling and anestrous groups showed a statistical difference of 5%, regarding the averages for T3 and T4 concentrations at the beginning of the breeding season (D0). Only T4 showed no significant difference 40 days later (D40)*,* as can be seen in Table 2. These results suggest that the hormones, T3 and T4, are related to the cyclicity of heifers bred in pastures, since the average of the concentrations were significantly higher in cycling females if compared with anoestrus animals regarding both hormones at the beginning of the breeding season. According to Saraiva et al*.* (2010), the hypothalamic-pituitary-thyroid and the hypothalamic-pituitary-ovarian axes act simultaneously on the control of folliculogenesis. Thyroid hormones may promote different effects on ovarian function, resulting in a regulation of fertility of bovines. The activity of iodothyronine deiodinases, enzymes responsible for the conversion of T4 into T3, is regulated by sex steroids; but the mechanisms involved are poorly defined. The heifers in this experiment were at puberty, and the lower levels of T3 found in the anoestrus group may be related to the gonadal hormones of this phase, as reported by Marassi et al*.,* (2007).

The highest levels of T4 at D0 in the cycling group coincide with higher estrogen levels at this phase. The administration of estradiol in ovariectomized rats increased the activity of the enzyme thyroid peroxidase (TPO) (Lima et al*.*, 2006). This enzyme catalysis' oxidation reactions and the organification of iodide in the presence of hydrogen peroxide; this suggests that estrogen stimulates the precursor reactions of T4, which is both the uptake of iodide by the thyroid and its organification.

Fortunato et al*.,* (2014) also observed the stimulatory effect of estrogen on the thyroid function in rats by increasing the uptake of iodide through the activity of TPO and by increasing the biosynthesis of thyroid hormones. These reports coincide with the dynamics of T3 and T4 studied in this research, in which the concentrations showed a positive relation with BCS and cyclicity. Table 3 shows the mean concentrations of T3 and T4 hormones of 248 Nelore heifers bred in pasture at the beginning of the breeding season and forty days later, regardless of the reproductive stage observed in gynaecological examinations. It is observed that the means of T3 concentrations differed significantly after 40 days, showing a decrease in concentration. This indicates a possible increase in the activity of iodothyronines, enzymes responsible for T4 deiodination, stimulated by gonadotropins secreted during the follicular period. The THI may also influence this change in the T3 concentration

Table 2. Mean and standard deviation of the concentration of the hormones triiodothyronine (T3) and thyroxine (T4) in Nelore heifers, cycling and anoestrus, raised on pasture at the beginning (D0) of the breeding season and forty days later (D40).

Day		Cycling (n=31)	Anoestrus (n=217)	p-value
D0	T3(ng/mL)	3.08 ± 0.15[b]	2.71 ± 0.05[a]	0.023
	T4(ng/mL)	151.14 ± 7.50[b]	122.66 ± 3.01[a]	0.048
		Cycling (n=187)	Anoestrus (n=58)	p-value
D40	T3(ng/mL)	2.75 ± 0.11[b]	2.50 ± 0.03[a]	0.003
	T4(ng/mL)	**140.35 ± 7.58[a]**	**128.79 ± 2.58[a]**	**0.464**

* Different letters in the same line indicate statistical difference (P<0.05).

Table 3. Mean and standard deviation of the concentrations of triiodothyronine (T3) and thyroxine (T4) of Nelore heifers at the beginning of the breeding season (D0) and 40 days later (D40).

Hormones	D0	D40	p-value
T3 (ng/mL)	2.77 ± 0.05[b]	2.54 ± 0.03[a]	≤0.001
T4 (ng/mL)	126.26 ± 2.89[a]	130.25 ± 2.47[a]	0.312

* Different letters in the same line indicate statistical difference at 5%.

Table 4. the breeding season of the control group and of the group treated on the first day of the experiment (D0) and 40 days (D40) after treatment with iodine.

Group	D0	D40
T3		
Treated (ng/mL)	2.69 ± 0.05 [bB]	2.46 ± 0.04 [aA]
Control (ng/mL)	2.73 ± 0.08 [aA]	2.55 ± 0.04 [aA]
T4		
Treated (ng/mL)	120.41 ± 3.82 [aA]	126.32 ± 3.48 [aA]
Control (ng/mL)	126.20 ± 4.78 [aA]	131.50 ± 3.82 [aA]

* Different lowercase letters in the same column indicate that there was statistical difference between groups, different uppercase letters in the same line indicate statistical difference between times (P<0.05)

found at D40. In the first 40 days of this experiment, temperature and humidity data were collected daily, resulting in an average THI of 91.83; this reflects high temperatures and humidity during this period. High environmental temperatures cause heat stress, which leads to an increased blood cortisol concentration and a decreased concentration of thyroid hormones (Starling et al., 2005). Consistent with these results is Coelho et al*.,* (2008) who observed that thermal stress promotes significant decreases only in the T3 plasma concentrations of goats; while T4 levels, in all breeds studied, remained unchanged.

Teixeira et al. (2008) reported this same behaviour in empty mares, in which variations in T3 and T4 levels were observed while maintaining a balanced correlation. Upon evaluating the effect of the application of iodine on heifers in anoestrus at the beginning of the breeding season, it was observed that the average concentration of T3 was statistically lower at D40 for the treated group (Table 4). The treatment with iodine may have influenced the serum level of T3 after 40 days. T3 levels reflect the functional status of the peripheral tissue more than the secreting performance of the thyroid gland. Table 5 shows the percentage of heifers cycling after 40 days, the pregnancy rate at the end of the breeding season and the average of the interval from the treatment calving. To study the effect of the application of iodine on animals that were gaining, losing or maintaining the body condition score, the animals were grouped according to the body condition score as observed between D0 and D40. Table 6 shows the results of mean T3 concentrations of different groups and their respective treatments during the two periods evaluated. The results in Table 6 show that animals that gained BCS during the first forty days of experiment and received iodine decreased the average concentration of T3. Table 7

Table 5. Cyclicity rate 40 days after treatment, pregnancy rate at the end of the breeding season and average number of days of treatment until delivery (LI) of the heifers from the control group and treated with iodine.

Parameter	Control	Treated
Cyclicity rate (%)	73.33[a]	73.39[a]
Pregnancy rate (%)	85.71[a]	79.82[a]
LI (Mean±standard deviation)	338.37±3.68[a]	337.61±3.58[a]

* Equal letters in the same line indicate that there was no statistical difference (P<0.05).

Table 6. Mean and standard deviation of the concentration of triiodothyronine (T3) of anoestrus Nelore heifers in the groups gaining, maintaining and losing body score condition between the first day (D0) of the experiment and forty days (D40) later, treated with iodine and placebo.

Groups	Treatment	D0 (ng/mL)	D40 (ng/mL)	p-value
Gaining	Iodine (n=49)	2.70±0.08 [aB]	2.41±0.05 [aA]	0.00
	Placebo (n=39)	2.62±0.14 [aA]	2.47±0.07 [aA]	0.35
Maintaning	Iodine (n=51)	2.72±0.09 [aA]	2.51±0.06 [aA]	0.07
	Placebo (n=55)	2.75±0.10 [aA]	2.60±0.06 [aA]	0.22
Losing	Iodine (n=10)	2.54±0.15 [aA]	2.45±0.15 [aA]	0.69
	Placebo (n=13)	3.12±0.33 [aA]	2.60±0.12 [aA]	0.16

* Different lowercase letters in the same column indicate that there was statistical difference between groups, different uppercase letters in the same line indicate statistical difference between times (P<0.05).

Table 7. Mean and standard deviation of the concentration of thyroxine (T4) of anoestrus Nelore heifers in the groups gaining, maintaining and losing BCS between the first day (D0) of the experiment and forty days (D40) later, treated with iodine and placebo.

Groups	Treatment	D0 (ng/mL)	D40 (ng/mL)	p-value
Gaining	Iodine (n=49)	119.3±6.0[acA]	126.6±5.2[aA]	0.36
	Placebo (n=39)	119.2±7.4[acA]	123±6.4[aA]	0.69
Maintaining	Iodine (n=51)	115.2±4.5[aA]	127.7±5.4[abA]	0.08
	Placebo (n=55)	125.5±5.8[abcA]	133±5.0[abA]	0.33
Losing	Iodine (n=10)	150.1±17.2[cA]	115.4±6.8[aA]	0.07
	Placebo (n=13)	149.9± 20.5[bcA]	150.4±11.5[bA]	0.98

* Different lowercase letters in the same column indicate that there was statistical difference between groups, different uppercase letters in the same line indicate statistical difference between times (P<0.05).

shows the results in relation to T4. Animals that gained BCS had lower mean concentrations of T4. These results agree with De Moraes et al*.,* (1998), who reported that induced hypothyroidism may result in improved weight gains and increased body condition. Table 8 shows the percentages of cycling and pregnant animals forty days after treatment and sixty days after the withdrawal of the bulls.

The results showed no statistical difference between heifer groups. This is different from Ferreira et al*.,* (2013) who observed an influence of BCS on the pregnancy rate of Nellore cows calved in pasture and subjected to timed artificial insemination, in which females with a lower body condition had lower pregnancy rates compared to those with a better condition (BCS ≥ 3). This study investigated the effect of the application of iodine on the plasma concentrations of thyroid hormones. However, there may be other effects on granulosa cells, such as an increased response to FSH and an improvement of follicle quality, as described by Cecconi et al*.,* (2004). Spicer et al*.* (2001) suggest that thyroid hormones may have direct stimulatory effects on the ovarian function of cattle, acting at the level of granulosa and theca cells. Figure 1 shows the influence of T3 and T4 concentrations on the

Table 8. Percentage of cycling and pregnant heifers forty days after the beginning of the experiment (D40) and final pregnancy in each group.

Groups	Treatment	Cycling (%) D40	Pregnant (%) D40	Final pregnancy (%) 60 days after the withdrawal of bulls
Gaining	Iodine (n=49)	81.63[a]	16.32[a]	79.59[a]
	Placebo (n=39)	74.36[a]	7.69[a]	79.48[a]
Maintaining	Iodine (n=51)	66.67[a]	5.88[a]	82.35[a]
	Placebo (n=55)	76.36[a]	7.27[a]	89.09[a]
Losing	Iodine (n=10)	60[a]	10[a]	70[a]
	Placebo (n=13)	53.84[a]	0[a]	84.61[a]

* Different lowercase letters in the same column indicate that there was statistical difference between groups (P<0.05).

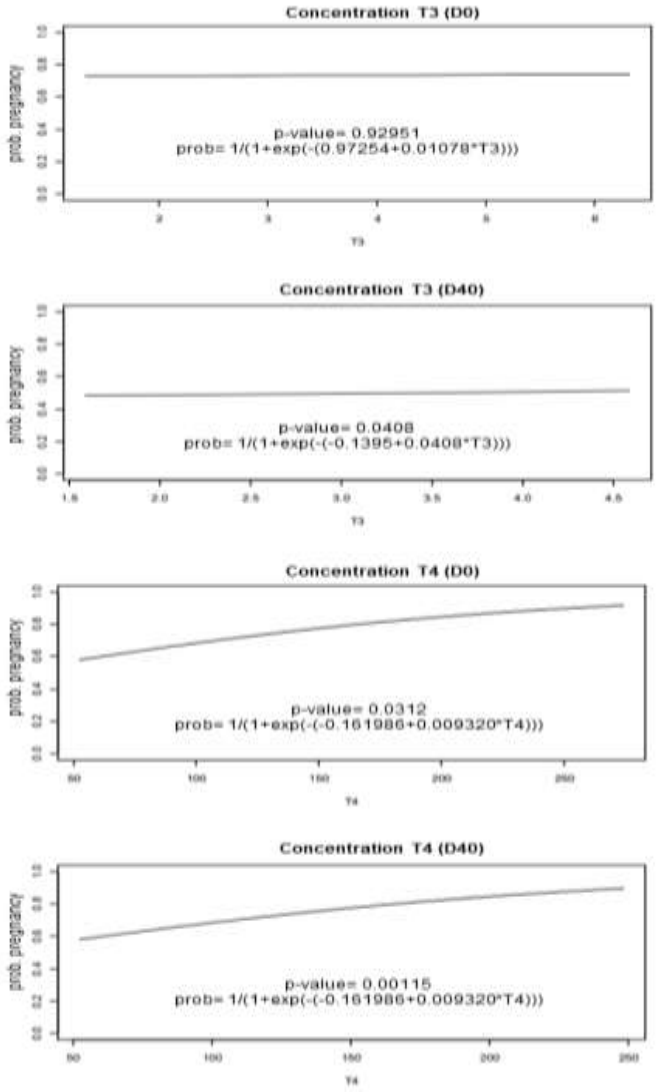

Figure 1. Influence of hormones concentrations of triiodothyronine (T3) and thyroxine (T4) at the beginning of the breeding season (D0) and forty days after (D40) on the probability of pregnancy of heifers raised on pasture 60 days after the late mating season.

probability of pregnancy at D0 and D40. It is noted that only the concentration of T3 at D0 was not significant, while the increase in other concentrations early in the breeding season increases the probability of pregnancy. These results reaffirm the importance of thyroid hormones to reproduction.

Conclusion

Thyroid hormones levels are related to the cyclicity of heifers, and they influence the probability of pregnancy and the treatment with iodine do not have effect on cyclicity, pregnancy rate and number of days between treatments and calving of Nelore heifers bred in pasture.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Comparison of antimicrobial properties of two spices commonly consumed in Nigeria and effect of temperature on their antioxidant properties

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The study compared the antimicrobial activities of essential oils (EOs) from *Piper guineense* (Uziza) and *Xylopica aethiopica* (Uda) using diffusion and dilution assays, and evaluated their antioxidant properties at different temperatures. Comparison of the antimicrobial activities was determined using well-in-agar diffusion, disc diffusion and broth dilution methods against *Escherichia coli, Salmonella* sp *and Bacillus cereus* as test isolates. The antioxidant activities at different temperatures (28, 50 and 70°C) were determined using 1,1- diphenyl-2-picrylhydrazyl (DPPH), ferric ion reducing antioxidant power (FRAP) and Fe^{2+} chelation methods. Their antimicrobial activities increased with increase in concentration. From the diffusion assays, *B. cereus* was more susceptible to the EOs than other isolates (19.5 and 18.0 mm). MIC values obtained from the broth dilution assay (8.0 – 16.0 mg/mL) were generally much lower than those obtained from disc diffusion and well-in-agar diffusion assays. Percent inhibition of isolates using broth dilution method ranged from 91.28 to 96.86%. Their antioxidant activities decreased with increase in temperature. *P. guineense* EO yielded higher DPPH scavenging activity (43.25, 46.64 and 33.36%, respectively) and Fe^{2+} chelating ability (25.60, 12.58 and 8.97 mg/g, respectively) at the different temperatures, while FRAP elicited the strongest antioxidant ability of the EOs (70.10, 37.51 and 26.55 mg/g, respectively). The EOs exhibited their strongest antioxidant abilities at 28°C.

Key words: Microbial isolates, antimicrobial susceptibility testing, antioxidant capacity, spices, essential oils.

INTRODUCTION

Essential oils (EOs) are secondary metabolites which are very rich in compounds with an isoprene structure, called terpenes. They can occur as diterpenes, triterpenes, and tetraterpenes (C_{20}, C_{30}, and C_{40}), or as hemiterpenes (C_5) and sesquiterpenes (C_{15}). Addition of oxygen to these compounds makes them terpenoids (Cowan, 1999). Several EOs derived from spices elicit varying degrees of antimicrobial effects (Swamy et al., 2016; Cui et al., 2015;

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Sonker et al., 2015; Beatovic et al., 2015) and also possess antioxidant properties (Beatovic et al., 2015) as well as have the ability to suppress cancer cell activity when applied on some human cancer cell lines such as tumors, glioma, breast cancer and leukemia (Adaramoye et al., 2011; Kuete et al., 2015; Sado Kamdem et al., 2015).

The use of essential oils as therapeutic agents and preservatives has become increasingly popular; however, research into the antimicrobial activities of these products has been hampered by lack of standardized and reliable screening methods, especially against food pathogens/food spoilage bacteria. Lack of standardized methods may also make direct comparison of results between studies impossible. Methods for antimicrobial assays are classified into three main groups, which are diffusion (Gulluce et al., 2006), dilution (Passos et al., 2002; Souza et al., 2003) and bio-autographic (Cos et al., 2006; Suleiman et al., 2010) methods. Agar-based disk diffusion is widely used because of its simplicity and low cost. The liquid-dilution method also allows determination of whether a compound or extract has bactericidal or bacteriostatic action at a particular concentration. A simple bioautographic procedure, involves spraying suspensions of the bacteria or fungi on thin layer chromatography (TLC) plates developed in solvents of varying polarities (Suleiman et al., 2010).

Piper guineense (*Uziza*) (Black pepper) and *Xylopica aethiopica* (*Uda*) (Guinea pepper) are spices which have over the years become popular amongst Nigerians due to their perceived health benefits. They are used in Nigeria for preparation of different cuisines which are sometimes considered delicacies (Ogueke et al., 2016). The use of these spices in traditional medicinal practice in Nigeria, Africa and other parts of the world are well documented in literature (Nwinyi et al., 2009; Ekanem et al., 2004; N'dri et al., 2009; Ezekwesili et al., 2010; EL-Kamali et al., 2007). In Nigeria and other parts of West Africa, hot extracts of the seeds and leaves of these spices, in addition to other plant parts, serve as tonic for women after childbirth to enhance uterine contraction, prevention of blood coagulation and expulsion of blood from the womb, draining of excess fluid to control weight, as well as an aphrodisiac (Ogueke et al., 2016; Abolaji et al., 2007).

Several researchers have studied the EOs of *P. guineense* from Cameroon and Nigeria (Amvam Zollo et al., 1998; Jirovetz et al., 2002; Tchoumgougnang et al., 2009; Oyedeji et al., 2005) which they found to be composed mainly of monoterpenoid alcohol (linalool, 52.2%) (Owolabi et al., 2013). Monoterpenes are the dominant oils present in *X. aethiopica* (El-Kamali and Adam, 2009). Antimicrobial activities of the EOs from these spices have been demonstrated on some microorganisms (Oyedeji et al., 2005; Okigbo and Igwe, 2007; Tatsadjieu et al., 2003; Okigbo et al., 2005;

Thomas, 1989; Asekun and Adeniyi, 2004; Schelz et al., 2006). However, a lot more microorganisms of food and clinical importance require to be screened for susceptibility to the EOs from these spices. The nutritional and health potential of the seed oils of the two spices have been evaluated by some researchers (Ogbonna et al., 2015; Dada et al., 2013) and have been shown to possess low toxicity (Fetse et al., 2016).

There is need to determine the antioxidant activities of these spices with a view to incorporation into food systems especially now that emphasis is on functional foods. It is also important to establish the antioxidant capacity of their EOs at varying temperatures. This is necessary since these spices are used in Nigeria and other African countries for preparation of a variety of dishes that require heating to elevated temperatures (80 to 100°C). Such data will guide users as to the appropriate temperature(s) for incorporation of the spices and their EOs during food processing to maximize their antioxidant capability. These Nigerian spices have been used for centuries as flavouring ingredients in many traditional dishes.

In this study, comparative evaluation of the antimicrobial activities of EOs from *X. aethiopica* and *P. guineense* was conducted using broth dilution assay, disc diffusion and well-in-agar diffusion methods commonly cited in literature for their ability to accurately assess their antimicrobial activities. Their antioxidant properties at varying temperatures were also determined.

MATERIALS AND METHODOLOGY

Collection of spices

Fruits of the Nigerian spices, *X. aethiopica* (Uda) and *P. guineense* (Uziza) were obtained from a farm in Obinze and Ihiagwa, Owerri West Local Government Area (latitude 5° 15'N - 5° 34'N and longitude 6° 52'E – 7° 05'E), Imo State, Nigeria in December, 2016. They were cleaned and sorted to remove sand, spoilt spices and dirt.

Preparation and extraction of essential oils (EOs)

Fruits of *X. aethiopica* and *P. guineense* were destalked, sorted and milled with attrition mill (Landers YCLA, S.A Colombia). The Eos were extracted using the Clevenger hydro distillation method (Selim, 2011). The spices (300 g of each milled spice) were put in a four litre flask (4 L) and 1.5 L distilled water added. The mixture was boiled for 3 h. After boiling, the EOs were collected and dried with anhydrous sodium sulphate. These were then stored at -10°C until required for use.

Preparation of different concentrations of EOs

The EOs were diluted using 5% dimethylsulphoxide (DMSO) solution to obtain the desired concentrations (20 – 100%). These were stirred thoroughly for complete homogeneity. Samples were plated out on nutrient agar and potato dextrose agar plates, and incubated at 37 and 30°C respectively for 24 h and 72 h to ensure sterility of the concentrations prepared.

Test microorganisms

Pure cultures of *Escherichia coli*, *Salmonella* sp. and *Bacillus cereus* were obtained from the laboratories of Federal Medical Centre Owerri (FMC), Imo State, Nigeria. They were used due to their frequent occurrence in clinical samples sent to the laboratories of FMC for analysis. These were re-identified and stored at 4°C on the appropriate agar slants until required for use.

Inoculum standardization

A loopful of the bacterial isolates was inoculated unto Mueller–Hinton Agar (MHA) plates and incubated at 37°C for 24 h. Discrete bacterial colonies were collected using sterile wire loop, inoculated into 10-mL Mueller–Hinton broth and incubated for 30 min. After incubation, the broth was centrifuged for 30 min, the top decanted and broth supernatant was washed with sterilized distilled water. This was centrifuged again and the procedure was repeated three times. After the centrifugation, the microorganisms were harvested and re-suspended in sterilized distilled water in sample bottles. The cultures were adjusted to 0.5 McFarland standards (approx 10^8 cfu/mL) and stored at 4°C, and this was used for the analysis.

Disc diffusion assay

Filter paper No. 1 was perforated to get paper discs of 5 mm diameter. These were sterilized at 110°C for 30 min, impregnated with the concentration of the EOs (20 - 100%) and placed on the surface of the Mueller-Hinton agar plates inoculated with the test bacteria. A control was set up with 0.1 mL dimethylsulphoxide, and the resulting plates were incubated at 37°C for 24 h. The zones of inhibition were measured using a meter rule to the nearest millimeter (mm).

Well-in-agar diffusion assay

This was determined using the method described by Kim et al. (1995) and Lino and Deogracious (2006). 17 mL of Mueller–Hinton Agar (MHA) was poured into sterile petri dishes and allowed to solidify. Using sterile cotton swabs, the cultures of bacteria as prepared above (shown in preparation of microbial strains) were swabbed uniformly on the surface of the plates and allowed to dry for 5 min. All plates were labeled properly and wells of 5.0 mm in diameter were made in the seeded agar plates using a sterile cork borer, sufficiently separated from each other to avoid overlap of growth inhibition zones. 0.01 mL of the different concentrations of the EOs was dispensed into the wells. Three plates were used for each concentration of EO. With the lids on, the plates were allowed to stand for 30 min at ambient temperature (30-32°C) to allow for proper diffusion of the EOs (Ogbulie et al., 2007). The MHA plates were incubated at 37°C for 24 h. The zones of clearance produced around the wells after incubation were observed, measured (diameter of agar well not included) and recorded as the diameter of zone of growth inhibition produced by the EOs. These were carried out in triplicates.

The minimum inhibitory concentrations (MIC) of the EOs using disc diffusion and well-in-agar diffusion assays were determined by plotting a graph of the natural log of concentration of extracts used (X axis) against the squares of the zones of inhibition of the extracts obtained (Y axis). The MIC was read out at the intercept of the regression line of the graph on the X axis. The antilog of the value at the intercept was taken as the MIC (Osadebe and Ukwueze, 2004; Ogbulie et al., 2007).

Broth dilution assay

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of EOs on bacterial isolates

The MIC and MBC of the EOs were determined by the modified method of Salah-Fatnassi et al. (2017), making double fold serial dilutions of the reconstituted extracts (0.625 - 50 mg/mL) in nutrient broth. Bacterial strains with culture suspensions adjusted to 0.5 McFarland standard turbidity (1.5 x 10^8 cfu/mL) were inoculated into the broth. All culture tubes were incubated at 37°C for 24 h. Growth was scored visually by the turbidity of the culture. The least concentration showing no visible growth was taken to be the MIC.

The MBC was determined by taking 0.1 mL inoculum from the tubes in which there was no growth and sub cultured on Mueller-Hinton agar plates. After incubation at 37°C for 24 h, the plates were observed for bacterial growth. The least concentration showing no growth was taken as the MBC.

Antioxidant properties of EOs

The antioxidant properties of the EOs were determined at 28, 50 and 70°C using 1,1-diphenyl-2-picryl-hydrazyl (DPPH) free radical scavenging assay, ferric ion reducing antioxidant power (FRAP) assay and Fe^{2+} chelation assay.

1,1-diphenyl-2-picryl-hydrazyl (DPPH) free radical scavenging assay

Evaluation of the total free radical scavenging capacity of the EOs against DPPH (1,1-diphenyl-2-picryl-hydrazyl) free radical was done according to the method described by Gyanfi et al. (1999). The DPPH radical solution was prepared by dissolving 2.4 mg DPPH in 100 ml methanol. A 1:10 dilution of the EOs was prepared and 1 ml of it was mixed with 1 mL, 0.4 mM methanolic solution containing DPPH radicals. The mixture was shaken vigorously and kept at ambient temperature for 30 min in the dark. Absorbance of the reaction mixture was measured at 516 nm wavelength using a Spectronic Genesys 20 spectrophotometer (Thermo Electron Corporation, USA). DPPH radical without antioxidant was observed and measured. These were performed in duplicate. The ability of the EOs to scavenge the DPPH radical was calculated as stated below using the following equation (Yen and Duh, 1994):

$$\text{DPPH Scavenged (\%)} = ((A_B - A_A)/A_B) \times 100 \quad (1)$$

Where, A_B = absorbance of blank at t = 0 min.
A_A = absorbance of the antioxidant at t = 30 min.

Ferric ion reducing antioxidant power (FRAP) assay

The ferric reducing activity of the EOs was estimated based on the method (FRAP) developed by Benzie and Strain (1996). The solutions for this assay consisted of the following: 300 mmol/L acetate buffer (pH 3.6), 20 mmol/L ferric chloride and 10 mmol/L TPTZ (2, 4,6-tripyridyl-s-triazine) in 400 mmol/L of HCl. The reagent for the assay was prepared fresh by mixing 10 parts of acetate buffer with 1 part of ferric chloride and 1 part of TPTZ solution. The analysis/assay was performed as follows: 2000 µL of freshly prepared FRAP reagent was mixed with 75 µL of sample, ethanol or hydroalcoholic solvent as appropriate for reagent blank. The absorbance was read at 593 nm using Spectronic Genesys 20 spectrophotometer (Thermo Electron Corporation, USA) after 30 min of incubation. The values obtained were expressed as ascorbic acid equivalent.

Table 1. MIC of *X. aethiopica* and *P. guineense* EOs on isolates using Disc diffusion and Well-in-Agar diffusion methods.

Essential oils	Microorganism	MIC (mg/mL)	
		Disc	Well-in-Agar
P. guineense	*B. cereus*	234.42	1.00
P. guineense	*E. coli*	81.28	69.18
P. guineense	*Salmonella* sp	128.82	251.19
X. aethiopica	*E. coli*	251.19	69.18
X. aethiopica	*Salmonella* sp	173.78	141.25
X. aethiopica	*B. cereus*	3.39	1.20

Fe^{2+} chelation assay

The Fe^{2+} chelating ability of the EOs was determined using the method of Minotti and Aust (1997), however, with slight modification by Puntel et al. (2005). Freshly prepared 500 µM $FeSO_4$ (150 µL) was added to a reaction mixture containing 168 µl 0.1 M Tris- HCl (pH 7.4), 218 µL saline and 100 µL of a 1:10 dilution of the essential oils. This was incubated for 5 min, before the addition of 13 µL 0.25% 1, 10- phenonthroline solution (w/v). The absorbance was subsequently measured at 510 nm wavelength; thereafter, the Fe^{2+} chelating ability was calculated.

Statistical analysis

The data were means of triplicate determinations and were analysed using Analysis of Variance (ANOVA). Statistical Package for Social Scientists (SPSS) version 20.0 was used for statistical analysis of data obtained. The means were separated using Fisher's Least Significant Difference (LSD) at 95% confidence level ($p<0.05$).

RESULTS

Antibacterial activity of EOs against isolates

Antibacterial susceptibility testing of *X. aethiopica* and *P. guineense* EOs against test isolates using well-in-agar diffusion and disc diffusion methods are shown in Figures 1 and 2 respectively, and Table 1. With well-in-agar diffusion method, *B. cereus* was observed to be more susceptible to the activity of both spices with a zone of growth inhibition measuring 19.5 and 18.0 mm for *P. guineense* and *X. aethiopica* EOs respectively at 100% concentration (Figure 1). However, the EOs had lower activities on *E. coli* both having a zone of growth inhibition of 9 mm at 100% concentration. *E. coli* was resistant to the activities of the EOs at concentrations lower than or equal to 33.3% of the EOs. Similar trend in antimicrobial activities was recorded using the disc diffusion method (Figure 2) although the values obtained were lower than those obtained with the well-in-agar diffusion method.

MIC values of the EOs obtained from disc diffusion and well-in-agar diffusion assays are shown in Table 1. The MIC values were generally high. The highest value (251.19 mg/mL) was produced by *P. guineense* and *X. aethiopica* on *Salmonella* sp. and *E. coli* respectively. With well-in-agar diffusion assay, *P. guineense* and *X. aethiopica* produced the lowest MIC values of 1.00 and 1.20 mg/ml respectively on *B. cereus*. However, with the disc diffusion assay, the EOs produced MIC values of 234.42 and 3.39 mg/mL respectively on the same bacterium.

Broth dilution assay

Results from the broth dilution assay showed MIC value of 8.0 mg/mL on the test isolates except *E. coli* where the activity of *P. guineense* produced MIC of 16.0 mg/mL. The MBC values ranged from 16-32 mg/mL (Table 2). Such low MIC values are of significance. Figures 3 to 8 show the graphs of percentage inhibition against concentration of EO. These revealed that the percentage inhibition increased with increase in concentration of the EOs. The values ranged from 91.28% as observed on *B. cereus* produced by *P. guineense* to 96.86% on *Salmonella* sp. produced by *X. aethiopica* at concentration of 32 mg/mL. For *E. coli,* the maximum percent inhibition was achieved at 94% at concentration of 32 mg/mL by *X. aethiopica* EO and 96% at a concentration of 32 mg/mL by *P. guineense* EO respectively (Figures 3 and 4). This further justifies the results obtained from the MIC and MBC using broth dilution assay.

Antioxidant properties of the EOs

The antioxidant properties of the EOs at different temperatures (28, 50 and 70°C) using the three methods (Tables 3 and 4) show that their activities decreased with increase in temperature. The abilities of the EOs to scavenge DPPH at different temperatures for *P. guineense* EO were 43.25, 28.06 and 23.89% respectively while *X. aethiopica* EO were 38.02, 25.09 and 21.55% respectively. *P. guineense* EO yielded higher DPPH

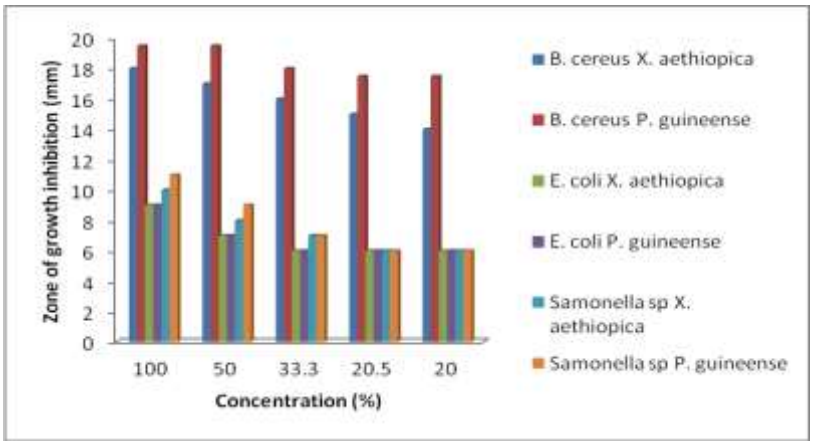

Figure 1. Antimicrobial susceptibility testing using well-in-agar diffusion method.

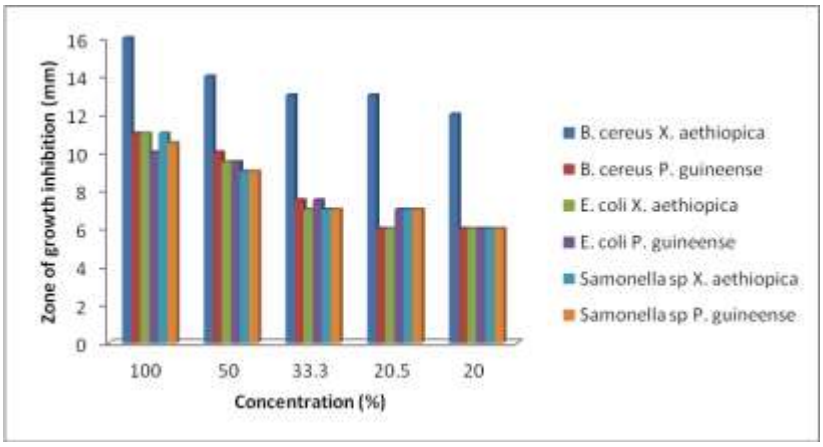

Figure 2. Antimicrobial susceptibility testing using disc diffusion method.

scavenging activity at the different temperatures than *X. aethiopica* EO. Fe^{2+} chelating ability of *P. guineense* EO was also higher at the temperatures studied (25.60, 12.58 and 8.97 mg/g, respectively) than the *X. aethiopica* EO. However, for the FRAP, *X. aethiopica* EO yielded higher reducing property than *P. guineense* EO with values of 70.10, 37.51 and 26.55 mg/g at 28, 50 and 70°C, respectively.

The values obtained significantly differed ($p<0.05$) among the methods and the temperatures adopted for the study. FRAP elicited the strongest antioxidant ability of the EOs while the optimum temperature at which the EOs exhibited the strongest antioxidant ability was 28°C.

DISCUSSION

Antimicrobial activities of extracts of spices have been studied by different researchers and their activities have been attributed to the phytochemicals they contain. The EOs tested in this study showed antimicrobial activity (bactericidal and bacteriostatic) on all the tested isolates which are common food pathogens (*E. coli*, *Salmonella* sp. and *B. cereus*), although differences were observed between their antimicrobial activities (Figures 1 and 2, Tables 1 and 2). Composition of critical chemical compounds in spices differs significantly due to variations and differences in genetic makeup, agronomical practices and environmental factors; thus, they may bring about differences in their antibacterial efficacy against food borne pathogens (Cowan, 1999; Chao et al., 1999; Hao et al., 1998). Such EOs could therefore be applied as antimicrobial preservatives in food (Pandey et al., 2017), although information in this line is minimal.

This study has shown that broth dilution assay (Table 2) is a better protocol for determining the antimicrobial efficacy of EOs from these spices than disc diffusion or well-in-agar diffusion methods (Table 1). MIC values obtained from the broth dilution assay were generally much lower than those obtained from disc diffusion and

Table 2. MIC and MBC of *X. aethiopica* and *P. guineense* EOs on isolates.

Essential oils	Microorganism	MIC (mg/mL)	MBC (mg/mL)
P. guineense	*B. cereus*	8	16
P. guineense	*E. coli*	16	32
P. guineense	*Salmonella sp*	8	32
X. aethiopica	*E. coli*	8	32
X. aethiopica	*Salmonella sp*	8	16
X. aethiopica	*B. cereus*	8	32

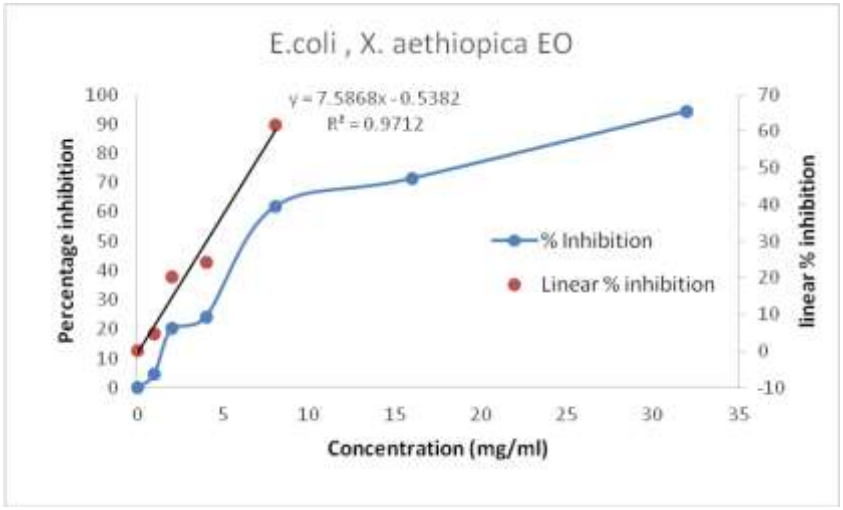

Figure 3. Graph of percentage inhibition against concentration of *X. aethiopica* on *E. coli*.

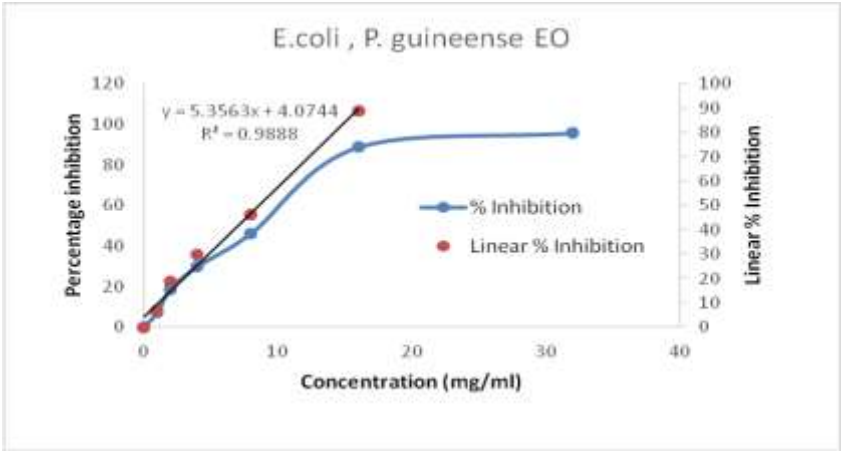

Figure 4. Graph of percentage inhibition against concentration of *P. guineense* on *E. coli*.

well-in-agar assays, thus indicating a stronger antibacterial activity against the tested isolates. The rate of diffusion in agar media and thickness of oils play a major role in the use of these protocols in assessing antimicrobial activities of oil containing extracts of plants such as the EOs from *P. guineense* and *X. aethiopica*.

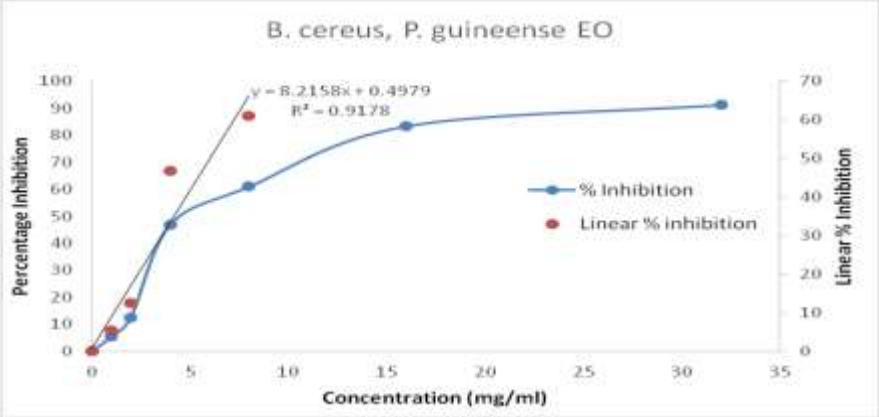

Figure 5. Graph of percentage inhibition against concentration of *P. guineense* on *B. cereus*.

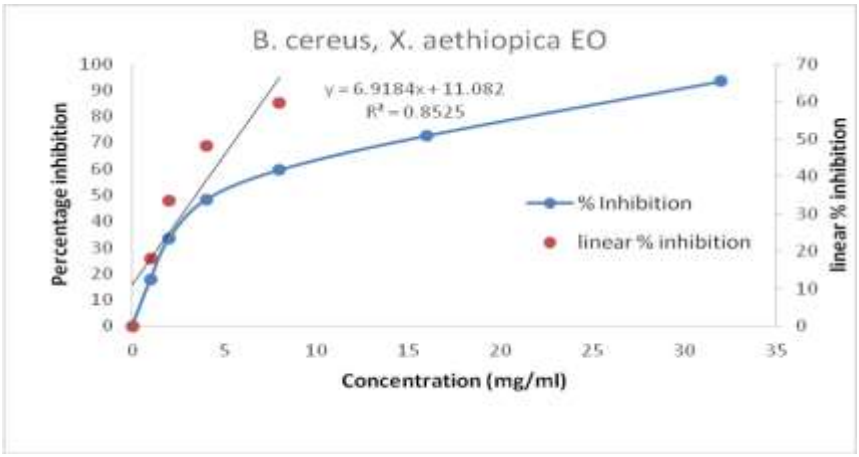

Figure 6. Graph of percentage inhibition against concentration of *X. aethiopica* on *B. cereus*.

Insolubility of active compounds in water and culture medium or the presence of inhibitors to the antimicrobial components also plays major roles in the activity of such EOs (Burt, 2004; Janssen et al., 1987; Kalemba and Kunicka, 2003; Okigbo and Ogbonnanya, 2006; Scorzoni et al., 2007). EOs are complex mixtures of a wide variety of components. Thus, their antimicrobial activity is related to their configuration, composition, amount and possible interaction which could be additive, antagonist or synergetic (Lis-Balchin et al., 1998; Wang et al., 2016). Inactivity of the EOs and other extracts of plants may be due to age of plant, extracting solvent, method of extraction and time of harvesting of plant materials (Okigbo et al., 2005; Okigbo and Ajalie, 2005). This corroborates the findings of Hadacek and Greger (2000) who reported that serial dilution test gave the most reproducible results for MIC and thus was recommended as a general standard methodology for testing of natural products.

Phytochemicals found in extracts of plants such as phenolics had been known to exhibit strong radical scavenging activity. Phenolic compounds in some cases have been found to be synergistic in their actions where they prevent the formation of reactive O_2 species, hydroxyl radicals, superoxide anions and hydrogen peroxide which usually cause oxidative damage to biomolecules (Satish et al., 2014). The study revealed that the EOs exhibited potent antioxidant properties (Tables 3 and 4). However, using the FRAP method, *X. aethiopica* EO exhibited the strongest reducing power. Such plant materials with high reducing power are usually good source of natural antioxidants. The antioxidant properties recorded for the EOs could be attributed to the bioactive compounds, phytochemicals and pigments they possess. *P. guineense* has been reported to contain safrole, 5-8% piperine, elemicine, 10% myristicine and dillapiol (Osuala and Anyadoh, 2006).

The observation that the antioxidant capacities of the

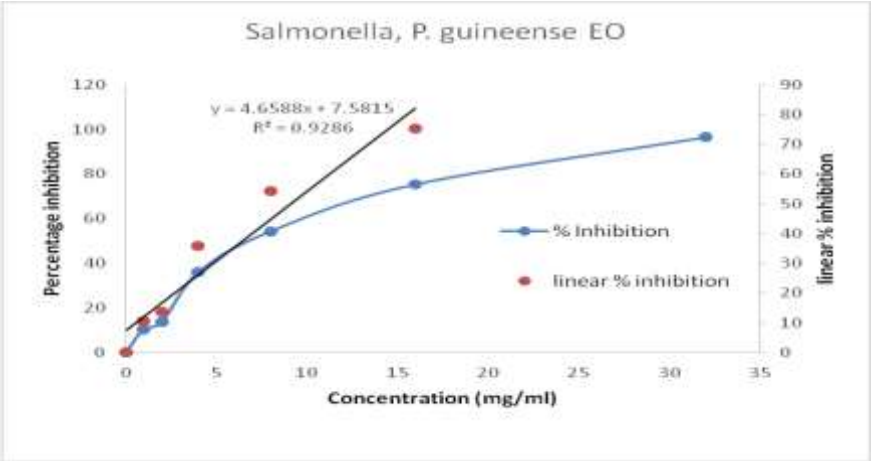

Figure 7. Graph of percentage inhibition against concentration of *P. guineense* on *Salmonella* sp.

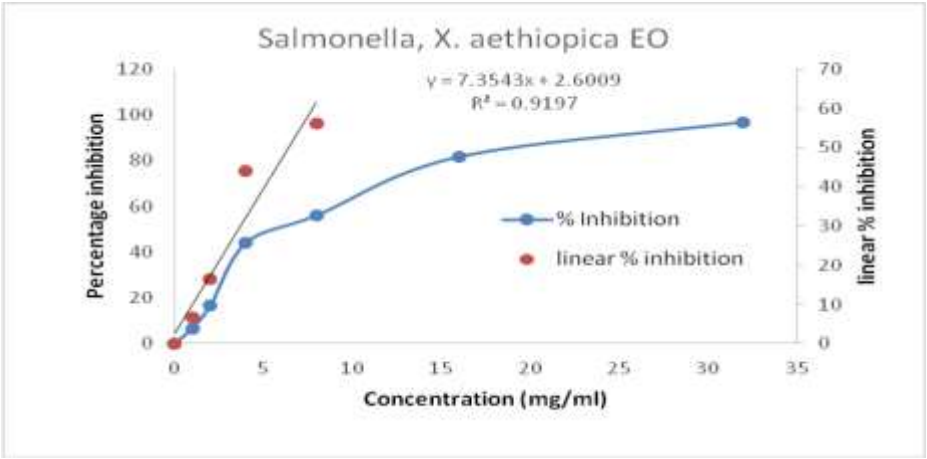

Figure 8. Graph of percentage inhibition against concentration of *X. aethiopica* on *Salmonella* sp.

EOs decreased with increase in temperature implies that when included in foods as preservatives such foods should be stored at temperatures not exceeding 28°C so as to obtain their maximum capabilities as antioxidants. Weaker antioxidant activity at higher temperatures could be attributed to the volatile characteristics of components of the EOs which are simultaneously lost through evaporation as temperatures increased. Polyphenolic compounds are known to boost antioxidant properties of natural extracts (Sun et al., 2007), thus increase in temperature could lead to loss of these polyphenolic compounds with attendant loss of antioxidant activity. The implication of this loss of antioxidant activities at higher temperatures is that food preparations requiring high temperature processing as is common with most dishes prepared with these spices in Nigeria, and other African countries may not provide the needed free radical scavenging/antioxidant ability. It is necessary, therefore, to develop method(s) for addition of the spices or their EOs after heating of the food product to ensure the antioxidant ability is not lost.

Conclusion

This study has demonstrated that the EOs from these spices (*X. aethiopica* and *P. guineense*) possess varying degrees of antimicrobial activities and antioxidant properties. Comparison of antimicrobial activities using well-in-agar diffusion, disk diffusion and broth dilution methods showed that broth dilution method/assay was a better protocol for determining the antimicrobial efficacy of the EOs from these spices than disc diffusion or well-in-agar methods. Analysis using different methods

Table 3. Antioxidant property of *P. guineense* EO at different temperatures (°C).

Method	Temperature (^oC)			LSD
	28	50	70	
Fe^{2+} (mg/g)	$^{1}25.60^{c}\pm0.08$	$^{2}12.58^{c}\pm0.01$	$^{3}8.97^{c}\pm0.01$	0.144
DPPH (%)	$^{1}43.25^{b}\pm0.01$	$^{2}28.06^{b}\pm0.01$	$^{3}23.89^{b}\pm0.01$	0.023
FRAP (mg/g)	$^{1}48.51^{a}\pm0.01$	$^{2}46.64^{a}\pm0.08$	$^{3}33.36^{a}\pm0.01$	0.146
LSD	0.032	0.144	0.023	

^{abc} Values with different superscripts along the columns differ significantly ($p< 0.05$)
^{1234} Values with different superscripts on the same row are significantly different ($p< 0.05$)
KEY:
DPPD = 1,1- diphenyl- 2- picrylhydrazyl free radical scavenging ability.
FRPA = Ferric ion reducing power assay.
Fe^{2+} = Ferrous chelating ability.

Table 4. Antioxidant property of *X. aethiopica* EO at different temperature (^oC).

Method	Temperature (^oC)			LSD
	28	50	70	
Fe^{2+} (mg/g)	$^{1}20.10^{c}\pm0.01$	$^{2}10.26^{c}\pm0.01$	$^{3}7.55^{c}\pm0.01$	0.131
DPPH (%)	$^{1}38.02^{b}\pm0.01$	$^{2}25.09^{b}\pm0.02$	$^{3}21.55^{b}\pm0.01$	0.136
FRAP (mg/g)	$^{1}70.10^{a}\pm0.01$	$^{2}37.51^{a}\pm0.01$	$^{3}26.55^{a}\pm0.01$	0.023
LSD	0.023	0.043	0.225	

^{abc} Values with different superscripts along the columns differ significantly ($p< 0.05$)
^{1234} Values with different superscripts on the same row are significantly different ($p< 0.05$)
KEY:
DPPD = 1,1- diphenyl- 2- picrylhydrazyl free radical scavenging ability.
FRPA = Ferric ion reducing power assay.
Fe^{2+} = Ferrous chelating ability.

showed that FRAP elicited the strongest antioxidant ability of the EOs. The antioxidant properties were also temperature dependent and exhibited their strongest antioxidant ability at 28°C. Further studies aimed at application of the EOs in food matrix are recommended to establish their actual activities in a food system.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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