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Cultural Variability of *Fusarium oxysporum* f. sp. *elaeidis* Isolates from Oil Palm in South Western Cameroon and Sensitivity to Four Plant Extracts

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Abstract

Oil palm (*Elaeis guineensis* Jacq.) of the Arecaceae is a perennial monocot of great importance in both small and large scale farming in most countries in the world. Vascular wilt caused by *Fusarium oxysporum* f. sp. *elaeidis* is one of the most damaging diseases of oil palm in Cameroon. It displays enormous cultural variability leading to difficulty in its control. This work aimed to study the cultural variability of *Fusarium oxysporum* f. sp. *elaeidis* isolates responsible for vascular wilt of oil palm in five Cameroon Development Corporation oil palm estates in South Western Cameroon and verify the bioactivity of five plant extracts to the fungus. A total of 107 isolates from oil palm rachis and rhizospheric soils of five estates were characterized culturally. These isolates showed variation in colony characters in Potato Dextrose Agar and Rose Bengal media. Neem and *Tithonia* leaf extracts showed inhibitory effects on the fungal isolates *in vitro.* This work therefore confirms the variability within the *Fusarium oxysporum* complex and the potential of producing anti-fungicidal sprays from *Tithonia* and neem leaf extracts in the control of this phytopathogenic fungus.

Key words – *Elaeis guineensis*– *In vitro* Control – Occurrence – Vascular wilt

Introduction

Oil palm (*Elaeis guineensis* Jacq.) of the Arecaceae is a perennial monocot of great importance in both small and large scale farming in most countries in the world (Ekhorutomwen et al. 2018). The oil palm originated in the tropical rain forest region of West Africa (Ntsomboh et al. 2015). It is produced by over 100 tropical and sub-tropical countries including Malaysia, Indonesia, Nigeria and Cameroon. Oil palm plantation in Cameroon is estimated to cover 76,000 ha (FAO 2007). The fact that oil palm produces fruit all year round makes it an important commodity in food security. The crop constitutes a major source of agricultural income for most countries (FAO 2002) and its production by multinational corporations such as the Cameroon Development Corporation provides employment for many. Oil palm is the most important and highest oil-producing crop in the world (Gascon et al. 1988). Palm oil has been reported to have activity for anodyne, antidotal,

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aphrodisiac, diuretic and vulnerary and it is also said to be a remedy for cancer, headaches and rheumatism (Duke & Wain 1981).

In 2001, Africa ranked second after Asia among world's oil producers (Bolivar & Cuellar-Mejia 2003). In Cameroon, seed production by the PNRPH (Programme National de Recherche sur le Palmier a Huile) doubled from1996 to 1999, passing from 800,000 to 1,700,000 pre-germinated seeds (Bell 2000) and by 2001, Cameroon produced some 159,000 tons of palm oil, thus being the third largest African producer (Fèvre 2003). Recent ranking schemes bring Cameroon to the 13th largest producing African country thus experiencing a drop in production. The steadily increasing interest in oil palm production has to be coupled with extension and improvement of the culture (Ntsomboh et al. 2012).

However, one of the oil palm's constraints is vascular wilt disease that is caused by *Fusarium oxysporum* f. sp. *elaeidis* (f.o.e), is the most damaging disease of oil palm in Africa (Tengoua & Bakoume 2008), causing up to 70% mortality of oil palm in plantations. Control of Fusarium wilt disease has been accomplished primarily by the application of chemical fungicides, long crop rotations, pasteurizing seedbeds with steam or fumigants. Nevertheless, the massive use of synthetic fungicides in crop defense has severe environmental impact. The inappropriate use of agrochemicals especially fungicides were found to possess adverse effects on ecosystems and a possible carcinogenic risk than insecticides and herbicides together. Moreover, resistance by pathogens to fungicides has rendered certain fungicides ineffective (Zhonghua & Michailides 2005). Due to the aforementioned considerations, there may be a need to develop new management systems to reduce the dependence on the synthetic agrochemicals. In this respect, plant extract may represent an ideal solution to the problem, as it can be easily tested in vitro. The aim of this research was to study the cultural variability of *Fusarium oxysporum* f. sp. *elaeidis* from five oil palm plantations in the Cameroon Development Corporation and to test the sensitivity of isolates to four plant extracts.

Materials & Methods

Sample collection

Sampling was done through purposive sampling; where collection of diseased palms was done by judging from symptoms. The outermost fresh frond was cut close to the stem with sharp knife. A piece of the rachis, about 4 cm was cut from the base of the rachis, placed in a zip lock bags, labeled and placed in a cool box and transported to the Life Sciences Laboratory in the University of Buea. Core soil samples were collected from three points (0-5 cm) around the base of the palm and bulked in separate zip lock bags, labeled and also transported to the Life Science Laboratory in the University of Buea. All collected samples were processed within 72 hours of collection. Table 1 below shows the number of samples collected per site.

Table 1 Distribution of F.o.e isolates from Palms in CDC.

Estate	**Samples collected**		
	Rachis	Soil	Total
Bota	25	25	50
Musaka	25	25	50
Mondoni	25	25	50
Debundscha	15	15	30
Idenau	10	10	20
Total	100	100	200

Isolation and Identification

Rachis from palm fronds was thoroughly washed under running tap, cut into segments (of 0.5 × 0.5 × 0.5 cm), immersed in 1% sodium hypochlorite (NaClO), 70% alcohol and rinsed with three

changes of sterile distilled water and plated in PDA and Rose Bengal media. Soil samples were cultured by means of broadcasting in PDA and Rose Bengal media. After 7 days of incubation, wet mounts were made from the mycelia growth, stained and observed under the microscope with an objective lens of 40x. The morphological characterization of isolates of *Fusarium oxysporum* f. sp. *elaeidis* was performed with the keys proposed by Leslie & Summerell (2006), which consisted of determining various characteristic parameters on plates after full growth (7-10 days) from both media.

Cultural and morphological identification

Isolates identification was based on the morphological characteristics of single-spores as described by Leslie & Summerell (2006). Colonies exhibiting the taxonomic features of *Fusarium oxysporum* were identified according to Nelson et al. (2004). Morphological identification was based on colony growth traits. The identity of the culture was further confirmed by the presence of macroconidia and microconidia. For microscopic characteristics, the isolates were cultured onto PDA for 7-10 days and observed under the microscope in order to check for the presence of "banana-shaped" microconidia. For macroscopic observation, the cultural appearances (colony colour and pigmentations, growth rate, colony margin, form and elevation) were observed on Potato Dextrose Agar (PDA) grown for 7-10days. Colony colour and pigmentations were determined by using the Munsell colour chart.

Sensitivity of Isolates to some Plant Extracts

Source of plant material and extraction

Four plant samples that are known to have antimicrobial properties (Shrivastava & Swarnka. 2014) were used; *Azadirachta indica* leaves and oil bought from Maroua, *Moringa oleifera* bought from Buea, *Withania somnifera* roots bought from Bamenda and *Tithonia diversifolia* leaves collected from Buea were used to screen for sensitivity assay for the fungal isolates. The collected material was washed thoroughly with running tap water and distilled water, the samples was shade dried and finely ground. The powdered plant material was weighed to 500 g and poured in a 1000 ml conical flask containing methylene chloride, stirred homogenously and then allowed to soak for 2 days. It was then filtered using Watman filter paper to clarity into round bottom flask and extracted with a Soxhlet apparatus and dried with a water bath and rotary evaporator respectively. This separated the solvent from the plant extract. The plant materials were first extracted with methylene chloride, then with methanol.

Screening and determination of Minimum Inhibition Concentration

Thirty-five fungal isolates having similar morphotype with a colony diameter of 50 mm and above were selected from the five sites and screened for sensitivity to the four plant extracts using the agar disc diffusion method. In this preliminary screening, five peripheral wells were made on PDA medium and each was filled with 0.2 g of crude plant extract and sterile distilled water was used as control. After screening for sensitivity to plant extracts and obtaining the plant extracts with the greatest and highest zones of inhibition against the plant pathogenic fungus, these extracts were dissolved in Dimethyl Sulfoxide (DMSO) and solutions of different concentrations (10 mg/ml, 20 mg/ml, 30 mg/ml, 40 mg/ml and 50 mg/ml) were prepared. The effect of these extracts on *Fusarium oxysporum* f. sp. *elaeidis* was tested *in vitro* by poison food technique (Bhawana et al. 2014). All petriplates were incubated at room temperature for seven days. After incubation, the growth inhibition of each concentration was determined by measuring the radial growth of *Fusarium oxysporum* f. sp. *elaeidis* in the test plate and compared with the control plate. The antifungal activity was assessed in terms of percentage inhibition. The percentage inhibition (I%) was calculated using the formula of Vincent (1947) thus;

$$I\% = \frac{Xc - XT}{Xc} \times 100$$

Where X= colony diameter (mm), C= Growth of mycelium in control plate (mm) and T= Growth of mycelium in treatment plate (mm) mean of three plates considered as final reading.

Results

Fusarium infected palms showed different symptoms based on the stage of the disease. Some of the primary symptoms observed were foliage with lower and older fronds desiccating and dying from the lower trunk toward the bud. Affected fronds died in a one-sided manner, from the lower leaflets (pinnae) and spines out to the frond tip. Dieback was continuous from the tip to the frond base on the other side of the rachis. Some leaves were dead from the frond tip back to the base on both sides of the rachis simultaneously. Linear brown stripes that had developed on the lower surface of the frond rachis extended a variable distance out from the frond base. Some pinnae and spines exhibited necrotic streaking as well. Vascular discoloration was evident in both cross and longitudinal sections of the rachis. Discrete pockets of brown tissue were observed in cross and longitudinal sections (Fig. 1).

Fig. 1 – Symptoms of *Fusarium oxysporum* f. sp. *elaeidis* from diseased palms. A Symptoms begins as a brown spot in the xylem. B Xylem tissue gets more infected as disease progresses. C One-sided dead of frond. D Leaves becomes weak forming skirt-like shape.

Identification of *Fusarium oxysporum* f. sp. *elaeidis*

From the 200 samples plated, 107 isolates were obtained. This gave an isolation frequency of 50.5%. Mungo Estate had the highest number of isolates with an isolation frequency of over 80%

and the least number of isolates came from Bota Estate with an isolation frequency of 30%. The soil samples yielded more isolates for three estates (Bota, Debundscha and Mondoni). Their isolation frequency ranged from 30% in Bota to 66% in Mondoni giving an overall isolation frequency of 44.2%. Idenau and Mungo had equal number of isolates from both soil and samples from the rachis with isolation frequencies of 40% and 80% respectively.125 samples out of 200 samples plated yielded F.o.e isolates across the study site after using Rose Bengal growth medium. This gave an isolation frequency of 59.3%. Mungo Estate had the highest number of isolates with an isolation frequency of 88% and the least number of isolates came from Bota Estate with an isolation frequency of 34%. The soil samples yielded more isolates for four estates (Bota, Debundscha, Mondoni and Mungo). Their isolation frequency ranged from 34% in Bota to 88% in Mondoni giving an overall isolation frequency of 61.7%. Idenau had more isolates from the rachis with isolation frequency of 50%. The remaining 75 samples plated yielded *Fusarium solani, Fusarium falciform* and *Fusarium camptoceras.* The F.o.e isolates showed highly variable macroscopic cultural characteristics, mainly in the pigmentation on PDA culture medium. Fig. 2 shows the "banana-shape" of the microconidia when viewed under the microscope and was in conformity to that of Leslie & Summerell (2006).

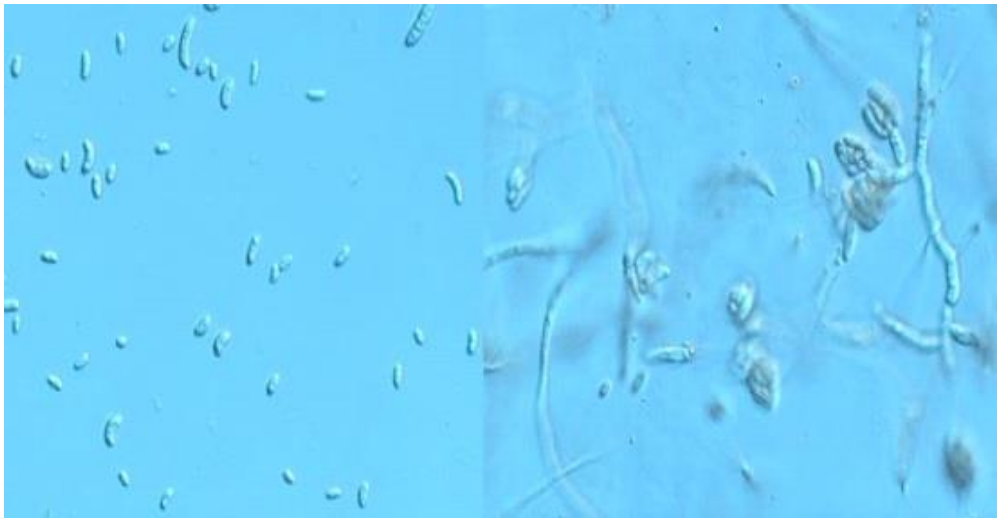

Fig. 2 – Microconidia of *Fusarium oxysporum* f. sp. *elaeidis* under wet mount.

Colony Diameter of *Fusarium oxysporum* f. sp. *elaeidis*

Most isolates obtained had colony diameter between 50-59mm and a majority was from Mondoni Estate. The least number of isolates had colony diameters between 20-29mm, having a majority from Bota Estate. The highest number of isolates from Rose Bengal produced cultures with colony diameter between 30-39mm and a majority of it came from Mungo Estate. These results can be seen in Fig. 3.

Colony Margin

The highest number of *Fusarium oxysporum* f. sp. *elaeidis* isolates having the same colony margin type was obtained from the rachis in Mungo and this colony margin type; undulate, was most predominant in all the sites (Figs 4, 5). The colony margin; entire (30%) had more *Fusarium oxysporum* f. sp. *elaeidis* isolates from soil samples of Mondoni and its least from Idenau.

Colony Elevation

Three forms of colony elevation (umbonate, raised and flat) were observed from all the sites with the raised elevation being the most frequent (Figs 6, 7).

Colony Form

The most predominant colony form was observed to be irregular. All sites except Bota had 2 isolates obtained from the rachis to be circular Table 2.

Surface Colony Colour

Four surface colony colour types were observed but the most predominant was salmon pink which could be seen more from the soil *Fusarium oxysporum* f. sp. *elaeidis* isolates of Mungo, followed by white, creamish white then pale pink Fig. 8.

Reversed Colony Colour

Four reversed colony colour types were observed but the most predominant types were violet (observed in soil *Fusarium oxysporum* f. sp. *elaeidis* isolates from Mungo Estate) and brown; observed in rachis *Fusarium oxysporum* f. sp. *elaeidis* isolates from Mondoni Estate (Figs 9, 10).

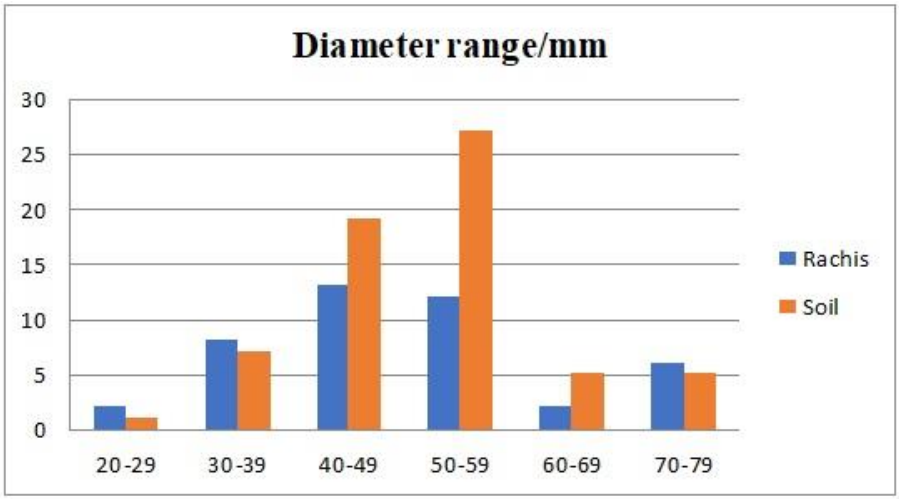

Fig 3 – Diameter range of Isolates obtained from samples collected

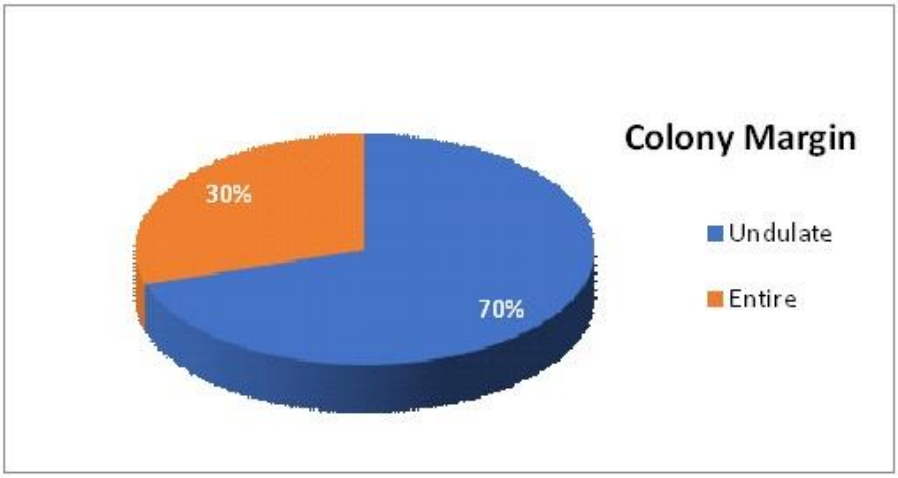

Fig 4 – Percentage Colony margin of *Fusarium oxysporum* f. sp. *elaeidis* isolates

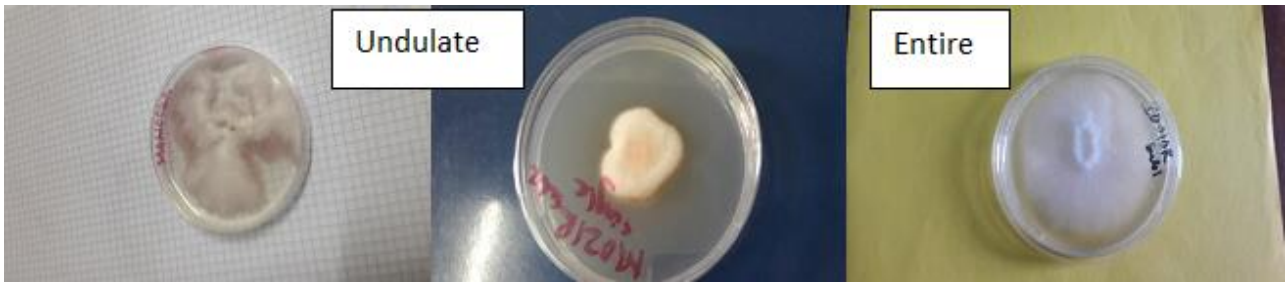

Fig 5 – Colony margin of *Fusarium oxysporum* f. sp. *elaeidis* Isolates

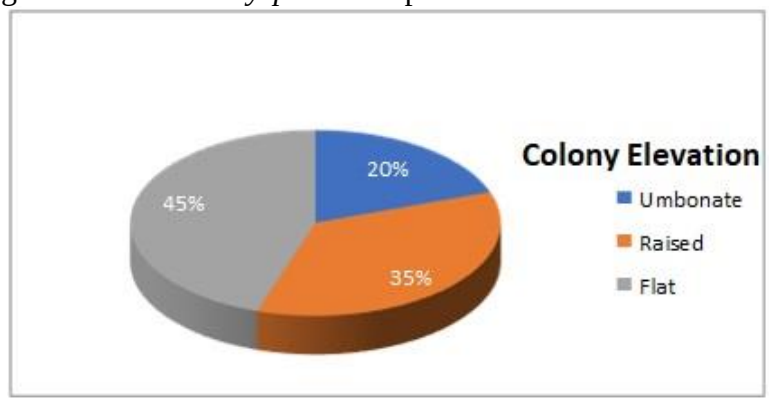

Fig 6 – Percentage Colony elevation of *Fusarium oxysporum* f. sp. *elaeidis* isolates

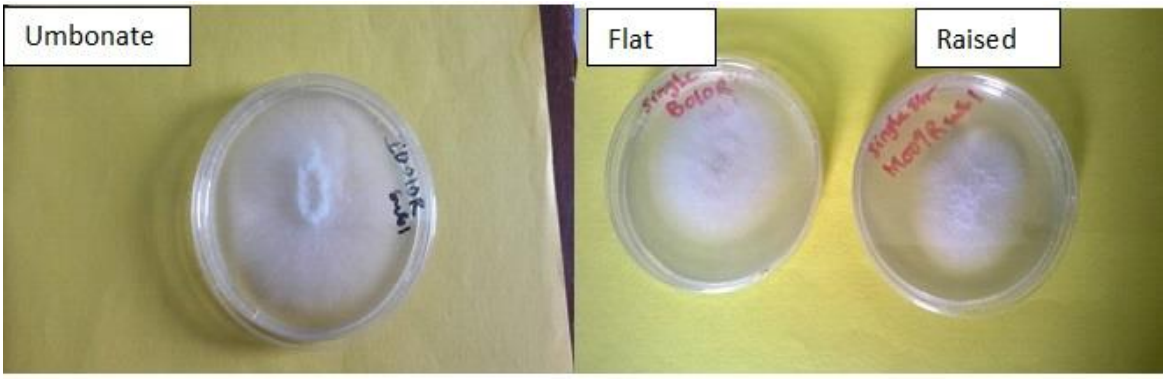

Fig 7 – Colony Elevation of *Fusarium oxysporum* f. sp. *elaeidis* Isolates.

Table 2 Colony Form of *Fusarium oxysporum* f. sp. *elaeidis* isolates obtained on PDA and RB

Site		**Colony Form**	
		PDA	**RB**
Bota	Soil	Irregular	Irregular
	Rachis	Circular	Irregular
Mungo	Soil	Irregular	Irregular
	Rachis	Irregular	Irregular
Mondoni	Soil	Irregular	Irregular
	Rachis	Irregular	Irregular
Debundscha	Soil	Irregular	Irregular
	Rachis	Irregular	Irregular
Idenau	Soil	Irregular	Irregular
	Rachis	Irregular	Irregular

Sensitivity to Plant Extracts

The agar disc dilution method of sensitivity testing to methylene chloride extracts proved all the plant extracts showed different level of antifungal activity as evidenced by a zone of inhibition (Fig. 11) with *Tithonia diversifolia* extract showing high zone of inhibition on selected isolate with morphotype irregular, umbonate, entire margin, salmon pink with reverse colour violet tested, Table 3.

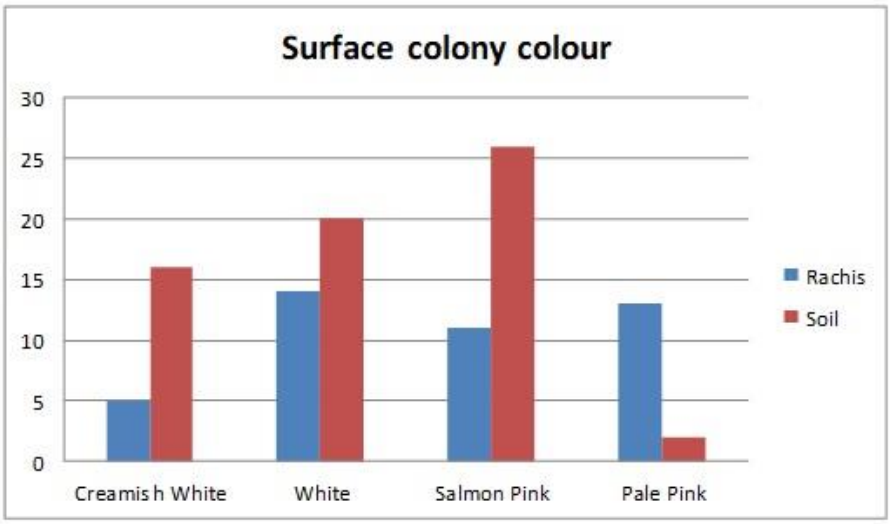

Fig 8 – Frequency of Surface Colony Colour of *Fusarium oxysporum* f. sp. *elaeidis* isolates

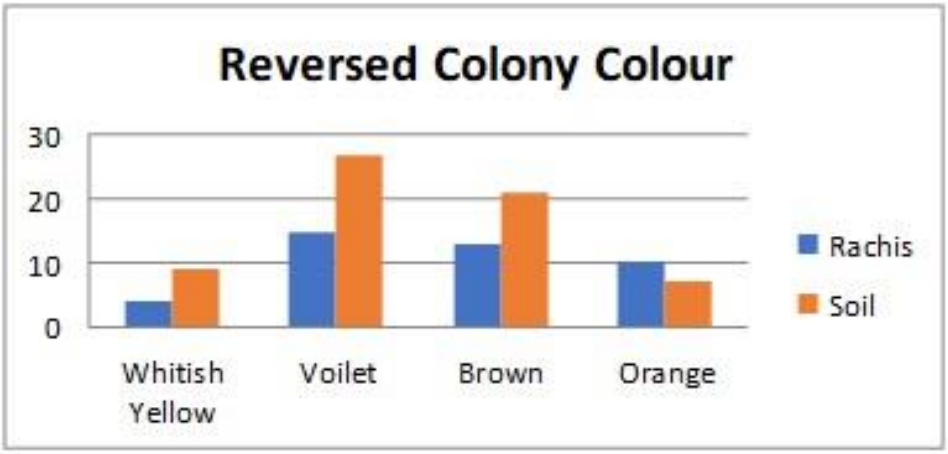

Fig 9 – Frequency of Reversed Colony Colour of *Fusarium oxysporum* f. sp. *elaeidis* isolates

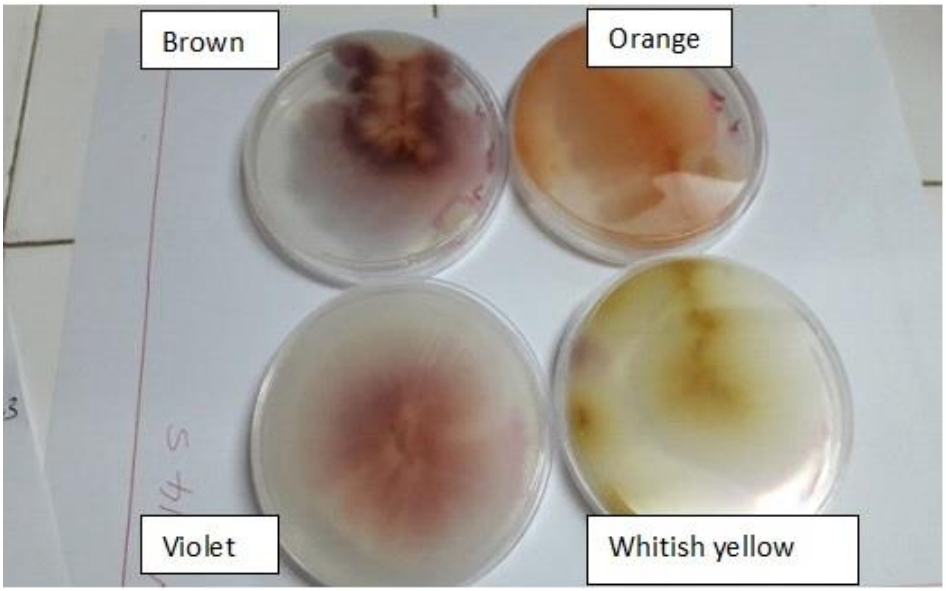

Fig 10 – Reverse Colony Colour of *Fusarium oxysporum* f. sp. *elaeidis* Isolates

Table 3 Degree of Sensitivity for F.o.e Isolates to five Methylene chloride plant extracts.

Site	Ginseng	Moringa	Neem leaves	Tithonia	Neem oil
Bota	-	-	-	+	-
Mungo	-	++	+++	+++	+
Mondoni	-	++	++	+++	-
Debundscha	+++	++	+++	+++	++
Idenau	++	++	+	-	+++

- = no inhibition, + = slight inhibition; ++ = medium inhibition; +++ = high zone of inhibition

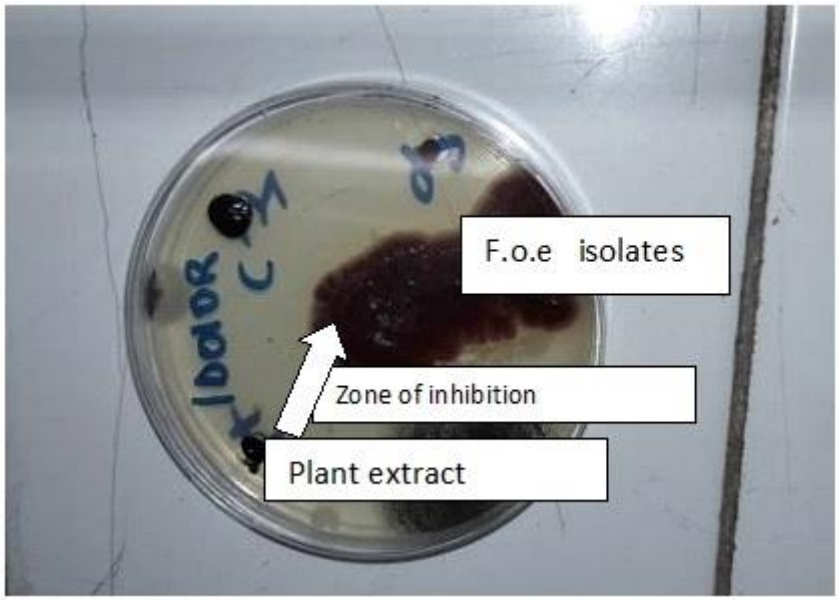

Fig. 11 – Preliminary sensitivity test showing zone of inhibition against F.o.e isolate

With methanol extracts, the most prominent extract that had high zones of inhibition was Neem leaves. Isolates of all the estates except Bota were sensitive to neem leaves, Table 4.

Table 4 Degree of Sensitivity for F.o.e Isolates to five Methanol plant extracts.

Site	Ginseng	Moringa	Neem leaves	Tithonia	Neem oil
Bota	+	+	+	+++	+
Mungo	+	+	++	-	-
Mondoni	+	+	++	+	-
Debundscha	++	+	+++	++	+
Idenau	+	+	++	+	-

- = no inhibition, + = slight inhibition; ++ = medium inhibition; +++ = high zone of inhibition.

Minimum inhibitory concentration (MIC)

All dilutions had an effect on the inhibitory diameter. There was a significant difference between the control and the four dilutions made. There was also a significant difference between 20 mg/ml and 50 mg/ml dilutions. An increasing dilution rate in the neem extract yielded more inhibitory effect on the fungus while for the *Tithonia* extract, as the dilution rate increased, a lesser inhibitory effect was encountered.

Discussion

Samples collected from Mungo Estate produced highest number of isolates followed by Mondoni, then Debundscha, Bota and Idenau. This could be due to the genetic origin of the planting material which is the most important factor in the occurrence of the disease (Ntsomboh et al. 2012), with the previous crop on primary forest, the disease appears late, whilst on soils impoverished by previous crops, and in second generation oil palm, vascular wilt can develop rapidly and crop techniques also has an effect. A culture-dependent approach was used to assess the cultural variability composition of F.o.e from different oil palm estates. *Fusarium oxysporum* f. sp. *elaeidis* was identified in samples that were collected based on their morphological and cultural characteristics. The method though inadequate, remains the most common and cheapest in the identification of fungi in developing countries as evident in other works (Kumar & Hyde 2004, Schmit & Lodge 2005). According to Leslie & Summerell (2006), F. *oxysporum* mycelium produces dark purple or dark magenta pigments when it is grown on PDA medium, never clear or whitish yellow. However, the pigmentation observed by Dubey et al. (2010) was violet, yellow and grey. Furthermore, Shahnazi et al. (2012) reported *Fusarium solani* isolates from black pepper producing pigmentation that was cream, white or whitish cream. The abundance of the *Fusarium oxysporum* f. sp. *elaeidis* in soil samples also confirms the soil borne nature of this pathogen. The location of *Fusarium oxysporum* f. sp. *elaeidis* both in the soil and in the plant renders it difficult to fight against the disease and the application of fungicide is expensive for large areas. Hence the importance of our study in bringing out the characteristics of the pathogen is a move towards devising a strategy to fight against it more efficiently through possible development of microbial antagonism or targeted biofungicide application. This is possible since the pathogen is known to spread according to soil type with sandy soils favoring its development. It also exists on rich volcanic soils like that of our study area and has been found on clay soils. Growth and morphology of all 107 strains studied varied with time and culture medium. However, this variability is inevitable as it could be influenced by light, quantity and composition of culture medium, laboratory conditions, and size of Petri dishes. This study would have been more consistent if the application of molecular techniques such as RAPD PCR (Diana et al. 1998) were applied to assess genetic variations between the *Fusarium oxysporum* f. sp. *elaeidis* isolates studied. Also if identification of the isolates was done through multigene analysis using the translation elongation factor alpha-1 gene region, the ITS and intergeneric spacer regions (O'Donnell et al.1998, Singh et al. 2006).

In this study, five concentrations of the methanol crude extract of Neem leaf effectively suppressed mycelial growth of the F.o.e. These results agree with Dissanayake (2013) who found out that methanol crude extracts of Neem, wild basil and aromatic ginger were found highly

effective in suppressing the growth of *F. oxysporum* even at 3.125% concentration. These findings also agree with Dissanayake (2012) who found that both ether and methanol Neem seed extracts gave the best results at 300 µlitre/100 ml concentrations but the methanol was found effective against *Aspergilus niger, Fusarium oxysporum* and *Trichoderma resii*. According to findings of Upasana et al. (2002), there are 15 plant extracts capable to inhibit completely conidial germination of *F. oxysporum*. Plant extracts exhibit significant fungicidal properties that support their traditional use as antiseptics. In the case of fungal infection, these mechanisms include synthesis of bioactive organic compounds Domenico et al. (2012) and antifungal proteins and peptides. Therefore, further studies are needed to determine the chemical identity of the bioactive compounds responsible for the observed antifungal activity. *Azadirachta indica* leaves possessed good anti-fungal activity, confirming the great potential of this plant in controlling *Fusarium oxysporum* f. sp. *elaeidis in vitro* because of its active compounds and it is useful for rationalizing the use of this plant in primary health care (Koona & Budida 2011). The extracts of Neem when used as medicinal plant, could be useful for the growth inhibition of the harmful fungus. The phyto-constituents; alkaloids, glycosides, flavonoids and saponins are antibiotic principles of plants. These antibiotic principles are actually the defensive mechanism of the plants against different pathogens Hafiza (2000).

Conclusion

The study has indicated the cultural variability of *Fusarium oxysporum* f. sp. *elaeidis* isolates and explored the possibilities of controlling *Fusarium oxysporum* f. sp. *elaeidis*, by using extracts of Neem and *Tithonia* leaves. The fungitoxic effects of the phyto- extracts indicates the potentials of these plant species as a source of natural fungicidal material. The findings of the present investigation could be an important step towards the possibilities of using natural plant products as anti-fungicidal sprays in the control of plant diseases caused by *Fusarium oxysporum* f. sp. *elaeidis*.

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