



Control of Maize Fungal Diseases using Essential Oils Extracted from Rosemary and Eucalyptus in Egerton University Main Campus Njoro, Kenya.

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Abstract-- Maize fungal pathogens pose a big risk to human life today. Some fungal pathogens have been linked with triggering tumorous growth in human cells. The present study was meant to isolate common fungal pathogens of stored maize grains and testing their sensitivity to essential oils extracted from rosemary and eucalyptus. The fungal pathogens were isolated using potato dextrose agar and characterised using cultural, morphological and biochemical assays. Sensitivity to essential oils was carried out using perpendicular method. The most common fungal pathogens were *Aspergillus* spp. (42%), *Penicillium* spp. (25%), *Fusarium* spp. (20%), and *Helminthosporium* spp. (13%). There was a relationship between heating time and yield of essential oils in rosemary ($r=0.99$) and eucalyptus ($r=0.99$). On the other hand, there was no significant difference in the amount of essential oils produced by rosemary and eucalyptus ($P=0.08$). In addition, there was a significant difference in growth inhibition of the fungal pathogens by essential oils from rosemary and eucalyptus ($P=0.000407$). The most prevalent fungal pathogens in maize seeds are *Penicillium* spp., *Helminthosporium* spp., *Aspergillus* spp. and *Fusarium* spp. Rosemary and eucalyptus spp. produce essential oils that are capable of controlling maize fungal pathogens. There is need to properly store maize to avoid fungal contamination which can lead to fatal human illnesses. In addition, the extraction of essential oils in large scale is highly recommended.

Keyword-- Egerton, Essential oils, Eucalyptus, Maize fungal diseases, Rosemary, Njoro.

I. INTRODUCTION

Maize is an important cereal crop in the world. It is a staple food to many populations especially in developing countries (Olga and Tibor, 2015). In addition, maize is a leading source of income to farmers. Unfortunately, cultivation of maize in the world and in Africa in particular has been curtailed by diseases. Grain losses from fungal diseases have been estimated as 11% of the total production (Peethambaran *et al.*, 2010).

In Kenya, the price of maize has tripled from 700 Kenyan shillings a bag to an average of 3000 shillings in the recent past (Camila *et al.*, 2015). The major challenge in the production of maize has been cited as prevalence of pests and diseases. Another limiting factor to maize production is post-harvest losses due to poor handling and storage (Binyam and Girma, 2016).

Among pathogens that cause diseases in maize, fungi rank second as the cause of deterioration and loss of maize (Mohammadi *et al.*, 2011). When conditions are favourable fungi cause about 50-80% of damage in stored maize. The control of maize diseases is important as a complementary technology to boost maize production (Lanubile, *et al.*, 2015).

Increased infestation of fungi has led to increased use of chemicals for their control (Christensen *et al.*, 2014). Indiscriminate use of chemicals has in turn lead to adverse effects on both human health and the environment. In humans it leads health problems like sneezing, throat irritation and coughing in addition to tumorous growth (Wu *et al.*, 2013). Most cases of human fungal poisoning have been from consumption of seed grains. Fungi also lead to environmental contamination by releasing chlorofluorocarbons and also water pollution which leads to destruction of marine life (Li *et al.*, 2011).

Due to overuse and mis-use of fungicides control of fungal pathogens has become a major problem (Rojas, *et al.*, 2014). The use of essential oils from plant has been cited as a possible method of averting this menace. Mohammadi *et al.* (2011) asserts that essential oils are concentrated hydrophobic liquid containing volatile aromatic compounds from plants. They give plants their distinctive smells, protect plants from pest and diseases and play a major role in plant pollination. Essential oils are more often extracted by distillation using steam and they have been shown to inhibit fungal growth on plants (Kabiru *et al.*, 2016).

This study aimed at first isolating the major fungal pathogens in maize followed by extracting essential oils from rosemary (*Rosmarinus officinalis*) and Eucalyptus (*Eucalyptus globulus*). In addition, the sensitivity test of the fungal pathogens was also studied.

II. MATERIALS AND METHODS

Research Area

The study was conducted at Egerton University, main campus Njoro in Kenya. Egerton University is located in Njoro Sub County with coordinates as 0 ° 23' south, 35 ° 35' and altitude of 2000m above sea level. Temperatures range between 17-22°C while the average annual rainfall is 1000mm (Amata *et al.*, 2014).

Collection of infected maize seed samples

Infected maize seeds were collected from farmers neighbouring Egerton University. The sample size was determined using a formula given by Navya *et al.* (2014).

$$n = \frac{Z^2 pq}{e^2}$$

Where n= sample size, Z= confidence level at 95% (1.96), p=estimated proportion of the sample population,

$$IF = \frac{\text{Number of samples of occurrence of fungi species}}{\text{Total number of samples}} \times 100$$

Cultural and morphological characterization of fungal pathogens

Identification of the fungal pathogens was carried out using cultural and morphological characteristics (Samson *et al.* (2010). The cultural characteristics used were colour of the colony, size and speed of growth in the culture medium. Morphological features considered were the size of the conidiophores, hyphae characteristics and elevation of the phialides.

Biochemical characterization of fungal pathogens Amylase plate assay

Screening of the fungal isolates for the amylase activity was done by plate assay (Balkan and Ertan, 2005). To obtain the proper conditions for growth, pH of the media was changed to 6.0 by use of 1 M HCl before autoclaving the media at 121 °C for 15 min. The isolates were separately inoculated on the medium and incubated at 28 ± 2 °C for 5-7 days. The zone of degradation of starch was determined which is an indicator of starch degrading activities of strains.

q= (1-p) and e= desired level of precision (0.05) with a standard value of 0.05. Substituting the values in the formula, the sample size was calculated as;

$$n = \frac{1.96^2(200)(1 - 200)}{0.05^2} = 130$$

The samples from various homesteads were mixed to make a composite sample prior to transportation to Egerton University, Department of Biological Sciences laboratory. Storage was done at 4°C until processing.

Isolation of the fungal pathogens

The fungal pathogens were isolated using Potato Dextrose Agar obtained from Oxoid (Basingstoke, UK). The maize seeds were placed in 10 % sodium hypochlorite for 2 minutes before rinsing using sterile water. The seeds were blot dried using previously sterilized Whatman filter papers and directly plated on Potato Dextrose Agar (PDA) media. The plates were incubated at a 28°C for up to 7 days (Henry *et al.*, 2015). This was followed by sub-culturing on PDA before storage in PDA slants at 4°C until further processing. Isolation frequency (IF) for each fungus was determined and expressed as percent by using the following formula (Binyam and Girma, 2016);

Lipase plate assay

The ability of the fungal isolates to produce lipase enzyme was performed by the method explained by Binyam and Girma (2016). Petri dishes containing the medium were separately inoculated with the fungal pathogens and incubated at 28 ± 2 °C for 7 days.

Pectinase plate assay

Pectinase activity of the isolates was determined according to the method given by Ortiz *et al.* (2010). Culture plates were separately inoculated with fungal pathogens and incubated for 3-5 days at 28 ± 2 °C. Lastly, the plates were flooded with 1% aqueous hexadecyltrimethyl ammonium bromide to precipitate the non-degraded pectin.

Protease plate assay

Protease activity of the fungal pathogens was determined by inoculating the strains in CZ medium amended with 1% skimmed milk powder (Suleiman and Omaf, 2013). Incubation was carried out in the dark at 28 ± 2 °C for 5 days.



Collection of plant samples

A pair of secateurs was used in cutting rosemary and eucalyptus samples from the host plants growing in Egerton University. The samples were separately placed in plastic containers to prevent drying and escaping of the volatile oils from the specimen. The samples were stored in a deep freezer at 4°C until processing.

Extraction of essential oils

A sample of 400g of fresh rosemary and eucalyptus leaves were separately loaded into 2-Litre round bottom flask containing 1.5 litre of water and placed on a heating mantle having power of 450 watt and timed.

The samples were boiled with water to release the oil within the leaves. The volatile oils evaporated along with the water into the condenser connected to a flask at 100°C and atmospheric pressure. The condensed steam and oils were collected in a separating funnel after which oil and water were separated. The water was drained off gently and the oils were separately collected in a 10ml measuring cylinder and measured after every 20 min for a period of 3h. The traces of water in the essential oils were removed by adding 1 gram of Magnesium Sulphate in the oil as a drying agent after which the yield obtained was calculated using the formula given below (Seid *et al.*, 2014);

$$\text{Yield of essential oil (\%)} = \frac{\text{amount of essential oil obtained (g)}}{\text{Amount of raw materials used (g)}} \times 100$$

Sensitivity test of the fungal pathogens to the essential oils

The antibacterial activity of the essential oils was determined by cross streak method (Mohammad *et al.*, 2009). Single streaks running at the centre of the petri plates containing Potato dextrose agar were separately made using the extracted essential oils from rosemary (*Rosmarinus officinalis*) and eucalyptus (*Eucalyptus globulus*). At right angle to this streak the fungal pathogens were inoculated followed by incubation of the plates at 27°C for 4 days. Antagonism was measured by determination of the size of the inhibition zone in millimeters.

Data analysis

Data analysis was carried out using Microsoft excel spreadsheet and Statistical Package for Social Sciences (SPSS) version 11.0 software. Pearson's correlation was used to determine the relationship between heating period and yield of essential oils while t-test was used in comparing yield of essential oils in rosemary and eucalyptus.

III. RESULTS

Cultural and morphological characteristics of the fungal isolates are presented in table 1. The most prevalent fungal pathogens were *Aspergillus spp.* (42%), *Penicillium spp.* (25%), *Fusarium spp.* (20%), and the least was *Helminthosporium spp.* (13%).

Biochemical characterization of the isolates

The *Fusarium spp.* isolated were amylase, pectinase and protease positive (Table 2). In addition, the isolate tested negative for lipase. Conversely, *Helminthosporium spp.* tested positive for lipase and pectinase and negative for pectinase and protease. On the other hand, *Aspergillus spp.* were positive for amylase, pectinase and protease while testing negative for lipase. Lastly, the isolated *Penicillium spp.* were positive for lipase, pectinase and protease but negative for amylase.

Table 1:
 Cultural and morphological characteristics of fungal pathogens

Fungal pathogens	Cultural Characteristics	Morphological Characteristics	Frequency
<i>Fusarium spp.</i>	Rapidly growing colonies, wooly to cottony growth, lemon and yellow	Multicellular distinctive sickle shaped macro <i>conidlia</i> .	20
<i>Helminthosporium spp.</i>	Colony olivaceous when young, blackening at maturity, velvety, slow growing	Hyphae branched, septate, pale brown, darkening with age, conidiophores septate, usually unbranched	13
<i>Aspergillus spp.</i>	Very common colours of colony (black and white)	<i>Conidia</i> borne in 360 arrangements covering the upper 2/3 of the <i>conidiophores</i> .	42
<i>Penicillium spp.</i>	Large fluffy white colonies almost covering the whole surface.	Non – septate branched hyphal enlarge at the apex to form <i>cornidophorex</i> they produce brownish black <i>conidia</i> in chains.	25

IV. EXTRACTION OF ESSENTIAL OILS

The yield of essential oils varied from 4.5% after the sampled were heat for 180 minutes to 0.6% after heating for 20 minutes in rosemary (Table 3). On the other hand, the percentage yield in eucalyptus ranged from 3.5 after the samples were heat for 180 minutes to 0.2 after heating for 20 minutes.

The weights of the plant samples, volume of distilled water and the heating temperature were maintained constant at 400g, 1.5 L, 100°C respectively. There was a relationship between heating time and yield of essential oils in rosemary ($r=0.99$) and leaves ($r=0.99$). Conversely, there was no significant difference in the amount of essential oils produced by rosemary and eucalyptus ($P=0.08$).

Table 2:
 Biochemical characteristics of the fungal isolates

Isolate	Amylase	Lipase	Pectinase	Protease
<i>Fusarium spp.</i>	+	-	+	+
<i>Helminthosporium spp.</i>	+	+	-	-
<i>Aspergillus spp.</i>	+	-	+	+
<i>Penicillium spp.</i>	-	+	+	+

Table 3:
 Yield of essential oils from rosemary and eucalyptus

Plant	Weight (g)	Distilled H ₂ O (L)	Heating time (Min)	Temperature (°C)	Yield (%)
Rosemary	400	1.5	20	100	0.6
	400	1.5	40	100	1.0
	400	1.5	60	100	1.7
	400	1.5	80	100	2.0
	400	1.5	100	100	2.8
	400	1.5	120	100	3.0
	400	1.5	140	100	3.2
	400	1.5	160	100	4.1
	400	1.5	180	100	4.5
	Eucalyptus	400	1.5	20	100
400		1.5	40	100	0.4
400		1.5	60	100	0.6
400		1.5	80	100	1.1
400		1.5	100	100	1.7
400		1.5	120	100	2.0
400		1.5	140	100	2.8
400		1.5	160	100	3.0
400		1.5	180	100	3.5

Sensitivity of the fungal pathogens to essential oils

The zone of inhibition of essential oils obtained from rosemary against *Fusarium spp.* was 18mm, *Helminthosporium spp.* (21mm), *Aspergillus spp.* (20mm) and *Penicillium spp.* (17mm) (Table 4). On the other hand, the zone of inhibition of eucalyptus essential oils on *Fusarium spp.* was 10mm, *Helminthosporium spp.* (12mm), *Aspergillus spp.* (13mm) and *Penicillium spp.* (09mm). There was a significant difference in growth inhibition of the fungal pathogens to the essential oils from rosemary and eucalyptus (P=0.000407).

V. DISCUSSION

Fusarium spp. is a soil borne pathogen. As a result, it is a serious pathogen of maize growing in soils having spores of the pathogen. The pathogen manifests itself as pale leaves, brown stem veins and wilted plants. The isolation of *Fusarium spp.* in the current study suggests wrong storage of maize because *Fusarium spp.* requires moisture for growth which agrees with a previous study carried out in Europe (Griffin, 2013) (Table 1).

Table 4:
 Sensitivity of the fungal pathogens to the extracted essential oils

Fungal pathogen	Zone of inhibition (mm)	
	Essential oils of rosemary	Essential oils of eucalyptus
<i>Fusarium spp.</i>	18	10
<i>Helminthosporium spp.</i>	21	12
<i>Aspergillus spp.</i>	20	13
<i>Penicillium spp.</i>	17	09

Fusarium spp. represents a diverse lineage of toxin producing strains in maize (Abdel, 2012). *Helminthosporium spp.* causes a lot of losses in poorly stored maize leading to huge losses (Charity *et al.*, 2010). Its negative effects start in the field where, if infected plants are detected should be cut and burnt to control it. The isolation of *Helminthosporium spp.* in this study agrees with findings of Navya *et al.* (2014). *Aspergillus spp.* has been known to produce a number of secondary metabolites some of which produce fatal conditions such as nervous poisoning in humans (Castellarie *et al.*, 2010). Its consumption has been shown to cause mass deaths of humans in many parts of the world (Abdullah *et al.*, 2016). The results of this study partly concurs with those obtained elsewhere (Prusky *et al.*, 2010). *Penicillium spp.* is one of the leading causes of post-harvest fungal pathogen that leads to great losses in cereals. It causes changes in pH of the affected tissues leading to rotting of the infected part (Abdullah *et al.*, 2016). The results of the current study differ with those of a previous study by (Camila *et al.*, 2015).

The results on biochemical tests of the fungal pathogens isolated in the current study are typical of the identified isolates (Table 2). This is in agreement with a study carried out in Nigeria (Onyeze *et al.*, 2013). However, the results on extraction of essential oils from rosemary and eucalyptus were higher than in a previous study (Kabiru *et al.*, 2016) (Table 3). This could be attributed to the ecological conditions in which the plants were growing. According to Kaskoniene *et al.*, (2013), soil fertility and high rainfall favours rosemary and eucalyptus growth and therefore production of essential oils.

The inhibitions of the fungal pathogens by essential oils obtained from rosemary and eucalyptus are presented in table 4. These results disagree with a previous study by (Vignesh *et al.*, 2016). These results of this study suggest that essential oils from rosemary are more effective in controlling fungal pathogens than those of eucalyptus.

The possible reasons could be that rosemary produces a wide variety of antimicrobials than eucalyptus (Kabiru *et al.*, 2016).

VI. CONCLUSION AND RECOMMENDATIONS

The most prevalent fungal pathogens in maize seeds are *Fusarium spp.*, *Helminthosporium spp.*, *Aspergillus spp.* and *Penicillium spp.* Rosemary and eucalyptus spp. produce essential oils that are capable of controlling maize fungal pathogens. There is need to properly store maize to avoid fungal contamination which can lead to fatal human illnesses. In addition, the extraction of essential oils on large scale is highly recommendation.

Conflict of interest

The authors declare no conflict of interest.

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International Journal of Recent Development in Engineering and Technology

Website: www.ijrdet.com (ISSN 2347-6435(Online) Volume 6, Issue 1, January 2017)

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