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Control of Ascochyta blight of Chickenpea using Essential Oils from Thyme (*Thymus vulgaris*), Sage (*Salvia officinalis L.*) and Rosemary (*Rosmarinus officinalis L.*) Plants

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Abstract

Chick pea is considered as the most important sources of cheap protein. However, the crop is endangered by a number of fungal diseases. Top in the list of these fungal diseases is ascochyta blight. This study was aimed at testing the sensitivity of *Ascochyta rabiei* to essential oils from thyme, sage and rosemary plant. Infected chick pea plant samples were collected from field seven in Egerton University. Fungal pathogens were isolated from the infected plants. Essential oils were extracted from thyme, sage and rosemary plant. Sensitivity of *Ascochyta rabiei* against extracted different essential oils was carried out, using agar well technique. The prevalence of *Ascochyta rabiei* in the plant samples from chick pea was 50%. The most active fungal pathogens were *Alternaria spp*. (45%), *Aschochyta rabiei* (50%), *Fusarium spp*. (22%), and *Penicillium spp*. (16%) and *Coletotrichum spp*. (17%).

There was a relationship in between yield of essential oils and heating time in rosemary (r=0.99) and leaves (r=0.99). Generally, no significant difference in the %yield of essential oils thyme, sage and rosemary (P=0.057) was seen. The percentage yield of essential oils ranged from β-Caryophyllene (1.9%) to Carvacrol (19.9%) in thyme; cis-Thujone (2.3%) to Camphenilone (25.2%) in sage and α-terpineol (2.5%) to Comphor (34.7%) in rosemary. The zone of inhibition in essential oils which was obtained from thyme against *Ascochyta rabiei* was 20 mm, sage (17 mm) and rosemary (19 mm). The minimum inhibition of essential oils from thyme leaves was 125 ± 0.02 mg/ml, sage (250 ± 0.01 mg/ml) and rosemary (250 ± 0.02 mg/ml). The essential oils from thyme, sage and rosemary have bioactive compounds that have antifungal properties against *Ascochyta rabiei*.

Keywords: Antagonism; *Ascochyta rabiei*; Chickpea; Rosemary; Sage; Thyme

Introduction

Chick peas (*Cicer arietinum*) have been described as an immensely nutritious pulse crop [1]. Its popularity lies in its high protein content which has led to the plant been branded as poor man's meat [2]. The plant thieves well in arid and semi-arid environmental conditions [3]. Chickpea is produced majorly by countries such as India, Turkey, Pakistan, Iran and Mexico [4]. Pakistan is one of the leading countries in production of the crop in the world. In Kenya the crop ranks third in cultivation after common bean and peas [5]. However, the acreage production of chickpea is very low in Kenya comparing to other countries throughout the world [6]. Regardless of the increasing demand of cheap protein sources, chickpea production is low with a world production of 0.8 t/ha [7]. About ninety percent losses in the yield occur due to root based diseases and pathogenic fungi such as ascomyceta blight [1]. Chickpea production is greatly influenced by root based pathogens in many countries of the world like India, Pakistan, Tunisia, Spain, Iran, Nepal and Burma [8].

Root infecting pathogenic fungi includes mainly *Fusarium oxysporum fsp. ciceris*, *Macrophomina phaseolina*, *Fusarium solani*, *Rhizoctonia solani* and *Phythium ultimum* [9]. These fungal pathogens are most prevalent because they produce spores which can persist in the environment without the host plant for more than six years [10]. Studies carried out elsewhere reported that ascomycota blight is responsible for 10%-15% yield losses in chickpea while black root rot damaged 60%-70%. Dry root rot caused by *Macrophomina phaseolina* damage over 500 host plant species of the tropical and temperate regions of the world [10].

Currently, many fungi including *Ascochyta rabiei* in chickpea have developed resistance to conventional anti-fungicides been used to day [11]. One of the remedies to the resistance is seeking for natural solutions to the problem such as the use of essential oils from different oil producing plants [12].

The term "essential oil" (Eos) was used for the first time in the 16th century by Paracelsus von Hohenheim, who used it to refer to the effective component of a drug as "Quinta essential" [13]. However, the first use of essential oils for therapeutic reasons was found in a document known as *Ebers papyrus* [14]. The document listed more than 800 EOs remedies and treatments of various diseases [15]. Myrrh was a favorite ingredient, often mixed with honey and other herbs, because of its high potential of inhibiting bacterial growth [16].

According to Fernandez DJ et al. essential oils are volatile oils that are complex mixtures of volatile constituents. They are synthesized by plants whose contents are made of at least two related groups which include terpenes and terpenoids and aromatic and aliphatic constituents, all of which have low molecular weight [17,18].

Thymus sp. is a well-known plant with aromatic characteristics which has been used since ancient times as a spice and herbal medicine [19]. Gharbi S et al. reported that essential oils from thyme (*Thymus zygis L.*) had a great inhibitory effect on *Botrytis cinerea* spore germination when compared with essential oils from other plants [20,21].

Another source of essential oils that has been exploited for a long time is members of the family *Cyperaceae* (sages) [20]. The sages are ubiquitous, but require experience to recognize because of their closeness to the grass family [21]. observed that four-fifths of the sedge plants do very well in damp or wet places, while one-fifth is found in drier regions, such as savannah grassland and sandy places including sand-dunes [21,22].

On the other hand, rosemary (*Rosmarinus officinalis L.*) originated from Southern Europe [23]. The plant has high antimicrobial and antioxidant properties [24]. Besides, the plant is used as a food flavoring agent because of its desirable aroma [25]. Egamberdieva D et al. reported that rosemary plants are rich sources of phenolic compounds with high antimicrobial activity against both Gram-positive and Gram-negative bacteria [26,27].

The aim of this study was to investigate the antifungal properties of essentials oils from thyme, sage and rosemary plant against *Ascochyta rabiei*.

Materials and Methods

The study 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 2000 m above sea level. Temperatures range between 17°C-22°C while the average annual rainfall is 1000 mm [28].

Collection of infected plant samples

One hundred and fifty samples of infected chick pea plant were randomly collected from field seven in Egerton University. The samples were weighed before being placed in sterile plastic bags, before storage in a refrigerator until mycological analysis.

Collection of thyme, sage and rosemary plant samples

A pair of secateurs was used in cutting rosemary, sage and rosemary samples from the host plants growing in Egerton University (Figure 1). 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.

Figure 1: Plant samples collected from thyme (A), sage (B) and rosemary (C).

Isolation of the fungi

The fungal pathogens were cultured in Potato Dextrose (Oxoid, Basingstoke, UK). The chickpea plant samples were placed in 10% sodium hypochlorite for 2 minutes before rinsing using sterile water. The chickpea plant samples 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. The fungal colonies were sub-cultured separately in PDA till pure cultures were obtained. The pure cultures were separately stored in PDA slants at 4°C until further processing. Isolation frequency (IF) for each fungus was determined and expressed as percent [29].

Cultural and morphological characterization of fungal pathogens

Identification of the fungal pathogens was carried out using cultural, morphological characteristics and fungi identification keys [30]. The cultural characteristics used in identification 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 philiades.

Extraction of essential oils from thyme, sage and rosemary plant samples

A sample of 400 g of fresh thyme, sage and rosemary leaves were separately loaded into 2 L round bottom flask containing 1.5 L of water

and placed on a heating mantle having power of 450 W 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. 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 10 ml measuring cylinder and measured after every 20 minutes for a period of 3 hours. The traces of water in the essential oils were removed by adding 1 gram of magnesium sulphate in the oil as a drying agent [31].

Sensitivity test of *Ascochyta rabiei* to essential oils extracted from thyme, sedge and rosemary

The antifungal activity of the essential oils was determined using agar well technique [3]. Wells 8 mm in diameter were made in PDA previously inoculated with *Ascochyta rabiei*. Separately, the essential oils from thyme, sage and rosemary plants were aseptically added using a micropipette. The petri dishes were placed in a refrigerator for 2 h to give the essential oils time to diffuse. The petri dishes were incubated at 27°C for 5 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 17.0 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.

Results

Isolation of fungal pathogens from chickpea plant

Cultural and morphological characteristics of the fungal isolates are presented in Table 1.

Fungal pathogens	Cultural characteristics	Morphological characteristics	Frequency (%)
Ascochyta rabiei	Large pink, rapidly growing cottony colonies.	The conidia were hyaline, rounded at both ends and occasionally two-celled under compound microscope.	50
Alternaria spp.	Large, brown colonies almost filling the whole plate.	Septate branched hyphae with brown conidia.	45
Fusarium spp.	Rapidly growing wooly to cottonly lemon and yellow.	Multicellular distinctive sickle shaped macro conidia.	22
Colletotrichum spp.	Large cottony growth, pink pigmented.	Conidia are one-celled, ovoid to oblong, slightly curved at one.	17
Penicillium spp.	Large fluffy white colonies almost covering the whole surface.	Non-septate branched hyphal enlarge at the apex to form cornidophorex they produce brownish black conidia in chains.	16

Table 1: Examples of pheromone-guided antimicrobial peptides (PG-AMP).

The prevalence of *Ascochyta rabiei* (Figure 2) in the plant samples from chickpea was 50%. The most prevalent fungal pathogens were *Alternaria spp.* (45%), *Fusarium spp.* (22%), *Coletotrichum spp.* (17%), and the least was *Penicillium spp.* (16%).

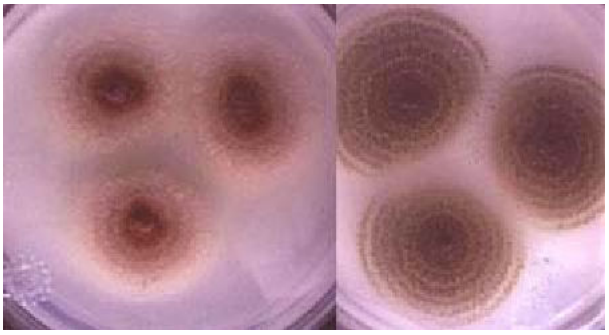

Figure 2: Pure cultures of *Ascochyta rabiei* growing on PDA.

Extraction of essential oils

Essentials oils from thyme, sage and rosemary had different colours (Figure 3).

Figure 3: Essential oil from thyme (A), sage (B) and rosemary (C).

The yield of essential oils varied from 0.6% after the samples were heat for 20 minutes to 4.5% after heating for 180 minutes in thyme (Table 2). On the other hand, the percentage yield in sage plant ranged from 0.1% after the samples were heat for 20 minutes to 3.5% after heating for 180 minutes. However, the yield of essential oils from rosemary varied from 0.1% after heating the samples for 20 minutes to 2.2% after the samples were heat for 140 minutes.

Plant	Weight (g)	Distilled H_2O (L)	Heating time (minutes)	Temperature (°C)	Yield (%)
Thyme	400	1.5	20	100	0.6
	400	1.5	40	100	1
	400	1.5	60	100	1.7
	400	1.5	80	100	2
	400	1.5	100	100	2.8
	400	1.5	120	100	3
	400	1.5	140	100	3.2
	400	1.5	160	100	4.1
	400	1.5	180	100	4.5
Sage	400	1.5	20	100	0.2
	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
	400	1.5	140	100	2.8
	400	1.5	160	100	3
	400	1.5	180	100	3.5
Rosemary	400	1.5	20	100	0.1
	400	1.5	40	100	0.6
	400	1.5	60	100	0.4
	400	1.5	80	100	1.2
	400	1.5	100	100	1.9
	400	1.5	120	100	1
	400	1.5	140	100	2.2
	400	1.5	160	100	2
	400	1.5	180	100	1.5

Table 2: Yield of essential oils from rosemary, sage and thyme.

The weights of the plant samples, volume of distilled water and the heating temperature were maintained constant at 400 g, 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 percentage yield of essential oils thyme, sage and rosemary (P=0.057).

Constituents of essential oils from leaves of thyme plant

The percentage composition of alpha pinene in thyme leaves was 11.3%, camphene (2.6%), beta pinene (9.5%), para cymene (11.2%), linalool (15.3%), borneol (10.9%), beta caryophyllene (1.9%), thymol (13.2%), carvacrol (19.9%), alpha terpinene (5.6%) (Table 3).

There was no significant difference in the percentage composition of the different compounds in the essential oils extract from thyme, sage and rosemary (F=0.75, P=0.48).

Compound	Composition (%)
Alpha Pinene	11.3
Camphene	2.6
Beta Pinene	9.5
Para Cymene	11.2
Linalool	15.3
Borneol	10.9
Beta Caryophyllene	1.9
Thymol	13.2
Carvacrol	19.9
Alpha Terpinene	5.6

Table 3: GC-MS analysis of essential oils from thyme leaves.

Constituents of essential oils from leaves of sage plant

The percentage composition of α-thujene in essential oil from sage leaves was 23.2%, α-pinene (15.7%), camphene (10.4%), β-pinene (9.6%), myrcene (15.8%), p-cymene (12.2%), 1,8-cineole (13.9%), camphenilone (25.2%), cis-thujone (2.3%), trans-thujone (4.1%) (Table 4).

Compound	Composition (%)
α-Thujene	23.2
α-pinene	15.7
Camphene	10.4
β-Pinene	9.6
Myrcene	15.8
p-Cymene	12.2
1,8-Cineole	13.9
Camphenilone	25.2
cis-Thujone	2.3
trans-Thujone	4.1

Table 4: GC-MS analysis of essential oils from sage (*Salvia officinalis L.*) leaves.

Constituents of essential oils from leaves of rosemary plant

The percentage composition of comphor in essential oil from sage leaves was 34.7%, α-pinene (21.6%), 1,8-cineole (14.4%), camphene (8.6%), borneol (7.7%), β-pinene (7.5%), verbenone (5.8%), β-caryophyllene (5.1%), limonene (3.8%), α-terpineol (2.5%) (Table 5).

Compound	Composition (%)
Comphor	34.7
α-pinene	21.6
1,8-cineole	14.4
camphene	8.6
Borneol	7.7
β-pinene	7.5
Verbenone	5.8
β-caryophyllene	5.1
Limonene	3.8
α-terpineol	2.5

Table 5: GC-MS analysis of essential oils from rosemary (*Rosmarinus officinalis L.*) leaves.

Sensitivity of *Ascochyta rabiei* to essential oils from thyme, sage and rosemary

The zone of inhibition of essential oils obtained from thyme against *Ascochyta rabiei* was 20 mm, sage (17 mm) and rosemary (19 mm) (Table 6).

Essential oil	Zone of inhibition (mm)
Thyme	20
Sage	17
Rosemary	19

Table 6: Sensitivity of the fungal pathogens to the extracted essential oils from thyme sage and rosemary.

Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of *Ascochyta rabiei* to essential oils from thyme, sage and rosemary

The minimum inhibition of essential oils from thyme leaves was 125 ± 0.02 mg/ml, sage (250 ± 0.01 mg/ml) and rosemary (250 ± 0.02 mg/ml) (Table 7).

Essential oil	MIC inhibition (mg/ml)	MFC (mg/ml)
Thyme	125 ± 0.02	125 ± 0.02
Sage	250 ± 0.01	250 ± 0.01
Rosemary	250 ± 0.02	250 ± 0.02

Table 7: MIC and MFC of essential oils from thyme, sage and rosemary plant.

In addition, the minimum fungicidal concentration of essential oils from thyme was (125 ± 0.02 mm/ml), sage (250 ± 0.01) and rosemary (250 ± 0.02). The minimum inhibition concentration of in essential oils from thyme tree leaves, sage and rosemary were equal to the minimum fungicidal concentration.

Discussion

The most common fungal pathogens in chickpea isolated in the current study were *Ascochyta rabiei* (50%), *Alternaria sp.* (45%), *Fusarium sp.* (22%), *Coletrotrichum sp.* (17%) and *Penicillium sp.* (16%). These results differed with those of a previous study carried out in China [17]. This can be attributed to differences in the environment in which the plants were growing. In addition [21] obtained results that differed with those of the current study. Further Eljounaidi K et al. explained that the spore content in the soil in which chickpea are growing greatly influences the fungal isolates obtained from plants growing in those areas [30].

However, the percentage yield of essential oils from leaves of thyme plant obtained in the current study concurred with a previous study carried out in Jordan by Abu-Darwish MS et al. [32]. These may be attributed to the species of thyme tree from which the essentials oils were extracted. In addition, the percentage yield of essential oils obtained from sage plant in this study differed with those obtained by Hamad YK et al. [33]. This may be attributed to the nutrient level of the soils in which the plant is growing in. Bernardes WA et al. explained that the soil nutrient level of a given area influences synthesis of essential oils in sage plant [34].

The percentage yield of essential oils from rosemary obtained from this study concurred with a study carried out by Moghtader M et al. [35]. This may be attributed to similarity of the species of rosemary that were being studied by Iqbal SM et al. [14]. Further Aljabeili HS et al. explained that the method of extraction of essential oils affects production of essential oils in *Rosemary spp.* [36].

The constituents of essential oils obtained from thyme leaves concurred with a previous study carried out in Pakistan [37]. Witkowska DI et al. asserted that the biochemical pathways used by thyme plant greatly influences the composition of essential oils produced [11]. The compounds obtained from essential oils of sage leaves in the present study agreed with a previous study carried out in India. This may be attributed to similarities in the study areas [38].

However, the constituents of essential oils from rosemary slightly differed with previous studies carried out in Brazil [39]. According to Bassolé IH et al. the species of rosemary from which the essential oils were obtained may have contributed to the observed results [40].

On the other hand, the zones of inhibition of the extracted essential oils from thyme, sage and rosemary on *Ascochyta rabiei* disagreed with a previous study by Pandey AK et al. [41]. This may be attributed to the type of solvents used to extract the essential oils [42]. Besides the minimum inhibition concentration (MIC) and minimum fungicidal concentration (MFC) obtained in the present study disagreed with a previous study [7]. This may be attributed to variations in the compounds present in the essential oils extracted [4]. In addition, MIC and MFC obtained in this study were equal. This suggested that the essential oils were fungicidal and not fungistatic.

Conclusion

Isolation of fungal pathogens from chickpea was carried out. Essential oils were extracted from thyme, sage and rosemary and their active ingredients determined. In addition, sensitivity test of the extracted essential oils to *Ascochyta rabiei* was carried out followed by determination of their MIC and MFC.

Recommendations

There is need to test the sensitivity of other fungal and bacterial pathogens to the extracted essential oils. Mass production of essential oils from thyme, sage and rosemary need to be carried out.

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