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APPLICATION OF ANISE AND ROCKET ESSENTIAL OILS IN PRESERVATION OF OLD MANUSCRIPTS AGAINST FUNGAL DETERIORATION

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Abstract

The main goal of this work was to evaluate the effectiveness of zinc sulphate as a microelement in combination with anis and rocket essential oils as alternative preservatives protecting old manuscripts. Two fungal isolates of Fusarium oxysporum and Trichoderma viride isolated from deteriorated manuscripts were chosen to evaluate the protective agents. The obtained results revealed that the concentration of 100mM of zinc ion completely inhibit the growth of F. oxysporum, whereas the growth of T. viride was reduced growth by 80.22%. The essential oil of anis and rocket were chemically analyzed by GC-MS. The main components of anis were anethole (91.06%) followed by Cyclooctasiloxane (2.29%), then Humulen (1.48%). While, the main component of rocket identified were 1-Isothiocyanato-4-(methylthio) butane (erucin = sulforaphane) (81.23%), followed by Carvacrol (5.27%) and Thymol (5.16%). The results showed that the main mechanical properties either elongation percent (%) or maximum force (N/mm^2) retained their values due to treatment with $ZnSO_4$ and fumigated by anise or rocket.

Keywords*: Essential oils; Metallic ion; Manuscripts; Fusarium oxysporum; Trichoderma viride.*

Introduction

Manuscripts constitute our most precious national and cultural heritage. Thus, the preservation of manuscripts is a serious problem for custodians throughout the world and this is why every possible effort must be taken to save these treasures for the future generation.

The responses of *Trichoderma* isolates to zinc ions were connected with concentrations of this metal. Zinc dosed 1000 and 3000ppm inhibited mycelial growth and *Trichoderma* spore germination, whereas lower concentrations of this metal stimulated *T. harzianum* growth [1], while Zn ion at 16ppm did not cause any effect on growth of *Trichoderma viride* [2], Zinc apparently inhibit the mycelial growth, whereas manganese ion stimulated spore germination of *T. viride* [3].

Anise (*Pimpinella anisum* L.), belonging to the *Umbelliferae* family is an annual herbaceous and a typical aromatic plant, which grows in several regions all over the world [4-6]. The chemical composition of essential oil of several Pimpinella species has been studied [7-11]. There are usually considerably variations in the major components within this species.

M. Embong et al. [7] established (E)-anethole (72.2%), (Z)-anethole (1.1%), anisyl ceton (0.9%), β-caryophyllene (0.8%) and carvone (0.3%); *M. Lawrence* [10] identified twenty-two

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components of the anise oil with the major components of (E)-anethole (85%), (Z)-anethole (2.2%) and methyl chavicol (1.02%); *F. Askari et al.* [6]identified (E)-anethole (90%), eugenyl acetate (2%), γ-gurjunene (1.85%) and estragole (1.04%).

Volatile oils used for culinary, pharmaceutical and perfumery purposes are composed almost entirely of two classes of compounds, terpenes and phenylpropenes [12].

So, this investigation was done to evaluate effectiveness of zinc sulphate as a microelement and anis and rocket essential oils as alternative preservatives fumigant protecting old manuscripts.

Materials and Methods

Effect of zinc sulphate microelement on fungal growth and cellulolytic enzyme activity

This experiment was performed *in vitro* to study the effect of $ZnSO_4$ metallic ion on the growth and cellulolytic enzyme activity of *Fusarium oxysporum* and *Trichoderma viride* isolates. Sterile PDA solid medium was amended with serial concentrations of $ZnSO_4$ metallic ion, at concentrations of 10, 50, and 100mM. The tested microelement was added to the medium before autoclaving and a set of three replicates were used. The efficacy of the microelement was expressed as % of inhibition of mycelial growth against control, calculated by using formula by [13]. Three replicates were performed for each particular treatment.

$$IP = [(C – T)/C] \text{ X } 100 \qquad (1)$$

where: IP - inhibitory percentage, C - Average colony diameter in check (control),
T - Average colony diameter in treatment

Detection of cellulases activity

The previously selected fungal isolates were propagated on slants of Czapek's agar medium for seven days to prepare the spore suspension as inocula for the culture medium.

The activity of the cellulases was assayed according to *N. Sidkey et al* [14], 250mL Erlenmeyer flasks each contains 100mL buffer (citrate phosphate buffer, pH = 5.5), and 1% Avicel, CMC or filter paper, and 1.5% agar were boiled until it's ingredients completely homogenized. An aliquot of 20mL of the above homogenized substrate were poured in each petri dish. After solidification of the plates make 2 holes on each side of the agar plate and 0.1mL of the above cell free filtrate (CFF) were filled in the well. After incubation period of 20h at 28±2°C, the plates were flooded with iodine solution (1% iodine: 2% potassium iodide). The clear zone around the well was measured in mm, as the diameter of the zone increase as the cellulases activity increase.

Preparation of essential oil

Seeds (250g) of Anise (*Pimpinella anisum*), Fennel (*Foeniculum vulgare*), Rosemary (*Rosmarinus officinalis*), Rocket (*Eruca sativa*) and Tea tree oil (*Melaleuca alternifolia*) were obtained from Department of Medicinal and Aromatic Plants Research, National Research Center, Egypt were subjected to hydrodistillation for 3hr. using a Clevenger type apparatus to obtain essential oil according to the Egyptian Pharmacopoeia [15]. Lower aqueous layer was discarded and upper layer of oil was collected. The resulted essential oil of each treatment was separately dehydrated with anhydrous sodium sulfate and preserved in a sealed vial at 4°C until further analysis [16].

Analysis of essential oil

The GC-MS analysis of the essential oil samples was carried out using gas chromatography-mass spectrometry instrument stands at National Research Center with the following specifications, instrument: a TRACE GC Ultra Gas Chromatographs (THERMO Scientific Corp., USA), coupled with a THERMO mass spectrometer detector (ISQ Single

Quadrupole Mass Spectrometer). The GC-MS system was equipped with a TG-WAX MS column (30m x 0.25mm i.d., 0.25μm film thickness). Analyses were carried out using helium as carrier gas at a flow rate of 1.0 ml/min and a split ratio of 1:10 using the following temperature program: 40ºC for 1 min; rising at 4.0ºC/min to 160ºC and held for 6min; rising at 6ºC/min to 210ºC and held for 1min. The injector and detector were held at 210ºC. Diluted samples (1:10 hexane, v/v) of 0.2μL of the mixtures were always injected. Mass spectra were obtained by electron ionization (EI) at 70eV, using a spectral range of m/z 40-450.

Components were preliminary identified by comparison of their mass spectra with those of a computer library or with authentic compounds and confirmed by comparison of their retention indices, either with those of authentic compounds or with data published in the literature. The retention indices were calculated for all volatile constituents using a homologous series of alkanes [17].

Conservation treatments for fungal control

Preparation of paper sheets

Paper sheets made of cotton pulp were prepared according to method described in Figure1.

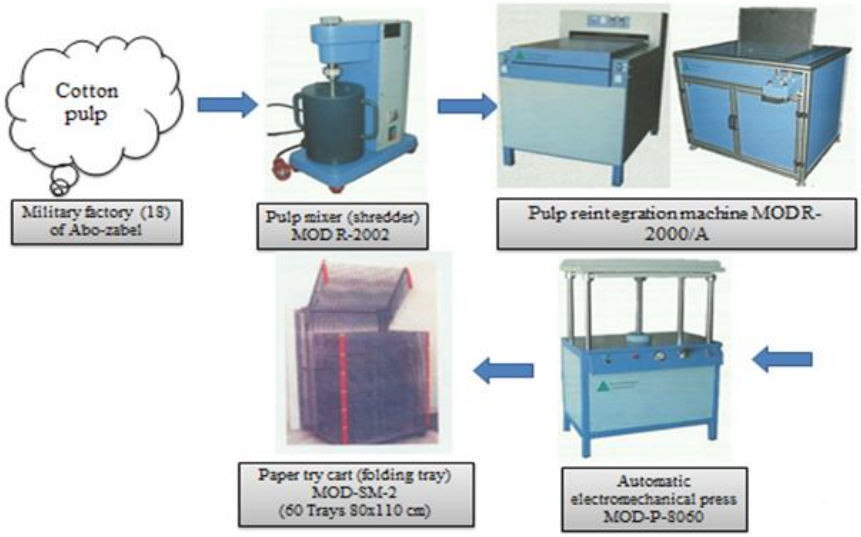


Fig. 1. Preparation of cotton paper to evaluate the effectiveness of zinc sulphate, anis and rocket essential oils

Treatment of paper sheet with protective materials (metallic ions and essential oils)

Eight paper sheets made of cotton pulp were cut into 15x20cm by using a scalpel, and sterilized by UV light exposure for one hour. Four sheets were soaked in a sterilized solution of $ZnSO_4$ at 0.05M concentration and left to dry under press. Spray 3 sheets with spore suspension of *Fusarium oxysporum* and other 3 sheets with spore suspension of *Trichoderma viride*. Incubate 2 sheets of paper for each fungus at a special jar containing anise essential oil and other 2 sheets with rocket essential oil as a fumigant, all sheets were incubated at 30ºC, for 30 days. At the end of the incubation period, the following physical properties were estimated:

Tensile strength

Sample of infected cotton paper was standardized to (1.5x15cm) and tensile strength was tested using universal testing machine at National Institute for Standards (NIS), Egypt according to *M. Brindha et al* [18].

Scanning electron microscope (SEM)

This examination was performed to evaluate the distribution and infection of fungi on the surface of treated and non-treated cotton paper samples by using SEM Model Quanta 250 FEG (Field Emission Gun), at the Egyptian mineral resources authority, Central Laboratories Sector [19].

Statistical analysis

The collected data were statistically computed using the software Mstate-c for Windows. Results were expressed with the standard error of the treatment means for 95% confidence limits.

Results and Discussions

Effect of Zinc ion on growth and cellulolytic activity

This experiment was performed *in-vitro* to study the effect of $ZnSO_4$ metallic ion on the growth and cellulolytic enzyme activity of *Fusarium oxysporum* and *Trichoderma viride* isolates.

Data in Table 1 and Figure 2a and b showed that $ZnSO_4$ completely inhibited the *F. oxysporum* growth at 100mM concentration, but the inhibition was only up to 37.41and 86.11% at 10 and 100mM concentration respectively. Whereas, $ZnSO_4$ amended solid medium at different concentrations of 10, 50 and 100mM of *T. viride* recorded percent inhibition to linear growth by 40.56, 57.22 and 80.22% respectively.

Also, all the cellulolytic activities including avicelase, carboxymethyl cellulases and filter paperase of *Fusarium oxysporum* as well as *Trichoderma viride* were adversely affected by the addition of $ZnSO_4$ to the medium. As, data of Avicelase enzyme showed that $ZnSO_4$ at 10, 50 and 100 mM added to the medium significantly decreased the enzyme activity of *F. oxysporum* by 12.47, 14.71 and 30.00% and enzyme activity of *T. viride* by 19.77, 24.42 and 27.33% respectively.

Table 1. Effect of Zinc ion on linear growth and cellulolytic activity of *Fusarium oxysporum* and *Trichoderma viride*

Metal ion	Conc. (mM)	linear growth (Ømm)		Cellulolytic activity {clear zone method (Ømm)}					
				Avicel$_{ase}$		CMC$_{ase}$		Fp$_{ase}$	
		F. oxysporum	*T. viride*	*F. oxysporum*	*T. viride*	*F. oxysporum*	*T. viride*	*F. oxysporum*	*T. viride*
Control		90.00 A	90.00 A	17.00 A	17.20 A	18.00 A	18.00 A	13.30 B	13.90 A
$ZnSO_4$	10	56.33 B	53.50 B	14.88 BC	13.80 BCD	14.30 B	13.30 BC	0 D	10.00 C
	50	12.50 E	38.50 C	14.50 BCD	13.00 CD	13.00 BC	12.50 C	0 D	10.00 C
	100	0 F	17.80 D	12.90 CD	12.50 D	13.00 BC	11.80 C	0 D	10.00 C

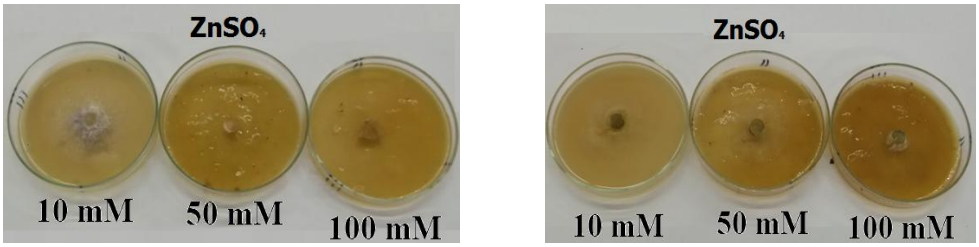
- Each figure represents average of three replicates, incubated at 30°C for 9days (solid) and 18days (liquid) Capek's medium.

- In each column, values followed by the same letters don't differ significantly (P≥ 0.05) (Duncan's multiple ranges test).

- Zero (0): No growth or no activity

Concerning, the effect of $ZnSO_4$ on the activity of CMCase of *F. oxysporum* as well as *T. viride* the same trends were also observed. However, the enzyme activity of Filter paperase, of *Fusarium oxysporum*, was completely inhibited by the addition of zinc sulphate at different

concentrations; on the other hand, it was found that, the addition of different concentrations of zinc ion led to the reduction of enzyme activity of *T. viride* by 28.06%. *G. Sanjeev and A. Eswaran* [20] and *G. Chand et al* [21] found the same trends, and agreement with other scientific researchers, of *G. Viniegra et al* [22] who stated that; the salts had been noticed directly related to metabolism, stimulation or inhibiting enzyme production in microorganism. Other scientists [23] reported that, cellulases enzymes of *T. reesei* were inhibited by Cu^{2+} (>20mM), Zn^{2+} (>1.0mM). However, metal cation of Zn^{2+} was necessary for cellulases synthesis by *T. viride* as reported by Mandels and Reese who hypothesized that, the metal may prevent some components necessary for induction from leaking out of the cells [24].



A **B**

Fig. 2. Effect of Zinc sulphate ($ZnSO_4$) on linear growth (mm) of *Fusarium oxysporum* (A) & *Trichoderma viride* (B) isolates

Effect of some essential oils fumigation and $ZnSO_4$ treatments on paper quality

Trials were made to find out the most suitable procedure for protecting old manuscripts and documents from different deteriorations, which suffer through aging.

Identification and quantification of the constituent of anise

The chemical composition of anise (*Pimpinella anisum*) essential oil obtained by GC/MS analysis is presented in Table 2 and Figure 3, the main component identified were Anethole (91.06%) followed by Cyclooctasiloxane (2.29%), then Humulen (1.48%).

Table 2. GC-MS of *Pimpinella anisum* (anise)

No.	Retention time	Area%	Compound Name
1	4.80	0.09	α-Pinene
2	7.71	0.10	Benzeneacetic acid, à,3,4tris[(trimethylsilyl)oxy], trimethylsilyl ester
3	8.71	0.03	α-Terpinen
4	10.34	0.25	Linalool
5	11.73	0.03	Geyrene
6	14.57	0.92	Benzene,1-methoxy-4-(2-propenyl)(P- Allylanisol)
7	16.85	0.30	Cyclohexasiloxane, dodecamethyl
8	**18.28**	**91.06**	**Anethole**
9	24.58	0.10	Aromandendrene
10	25.28	0.10	4,4-Dimethyl-3-(3-methylbut-3-enylidene)-2-methylenebicyclo[4.1.0] heptane
11	25.78	1.48	Humulen(v1)
12	26.64	0.06	α-Longipinene
13	27.39	0.08	Isolongifolene, 4,5dehydro
14	30.46	2.29	Cyclooctasiloxane, hexadecamethyl- CYCLOOCTA SILOXANE
15	36.11	0.71	Cyclononasiloxane, octadecamethyl
16	40.97	0.28	Octasiloxane,1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15 hexadecamethyl
17	49.42	0.15	Cyclonona siloxane, octadecamethyl
18	56.68	0.15	Heptasiloxane, hexadecamethyl

The same results were also reported before [5, 6, 16, 25-27]. Moreover, each of the essential oil components has its own contribution on biological activity of the oil. For example, Anethole was found in anise as the main compound, and this compound has more fungicidal effect [28].

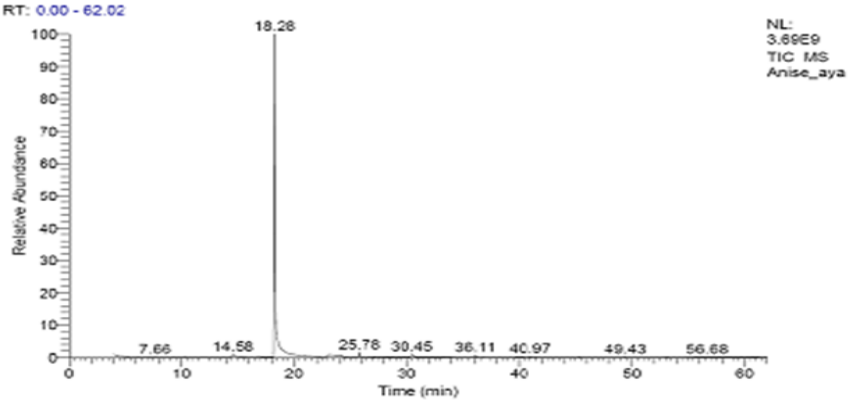


Fig. 3. GC/MS of *Pimpinella anisum* (anise) showed the highest peak (anethole)

Identification and quantification of the constituent of rocket essential oil

The chemical composition of rocket essential oil obtained by GC/MS analysis are presented in Table 3 and Figure 4, the main component identified were 1-Isothiocyanato-4-(methylthio) butane (erucin = sulforaphane) (81.23%), followed by Carvacrol (5.27%) and Thymol (5.16%). This result was also confirmed by [29-31].

Also, *B. Sabry* [32] reported that, erucin, which accounted for approximately 78.69% of the rocket extracts which play an important role as an antifungal agent

Table 3. GC/MS of *Eruca sativa* (rocket)

No.	Retention time	Area%	Compound Name
1	4.02	0.16	Allyl Isothiocyanate
2	6.28	0.94	9,10 Dideutero octadecanal
3	7.52	0.18	Cymene
4	7.74	0.23	Eucalyptol(1,8Cineole)
5	8.20	0.47	Undecane
6	8.39	0.19	Dodecane
7	9.89	0.29	I-Gala-I-ido-octose
8	10.42	0.16	Linalool
9	12.30	0.49	Camphor
10	12.83	0.18	Verbenol
11	13.28	0.24	Borneol
12	13.61	1.14	Terpinen-4-ol
13	15.08	1.94	B-D-Glucopyranose,1thio-,1[N-hydroxy-5 (methylthio)pentanimidate]
14	16.54	0.85	Carvone
15	17.03	0.32	Eicosane
16	17.87	0.28	l-Gala-l-ido-octose
17	18. 41	5.16	Thymol
18	18.77	5.27	Carvacrol
19	**24.45**	**81.23**	**Erucin (1-Isothiocyanato-4-(methylthio)butane)**
20	25.87	0.32	β-D-Glucopyranose, 1-thio-1-[N-hydroxy-5-(methylthio) Pentanimidate] (Thiofanox)
21	27.72	0.29	1, 4, 7-Triazaheptane,1,7-Bis(1-Methyl-1- phosphonato)Ethyl

Therefore, the effects of both essential oils and metallic ion on the susceptibility of recently paper to infection with fungi and on main mechanical properties were tested.

Results given in Table 4 and Figure 5 illustrated that, the infection of paper with *F. oxysporum* affected greatly the main mechanical properties both elongation percent (%) and maximum force (N/mm^2) of the paper as they decreased from 0.675% and 11.5 to 1.644% and 23.16, respectively.

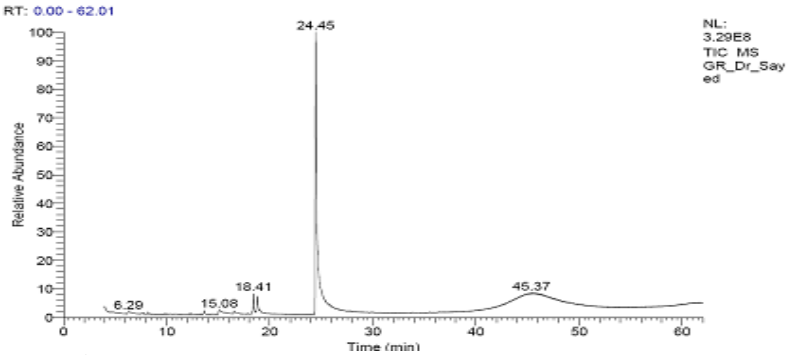


Fig. 4. GC/MS of *Eruca sativa* (rocket) showed the highest peak (1-Isothiocyanato-4 (methylthio) butane)

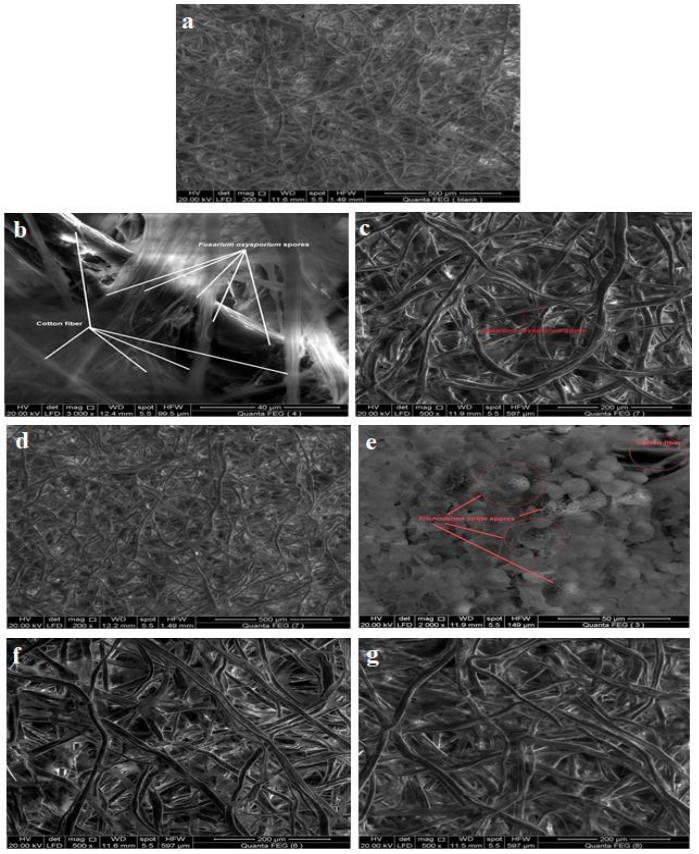


Fig. 5. SEM photographs showing the effect of $ZnSO_4$ and anise, rocket essential oil on cotton paper preservation: a - SEM picture at 200X. (control); b - SEM picture at 3000X. Cotton paper infected with *F. oxysporum* after 30days of infection. Arrows indicate the fungal spores (macro and micro conidia), c - SEM picture at 500X. Cotton paper infected with *F. oxysporum* and treated with $ZnSO_4$ and fumigated by anise. Arrows indicate fungal spore after 30 days of infection, d - SEM picture at 200X. Cotton paper infected with *T. viride* and treated with $ZnSO_4$ and fumigated by anise after 30 days of infection, e - SEM picture at 2000X. Cotton paper infected with *T. viride* after 30days of infection. Arrows indicate the fungal hypha and germinated spores, f - SEM picture at 500X. Cotton paper infected with *F. oxysporum* and treated with $ZnSO_4$ and fumigated by rocket after 30days of infection, g - SEM picture at 500X. Cotton paper infected with *T. viride* and treated with $ZnSO_4$ and fumigated by rocket after 30 days of infection.

Table 4. Effect of treatment with $ZnSO_4$ and fumigation by anise and rocket essential oils on the percentage of infection by either *Fusarium oxysporum* or *Trichoderma viride*, and subsequently on the physical properties of paper

Microorganism	*Fusarium oxysporum*		*Trichoderma viride*	
	Physical properties			
Physical properties	**Elongation%**	**Maximum force (N/mm^2)**	**Elongation%**	**Maximum force (N/mm^2)**
Control	1.644	23.16	1.644	23.16
Infected Paper	0.675	11.52	0.985	12.59
Paper treated with $ZnSO_4$ and fumigated by anise	**2.182**	**25.42**	**1.164**	**13.62**
Paper treated with $ZnSO_4$ and fumigated by rocket	**1.652**	**25.75**	**2.302**	**28.92**

Moreover, the main mechanical properties, either elongation percent (%) or maximum force (N/mm^2) retained their values due to treatment with $ZnSO_4$ and fumigated by anise or rocket. Since, infected paper with *F. oxysporum* treated with $ZnSO_4$ + anise, the elongation (%) and maximum force (N/mm^2) attained 2.182% (223.3% increase) and 25.42 (120.65% increase) than the control, respectively. The same trend was also observed with rocket essential oil as the elongation (%) and maximum force (N/mm^2) in the treated paper were increased by (144.7, 123.52 % increase), respectively.

Whereas, infected sheets with *T. viride* applied with $ZnSO_4$ and fumigated by anise or rocket essential oils revealed the same trend , as the elongation (%) was increased from 0.985% to 1.164% (18.2% increase) in anise and from 0.985% to 2.302% (133.7% increase) in rocket treatment, respectively. It is clear that almost all the maximum force and elongation values of the treated samples were higher than that of the infected paper with both fungi. The improvement in elongation and maximum force as a result of essential oils and metal ions may be due to the inhibition of fungi by essential oils and metal ions.

Scanning electron microscope (SEM)

The distribution and infection of *F. oxysporum* and *T. viride* on the surface of treated and non-treated cotton paper samples were observed in Figure 5.

Conclusion

It was revealed from the study that both fungicides and extracts of plant origin caused inhibition in mycelial growth and spore germination of *F. oxysporum* as well as *T. viride.*

So, it is worthy to state that, the recommendation to employ such treatments is not only due to their vital effectiveness in protecting treated paper sheets against biodegradation, but also to their advantage in elimination of harmful microorganisms which cause various health hazards in man and other organisms.

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