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SYNERGETIC ANTIFUNGAL ACTIVITIES OF THREE THYMUS SPECIES ESSENTIAL OILS AGAINST CHICKPEA BLIGHT

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Abstract

The objective of this study is to find out the antifungal and synergistic activity of three essential oils of thyme species: *Thymus capitatus* (TC), *Thymus bleicherianus* (TB) and *Thymus satureioides* (TS) against chickpea blight (*Ascochyta rabiei*). After obtaining the essential oils by hydrodistillation, they were tested in vitro against the fungus *Ascochyta rabiei* on agar media, in order to obtain percentages of growth inhibition (PGI) which can explain their activities. All the results found show the good activity of the three essential oils, particularly of *Thymus capitatus* of PGI equal 30.88% at the concentration of 5% and the mixture of 3 essential oils of PGI equal 32.04% at the concentration 2:2:1 (TC% / TB% / TS%). The activities mentioned during this work result from the major compounds of the essential oils of three species of Thymus, particularly thymol and carvacrol, which have known through their antimicrobial powers listed in several previous works.

Keywords: *Antifungal activity, chickpea blight, essential oils, synergetic effect, Thymus species.*

Introduction

Chickpea (*Cicer arietinum L.*) is one of the most consumable food leguminous plants in different regions of the world and is an important source of protein mainly in Africa [1-2]. Its cultivation area is currently around 11.5 million hectares worldwide, mainly in Mediterranean areas [3]. Morocco is considered to be the first producers of chickpeas in Africa, being considered a leader in the world market [4]. Ascochyta blight caused by *Ascochyta rabiei* is a fungal disease of chickpea [5], it is considered the most devastating. It affects the stems, leaves and pods of plants producing lesions and breaks in the shoots, so they can affect seeds in wetland weather conditions and diseases can develop rapidly from spore germination through the development of the individual leaves of the plant and spreading rapidly to all chickpea plants and even the destruction of the entire crop [6-8]. Spread and development of Ascochyta blight can occur by spattering and airborne conidia and/or ascospores as well as by industrial distribution of plant material or seeds [9-10].

Prophylactic measures and the application of chemical fungicides having a negative impact on human health and the environment are the main methods adopted against this disease [11-14]. the main fungicides used are: Thiram [15], Benomyl [16] and Thiabendazole [17], their uses remain limited by the presence of several disadvantages which are often ineffective in the case of exceeding the recommended doses in the fields [18-20]. Biological control and natural biofungicides are an alternative to chemical fungicides which can overcome all environmental problems especially the toxicity to human health [21-22]. The research of natural products is one of the major fields of scientists in agriculture, among these most famous substances we find essential oils which are a mixture of chemical compounds have antimicrobial power and can therefore be used as biofungicides in the fight biological [23-26].

The work of this study aims at the synergistic measurement of three essential oils of thyme such as: *Thymus capitatus* (TC), *Thymus bleicherianus* (TB) and *Thymus satureioides* (TS) by the antifungal activity against *Ascochyta rabiei* the fungus responsible for development of Ascochyta blight.

Material and methods

Plant material

The aerial parts of three species of Thymus plants: *Thymus capitatus* (TC), *Thymus bleicherianus* (TB) and *Thymus satureioides* (TS) were collected in April 2020 at different regions of Morocco, respectively Hight Atlas, Midle Atlas and Rif. These species were verified by a team of two botanists specialized in Sultan Moulay Slimane University (Morocco).

Extraction of the essential oils

To produce the essential oils of the three selected plants, the aerial parts of each species were dried in an oven at 60 ° C, then a hydrodistillation process was carried out for 4 hours using an apparatus of Clevenger type. The essential oils obtained are dried with anhydrous sodium sulfate Na_2SO_4 before being stored in a refrigerator at 4 °C in small brown vials [27].

Antifungal activity

The pure culture of the test fungal species *Ascochyta rabiei* was obtained from the Regional Center for Agronomic Research in Settat (Morocco). The culture was maintained on agar medium with malt extract (MEA).

The method used for antifungal activity is described by Jabeen & Javaid (2010) [28]. Malt extract agar (MEA) medium was prepared by autoclaving at 121°C and cooled to 50–55 °C (still in liquid form). To 7.5 mL of MEA medium, 2.5 mL of stock solutions 20% (w/v) (essential oil + distilled water + Tween 80) were added to prepare 5% w/v concentration of the essential oils in the medium. The lower concentrations of 4, 3, 2 and 1% were prepared by adding 2.0, 1.5, 1.0 and 0.5 mL of the stock solutions to 7.5 mL of MEA plus appropriate quantities of distilled water control mixtures, to make the final volume 10.0 mL. Control treatments were without any addition of the essential oils and received 2.5 mL of distilled water control mixtures to 7.5 mL of MEA medium only. The essential oils were thoroughly mixed with the medium. Ten milliliters of each medium was poured into each 90 mm diameter sterilized Petri dishes. Mycelial discs were prepared using a pre-sterilised cork borer of 5 mm diameter from the tips of 3 – 7 days old culture of *Ascochyta rabiei*, and were placed in the centre of each Petri dishes after solidification of the MEA medium. Each treatment was replicated three times. dishes were incubated at 25 °C for 7 days. Fungal growth was measured by averaging the three diameters, taken at right angles, for each colony. Percentage growth inhibition of the fungal colonies (PGI) was calculated by applying the following formula:

$$\text{PGI (\%)} = \frac{\text{Growth in control} - \text{Growth in treatment}}{\text{Growth in control}} \times 100$$

The same procedure was carried out for the study of the effect of the synergistic activity of three essential oils, from which the concentrations of the binary and ternary mixtures of oils were tested such as: 3:1:1, 1:3:1, 1:1:3, 2:2:1, 2:1:2, 1:2:2, 4:1:0, 1:4:0, 3:2:0, 2:3:0, 4:0:1, 1:0:4, 3:0:2, 2:0:3, 0:4:1, 0:1:4, 0:3:2 and 0:2:3, with the 1st index corresponding to the concentration of *Thymus capitatus*, the 2nd index corresponding to the concentration of *Thymus bleicherianus* and 3rd index corresponding to the concentration of *Thymus satureioides*.

Results and discussion

The three essential oils of Thymus species are the subject of previous work by our team which are mentioned in the publication by Ainane et al. **[29]** (Table 1). The extraction yields of these oils are 1.43% for *Thymus capitatus*, 1.71% for *Thymus bleicherianus* and 0.69% for *Thymus satureioides*. The chemical characterization was made by gas chromatography coupled by mass spectrometry (GC-MS) by a simple method of characterization of essential oils based on the determination of the experimental retention indices compared with the theoretical ones. 35 compounds have been identified in 100% of the essential oil of *Thymus capitatus*, or three major molecules: carvacrol, α-terpinene, and β-ocymene presentet a percentage of 85.09%. 38 compounds have been identified in the essential oils of *Thymus bleicherianus* with a percentage of 99.10%, with four major molecules representing 79.57% such as: thymol; β-ocymene; camphor and o-cymene. 37 compounds were detected in the essential oil of *Thymus satureioides* with a percentage of 99.54%, where the five major molecules present 74.79% such as: thymol; α-terpinene; e-β-ocymene; camphor and borneol.

All the results obtained during the study of the effect of essential oils on the growth of the colony of the fungus of *Ascochyta rabiei* are mentioned in Figure 1 and Table 2. The experimental results of the antifungal tests of three oils essential species of Thymus: *Thymus capitatus*, *Thymus bleicherianus* and *Thymus satureioides* show significant activity, the higher the concentrations, the diameters of fungal colonies are reduced which results in the good inhibitory effect of the oils studied, particularly at the 5% concentration. The values of the percentage growth inhibition of *Ascochyta rabiei* varied from 10.29% to 30.88% for *Thymus capitatus*, from 1.49% to 14.71% for *Thymus bleicherianus* and from 2.90% to 17.39% for *Thymus satureioides*. It is noted that the essential oil of *Thymus capitatus* exhibited a very interesting activity compared to the other activities of the two essential oils of *Thymus bleicherianus* and *Thymus satureioides*.

The synergistic study of the antifungal activities against *Ascochyta Rabiei* of the three essential oils was made by the combination of three essential oils in the form of mixtures of binary and ternary concentrations. The values of the growth inhibition percentages are displayed in Table 3. All of the mixtures prepared based on the 3 essential oils show significant activities around an average of PGI around 22%. It is noted that most of the blends that contain *Thymus capitatus* essential oil have activity against chickpea blight, hence the 2:2:1 (TC%/TB%/TS%) mixture gave a high potency value.

Dozens of studies have documented the activity of Thymus species on antifungal activity against chickpea blight. Waithaka et al. (2018) **[30]** worked on three essential oils, among them the essential oil from the leaves of *Thymus vulgaris*. Chemical characterization has shown that this oil contains major molecules such as: carvacrol (19.9%), linalool (15.3%), thymol (13.2%), α-pinene (11.3%), p-cimene (11.2%) and borneol (10.9%). The antifungal activity against chickpea blight was carried out by the technique of agar wells, the zone of inhibition of which is 20 mm. Ennouri et al. (2020) **[31]** studied the activity of five essential oils against chickpea blight, of which the essential oil of *Thymus vulgaris* showed significant activities by liquid microdilution technique, so the results of the study target two molecules: thymol and carvacrol as compounds responsible for the activity through their presence in all the oils studied.

The three essential oils of our study, know these last two molecules mentioned in the two literatures which proves their positive effects against chickpea blight. Thymol and carvacrol are two isomers which exhibit interesting biological activities particularly antimicrobial activity against several pathogenic strains and low toxicities **[32]**. The specific properties of these two molecules in several medical fields are aimed at their uses in the treatment of chickpea blight either in the form of synthetic molecules or in the form of combinations of essential oils of Thymus species.

Conclusion

The three essential oils of Thymus species show an important activity at different concentrations from 1% to 5%, going up to a PGI 30.88% at the concentration 5% of the essential oil of Thymus capitatus. Also, positive results were seen during the measurement of the synergistic activity of three essential oils, where the PGI is from 10% to 32% for all mixtures. These performances of thyme essential oils have been proven by the presence of the majority compounds in particular: thymol and carvacrol, these last two molecules are responsible for the mechanisms of the fight against chickpea blight.

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Table 1. The major molecules identified in the three essential oils of Thymus species.

Species	Yield	Major compounds	Percentages of major compounds
Thymus capitatus	1.43%	Carvacrol; α-Terpinene and β-Ocymene.	85.09 %
Thymus bleicherianus	1.71%	Thymol; β-Ocymene; Camphor and o-Cymene.	79.57 %
Thymus satureioides	0.69%	Thymol; α-Terpinene; E-β-Ocymene; Camphor and Borneol	74.79%

Table 2. Percentage inhibition of the growth of different concentrations of essential oils of thymus species.

Spieces	Concentration	PGI* (%)
Thymus capitatus	0%	0.00 ± 0.00
	1%	10.29 ± 3.20
	2%	14.71 ± 3.67
	3%	16.18 ± 4.07
	4%	23.53 ± 4.54
	5%	30.88 ± 4.97
Thymus bleicherianus	0%	0.00 ± 0.00
	1%	1.49 ± 0.23
	2%	7.46 ± 1.95
	3%	8.96 ± 2.11
	4%	10.45 ± 3.24
	5%	14.71 ± 3.66
Thymus satureioides	0%	0.00 ± 0.00
	1%	2.90 ± 0.00
	2%	7.25 ± 1.81
	3%	8.70 ± 2.02
	4%	13.04 ± 3.17
	5%	17.39 ± 4.17

*PGI: Percentage growth inhibition.

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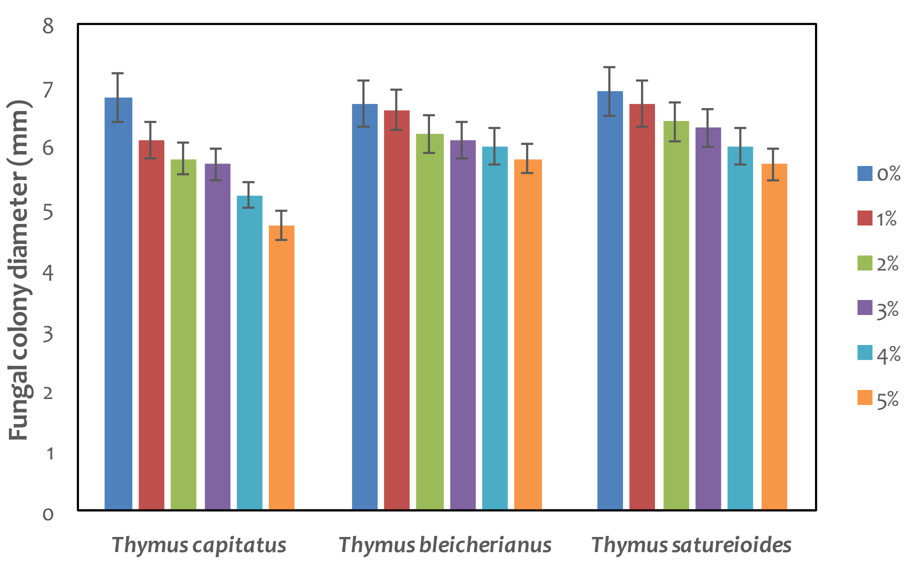
Table 3. Synergetic antifungal activity of the three essential oils in combination.

Concentration (TC% / TB% / TS%)	PGI* (%)
3:1:1	21.37 ± 3.66
1:3:1	23.15 ± 4.41
1:1:3	22.88 ± 4.07
2:2:1	32.04 ± 5.12
2:1:2	23.45 ± 4.42
1:2:2	25.65 ± 4.20
4:1:0	25.29± 4.11
1:4:0	22.64 ± 3.95
3:2:0	23.54 ± 4.46
2:3:0	22.86± 4.06
4:0:1	26.44± 4.78
1:0:4	23.32 ± 4.33
3:0:2	25.33 ± 4.16
2:0:3	22.21± 4.45
0:4:1	11.63 ± 3.55
0:1:4	14.34± 3.57
0:3:2	16.21± 3.89
0:2:3	16.71 ± 3.92

*PGI: Percentage growth inhibition.

Figure 1. Effect of essential oils of three species on growth of A. rabiei.



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