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Research Article

Investigating the combined effects of binary mixtures comprising essential oils and Azole fungicides on some plant pathogenic fungi

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Abstract: Combinations of traditional fungicides and natural compounds have the potential to produce more effective antifungal activity at sufficiently low concentrations. The potential fungicidal activity of binary mixtures comprising *Coriandrum sativum* and *Foeniculum vulgare* essential oils (E.O.s), as well as the two azole fungicides, Difenoconazole and Tebuconazole, were examined employing the *n.n* design. The concentration addition (C.A.) and independent action (I.A.) models were used to analyze the combined effects of the mixtures. In most mixtures, the essential oil enhanced the fungicidal activity of the azole fungicides. Mixtures containing Tebuconazole and *F. vulgare* exhibited a strong synergism, with an EC_{50} value of 43.12 for Mix. 9. The model deviation ratio (M.D.R.) for the tested mixtures ranged from 0.61 to 13.61, indicating the absence of antagonistic interaction among the mixtures components. It was observed that the I.A. model tended to underestimate the fungicidal activity, while the C.A. model provided a more accurate prediction. Further studies are required to investigate the primary natural products in these essential oils responsible for the synergistic effect on the azole fungicides.

Keywords: Difenoconazole, Tebuconazole, *Foeniculum vulgare*, *Coriandrum sativum*, Synergism

Introduction

The continuous utilization of synthesized pesticides has the potential to induce the emergence of new strains of pathogens resistant to the pesticides, thus making their management challenging (Nicholson, 2007). In recent decades, there has been a concerted and intensive effort to explore alternative options to synthetic chemicals. There is a growing interest in utilizing plant extracts and essential oils as a valuable source of bioactive compounds for developing pesticides (Amadioha *et al.*, 1998; Al-Samarrai *et al.*, 2012). Natural sources of chemicals have comparatively fewer adverse effects. They may be considered favorable alternatives to synthetic pesticides, particularly when it comes to protecting crops, the environment, and non-target organisms from the hazards of residues (Al-Samarrai *et al.*, 2012; Zaker, 2016; Santra and Banerjee, 2020). Essential oils and natural products demonstrate the capacity to prevent the onset of fungal and microbial diseases effectively, whether used alone (Abbod *et al.*, 2020; Gupta *et al.*, 2010) or

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in combination with other chemical compounds (Karaca *et al*., 2023; Sadhasivam et al., 2019; Zaidi *et al*., 2018).

The utilization of a combination of chemicals is a favorable approach for delaying the emergence of fungicide resistance (Van Den Bosch *et al*., 2014). It is important to note that the employment of a mixture with a diverse mode of action can ensure efficient control by targeting multiple active sites (Belden *et al*., 2007; Van Den Bosch *et al*., 2014; Oliver and Hewitt, 2014).

The combination of chemicals within a mixture can potentially enhance their respective bioactivities, resulting in a phenomenon known as synergistic effects. Conversely, certain compounds may decrease the activity of others within the mixture, leading to an antagonist interaction (Altenburger *et al*., 2003; 2004; Belden *et al*., 2007). Various models have been employed to investigate the interactions between constituent components of mixtures. The concentration additive (C.A.) model is frequently used in the case of mixtures with the same mode of action, while the independent action (I.A.) model is more appropriate for mixtures with multiple modes of action (Altenburger *et al*., 2003; 2004).

The current investigation examined the potential fungicidal effectiveness of binary mixtures consisting of essential oils and azole fungicides. Furthermore, the combined bioactivity of these substances was evaluated using both the C.A. and I. A. models.

Materials and Methods

Essential oils (E.O.s) and extraction

The leaves of *Coriandrum sativum* and *Foeniculum vulgare* were collected from Homs Province. Accurate species identification was performed in the Department of Field Crops, Al-Baath University, Homs, Syria. Plant materials were air-dried for 10 days, and dried materials were maintained at 4 °C until being used for essential oil extraction. For essential oil extraction, 100 g of dried materials were finely milled and exposed to hydrodistillation for 4 h in a Clevenger-type apparatus using 6.0 mL water as a solvent. The essential oil was dehydrated with anhydrous sodium sulfate and filtered, and the solvent was removed by reduced pressure.

The oil was then preserved at 4 °C for further tests (British Pharmacopoeia, 1963).

Azole fungicides

Difenoconazole (purity 98%, [C.A.S.] number 119446-68-3) and Tebuconazole (purity 99%, [C.A.S.] number 107534-96-3) were purchased from Agricore Chemical Industry Co., Ltd. (A.C.I), China.

Fungal strains

The fungal strains used in the study included *Macrophomina phaseolina* Mph44, and *Fusarium oxysporum* f sp l*ycopersici*, which were isolated and characterized previously in the Department of Plant Pathology of Tarbiat Modares University, Tehran, Iran.

Signal antifungal activity

The poisoned food technique was employed to assess the efficacy of each individual against the mycelial growth of *M. phaseolina* and *F. oxysporum*, (Schmitz, 1930). Increasing concentrations of the tested compounds were carefully mixed with 20-25 ml of melted warm P.D.A. medium in a Petri dish. A 6 mm diameter agar disc of the 7-day-old culture of the pathogen was aseptically transferred to the center of the Petri dish, which was then inoculated with P.D.A. plates. Each treatment was replicated three times. A basal medium (P.D.A.) serves as the control. The inoculated plates were incubated at a constant temperature of 25 °C, and the colony diameter was measured and recorded after 7 days. The percentage of mycelial growth inhibition was determined using the following equation:

$$\text{Mycelial growth inhibition \% (I \%)} = (C-T)/C \times 100 \quad (1)$$

Where C is the diameter of the fungal colony (mean) in control, and T is the diameter of the fungal colony (mean) in the presence of the synthesized compound. The respective dose-response curves and effective concentration

(EC_{50}) values were calculated using non-linear regression analysis by GraphPad Prism® version 7 (GraphPad Software Inc.)

Mixture design

Mixture toxicity assessments were conducted utilizing the *n.n* design based on a constant concentration of the azole fungicide ($1 \times EC_{50}$) and escalating concentrations of the essential oil ($0.5 \times EC_{50}$; $1 \times EC_{50}$) in the binary mixture and vice versa (Fai *et al.,* 2017). The calculation of the percentage of each component within the mixture is demonstrated in Table 2. The pathogen was exposed to eight concentrations of each mixture with a fixed ratio in accordance with the protocols outlined for signal antifungal activity testing. Dose-response curves were generated using Prism 7.

Combined effects of mixtures

The expected EC_{50} of the mixtures was calculated using the concentration addition model (C.A.) (Altenburger *et al.,* 2004) according to (Eq. 2)

$$EC\chi_{mix} = \left(\sum_{i=1}^{n} \frac{p_i}{EC\chi_i} \right)^{-1} \quad (2)$$

Where ECx_{mix} is the total concentration of the mixture provoking χ% effect; ECx_i is the concentration of component *i* provoking the x% effect when applied singly, and p_i denotes the fraction of component *i* in the mixture.

For the independent action (I.A.) model (Altenburger et al., 2004), the following equation (Eq3.) was used:

$$EC_{mix} = 1 - \prod_{i=1}^{n} \left(1 - (p_i Ec_i)\right) \quad (3)$$

Where EC_{mix} is the total effect of the mixture, and Ec_i is the effect expected from component i.

The model deviation ratio (M.D.R.) (Belden *et al*., 2007) was utilized to assist the deviation of observed toxicity from the toxicity predicted by models, in which:

$$MDR = \frac{Expected}{Observed} \quad (4)$$

Where *Expected* is the effective concentration of the mixture that the model would predict, and *Observed* is the effective concentration for the mixture obtained from toxicity testing (Belden *et al*., 2007). Based on the M.D.R. value, the types of interactions between mixture components are divided into three groups: synergistic (M.D.R. > 2), additive ($0.5 \leq$ M.D.R. ≤ 2), and antagonistic (M.D.R. < 0.5) (Belden *et al*., 2007; Cedergreen, 2014).

Results

Signal toxicity test

Essential oils of *C. sativum* and *F. vulgare* and the azole fungicides were tested individually against *M. phaseolina* and *F. oxysporum*. Figure (1) shows the dose-response curve for difenoconazole, tebuconazole, and E.O.s. Regarding *M. phaseolina*, the testes E.O.s exhibited negligible ant-fungal activity with EC_{50} > 2500 mg/l for *C. sativum*, and >3000 for *F. vulgare.*

Both azoles demonstrated a highly antifungal activity against both pathogens. Tebuconazole has a higher bioactivity than difenoconazole, with EC_{50} of 8.714 and 1.228 mg/l for *F. oxysporum* and *M. phaseolina*, respectively. It was clear that azoles were observably more effective than the E.O.s in preventing mycelium growth *in vitro* (Table 1).

Table 1 Fungicidal activity of single toxicity test.

Antifungal agent	Pathogen	EC_{50}, (mg/l)	$EC_{50,\ upper}$ (mg/l)	$EC_{50,\ lower}$ (mg/l)
Difenoconazole	F	8.714	5.519	12.53
Difenoconazole	M	10.18	6.099	16.12
Tebuconazole	F	0.706	0.551	0.903
Tebuconazole	M	1.228	0.8772	1.711
C. sativum	F	455	415.3	496.8
C. sativum	M	> 2500	-	-
F. vulgare	F	590	525.0	855.5
F. vulgare	M	> 3000	-	-

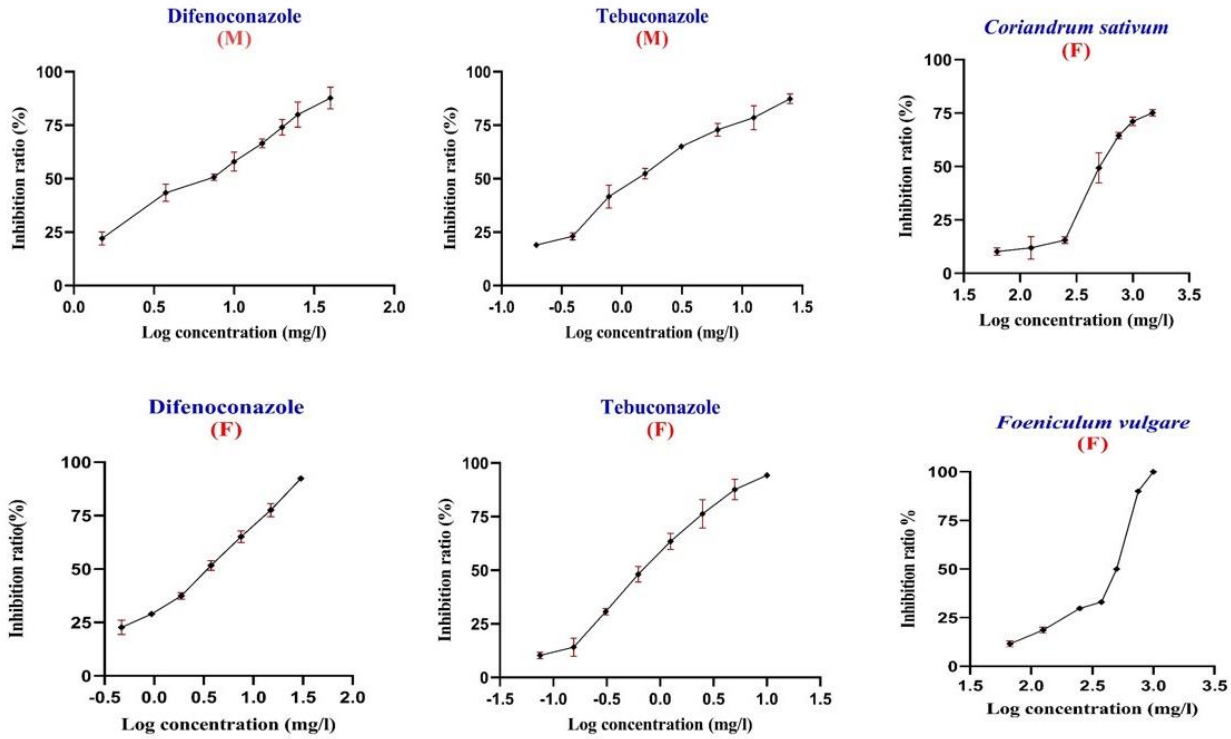

Figure 1 Dose-response curves of signal fungicidal activity of the tested compounds. (M) refers to *M. phaseolina*, while (F) refers to *F. oxysporum.*

Table 2 Concentrations and ratios (*Pi*) of mixtures tested using *n·n* design.

Mixture No.	Mixtures ratio in the *n.n* design [a]	Concentrations of mixture components (mg/l)	Components percentage (*Pi*)
Mix. 1	1 × EC_{50}(F) : 1 × EC_{50}(D)	590 (F) : 8.714 (D)	0.985 (F) : 0.015 (D)
Mix. 2	1 × EC_{50}(F) : 0.5 × EC_{50}(D)	590 (F) : 4.357 (D)	0.99 (F) : 0.01(D)
Mix. 3	0.5 × EC_{50}(F) : 1 × EC_{50}(D)	295 (F) : 8.714 (D)	0.97 (F) : 0.03 (D)
Mix. 4	1 × EC_{50}(C) : 1 × EC_{50}(D)	455 (C) : 8.714 (D)	0.98 (C) : 0.02 (D)
Mix. 5	1 × EC_{50}(C) : 0.5 × EC_{50}(D)	455 (C) : 4.357 (D)	0.99 (C) : 0.01 (D)
Mix. 6	0.5 × EC_{50}(C) : 1 × EC_{50}(D)	227.5 (C) : 8.714 (D)	0.963 (C) : 0.037 (D)
Mix. 7	1 × EC_{50}(F) : 1 × EC_{50}(T)	590 (F) : 0.706 (T)	0.999 (F) : 0.001 (T)
Mix. 8	1 × EC_{50}(F) : 0.5 × EC_{50}(T)	590 (F) : 0.35 (T)	0.9995 (F) : 0.0005(T)
Mix. 9	0.5 × EC_{50}(F) : 1 × EC_{50}(T)	295 (F) : 0.706 (T)	0.997 (F) : 0.003 (T)
Mix. 10	1 × EC_{50}(C) : 1 × EC_{50}(T)	455 (C) : 0.706 (T)	0.999 (C) : 0.001 (T)
Mix. 11	1 × EC_{50}(C) : 0.5 × EC_{50}(T)	455 (C) : 0.35 (T)	0.9992 (C) : 0.0008 (T)
Mix. 12	0.5 × EC_{50}(C) : 1 × EC_{50}(T)	227.5 (C) : 0.706 (T)	0.997 (C) : 0.003 (T)

[a:] the symbol (F) refers to *F. vulgare*, (C) *C. sativum*, (D) Difenoconazole, and (T) Tebuconazole.

Regarding the weak fungicidal activity of E.O.s on *M. phaseolina*, the mixture of these E.O.s with azoles was designed and tested only on the fungus *F. oxysporum.*

Combined fungicidal activity

According to the EC_{50}s obtained for the tested azoles and E.O.s (Table 1), the mixtures were assessed on *F. oxysporum* as the individual E.O.s showed negligible antifungal activity against *M. phaseolina*. The fixed ratio *n.n* design was used for the combinatory effect assay based on the same methodology used in the signal compounds test. The ratios of the mixture components are shown in Table (2).

Based on the *n.n* design, 12 mixtures were tested against *F. oxysporum*, and displayed a diverse range of fungicidal activity. The EC_{50} values of mixtures consisting of difenoconazole and *C. sativum* (**Mixs. 4–6**) varied from 67.3 to 305.7 mg/l. Furthermore, the EC_{50}s of Tebuconazole/*C. sativum* mixtures (**Mixs. 10–12**) fell within the range of 107 to 209.1 mg/l, as shown in Table 3 and Fig. 2. According to the C.A. and I.A. models, it was notable that antifungal activities of difenoconazole/*C. sativum* mixtures (**Mix. 4** and **Mix. 6**) were better than those of individual components, except **Mix. 5**, which showed an additive effect by both models. **Mix. 6** demonstrated a significant synergistic effect between *C. sativum* essential oil and difenoconazole with EC_{50} = 67.37 mg/l. The **Mix. 10** exhibited a synergistic effect according to both models with M.D.R. of 2.03 and 3.34 for C.A. and I.A. models, respectively; however, **Mix.12** demonstrated additive interaction between components based on the C.A. model (M.D.R. = 1.45), while I.A. model revealed an underestimate prediction of fungicidal activity with M.D.R. = 4.23 (Table 3). The mixtures of difenoconazole and *F. vulgare* (**Mixs. 1–3**) showed either additive or synergistic interaction between fungicide and E.O. **Mix. 2** exhibited strongly synergistic interaction with EC_{50} of 69.74 mg/l, and M.D.R. values of 5.08 and 7.65 for C.A. and I.A., respectively. The IA model demonstrated an underestimation of fungicidal activity compared to the C.A. model (Table 3, Fig. 2). The mixtures of tebuconazole and *F. vulgare* (**Mixs**. **7–9**) revealed a robust synergistic effect between the essential oil and fungicide. M.D.R. values for **Mix. 9** recorded 3.91 and 13.61 for C.A. and I.A. models, respectively (Table 3).

Based on M.D.R. values, it was observed that I.A. model tended to underpredict fungicidal activity, and C.A. model was more accurate in predicting the fungicidal activity of the studied mixture.

Overall, the E.O.s. did not exhibit any antagonist interactions with both fungicides, meanwhile, in most mixtures, the essential oil synergizes the fungicidal activity of the azole fungicides (Table 3).

Table 3 EC_{50} values and interaction types of tested mixtures.

Mixture	$EC50_{(obs.)}$ mg/l	$EC50_{(CA)}$ mg/l	$EC50_{(IA)}$ mg/l	$MDR_{(CA)}$	Interaction type $_{(C.A.)}$[a]	$MDR_{(IA)}$	Interaction type $_{(I.A.)}$
Mix. 1	481.0	294.91	505.32	0.61	AD	1.05	AD
Mix. 2	69.7	353.91	533.29	5.08	S	7.65	S
Mix. 3	67.4	196.59	422.95	2.92	S	6.28	S
Mix. 4	169.7	224.77	368.36	1.33	AD	2.17	S
Mix. 5	305.7	300.90	411.29	0.98	AD	1.35	AD
Mix. 6	67.4	157.17	297.22	2.33	S	4.41	S
Mix. 7	96.1	321.58	588.99	3.34	S	6.1۲	S
Mix. 8	177.3	416.20	589.50	2.35	S	3.32	S
Mix. 9	43.1۲	168.37	586.99	3.91	S	13.61	S
Mix. 10	136.1	276.85	454.22	2.03	S	3.34	S
Mix. 11	209.1	300.37	454.38	1.44	AD	2.17	S
Mix. 12	107.0	155.27	452.68	1.45	AD	4.23	S

[a]: **AD** = additive, **S** = synergistic.

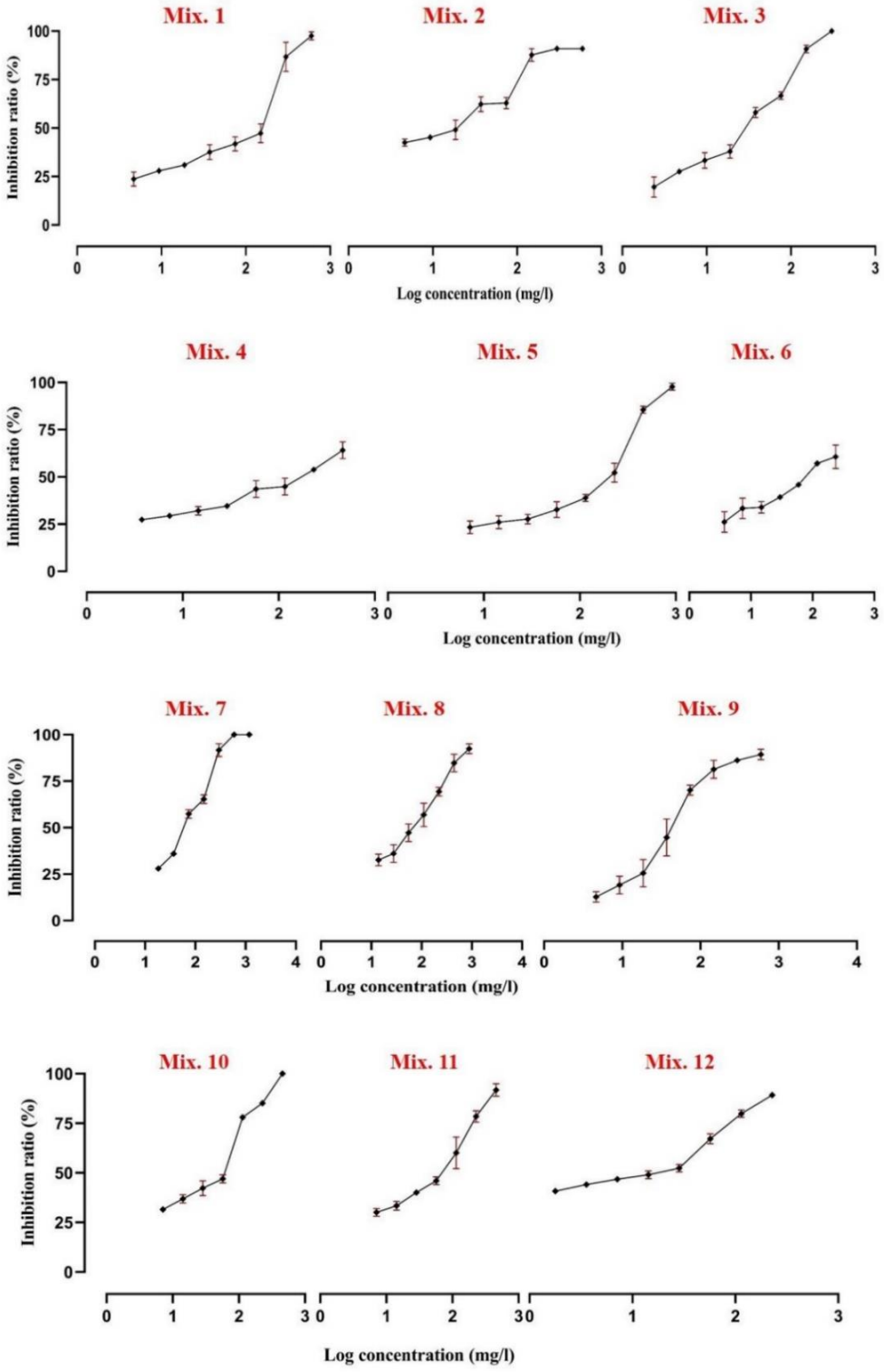

Figure 2 Dose-response curves of the tested mixtures against *F. oxysporum.*

Discussion

The combination of fungicides is a beneficial method for delaying the onset of resistance to fungicides (Van Den Bosch *et al.*, 2014). Plant essential oils are valuable for creating effective fungicide mixtures (Amadioha *et al.*, 1998; Al-Samarrai *et al.*, 2012). This study explored the fungicidal effects of essential oils from *Coriandrum sativum* and *Foeniculum vulgare*, individually and combined with Difenoconazole and Tebuconazole. The findings demonstrated that these oils boost the fungicidal efficacy of azole fungicides without any antagonistic interactions observed through the C.A. and I.A. models. The results showed that the C.A. model offered a more accurate prediction of mixture components. Belden et al. (2007) examined the predictive precision of C.A. and I.A. models for pesticide mixture toxicity. They found that the C.A. model was more precise for mixtures with pesticides sharing the same mode of action, while the I.A. model tended to underestimate toxicity (Belden et al., 2007).

Mixtures containing essential oils derived from basil, marjoram, clove, cumin, and caraway, or their active components, have been found to exhibit a synergistic effect against both *Candida albicans* and *Aspergillus niger* (Hassan *et al*., 2020). Natural essential oils generally possess a significant aromatic nucleus and an O.H. group, and phenolic compounds which can have an impact on the interactions with the fungal essential pathway, thereby enhancing their fungicidal activity (Farag *et al*., 1989; Hassan *et al*., 2020; Cox *et al*., 2001). The essential oil of C. *sativum* is composed of compounds such as linalool, α-pinene and γ-terpinene (Satyal and Setzer, 2020) and other monoterpenes (Freires *et al*., 2014) and which exhibited antifungal activity against *Candida albicans.* The essential oil derived from the seeds of *F. vulgare* contains D-limonene, menthol, estragole, and 2-decenal. Khaleil *et al*. (2021) have reported that this oil exhibits antifungal activity against Fusarium root rot disease in *Vicai faba*. The presence of these natural compounds in the essential oils may potentially explain the strong synergistic interactions observed when combined with azole fungicides.

Conclusion

The present investigation demonstrated the combined interactions between the essential oils of *Coriandrum sativum* and *Foeniculum vulgare and* the azoles fungicides Difenoconazole and Tebuconazole. The concentration addition (C.A.) and independent action (I.A.) models were utilized to examine the joint impacts of the mixtures. In most mixtures, the essential oil enhanced the fungicidal activity of the azole fungicides and demonstrated a strong synergistic interaction with the azoles. It was observed that the I.A. model tended to underestimate the fungicidal activity, whereas the C.A. model provided a more precise prediction. Further investigations should be conducted to determine the natural molecules responsible for the synergistic effects.

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Author contributions: M. Abbod supervised this study. M. Abbod designed the experiments. M. Abbod wrote the manuscript and performed the fungicidal activity study. E. Khallouf prepared the E.O.s. M. Abbod and E. Khallouf participated in the analysis of the results and reviewed the manuscript.

Availability of data and materials: All data generated or analyzed during this study are included in the article main text.

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بررسی اثرات ترکیبی مخلوطهای دوتایی اسانس‌های گیاهی و قارچکش‌های گروه آزول روی برخی از قارچ‌های پاتوژن گیاهی

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چکیده: ترکیب قارچکش‌ها با ترکیبات طبیعی پتانسیل تولید فعالیت ضدقارچی مؤثرتر را در غلظت‌های پایین دارند. فعالیت قارچکشی بالقوه مخلوطهای دوتایی شامل اسانس گشنیز *Coriandrum sativum* و رازیانه *Foeniculum vulgare* و همچنین دو قارچکش آزول، دیفنکونازول و تبوکونازول، با استفاده از طراحی n.n بررسی شد. مدل‌های افزودن غلظت (C.A.) و عمل مستقل (I.A.) برای تحلیل اثرات ترکیبی مخلوطها استفاده شد. در بیش‌تر مخلوطها، اسانس فعالیت قارچکشی قارچکش‌های آزول را افزایش داد. مخلوطهای حاوی تبوکونازول و رازیانه هم‌افزایی قوی را نشان دادند و مقدار EC_{50} برای مخلوط ۹ برابر ٤۳/۱۲ بود. نسبت انحراف مدل (M.D.R.) برای مخلوطهای آزمایش شده از ۰/٦۱ تا ۱۳/٦۱ متغیر بود که نشان‌دهنده عدم وجود تعامل متضاد بین اجزای مخلوطها است. مشاهده شد که مدل I.A. تمایل به‌دست کم‌انگاری فعالیت قارچکشی داشت، درحالی‌که مدل C.A. پیش‌بینی دقیق‌تری را ارائه داد. مطالعات بیش‌تری برای بررسی ترکیبات موجود در اسانس که مسئول اثر هم‌افزایی روی قارچکش‌های آزول هستند، مورد نیاز است.

واژگان کلیدی: دیفنکونازول، تبوکونازول، گشنیز، رازیانه، هم‌افزایی