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(Original Article)

Preparation and in Vitro Antifungal Evaluation of Difenoconazole Nanoemulsion for Efficient Transport into Fungal Cells

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Abstract

Recent advances in nanotechnology have paved the way for the development of nano-delivery systems. These systems enable the efficient delivery of fungicides to target pathogens, enhancing their bioavailability while minimising environmental and human health risks. A difenoconazole nanoemulsion was prepared using an ultrasonic method. The nanoemulsion (NE) is stable at room temperature, cold and heat storage, and many technical indicators meet the requirements. A transparent NE material with a narrow size distribution, suitable pH and viscosity was obtained. Scanning electron microscopy (SEM) analysis revealed the formation of discrete nanodroplets with sizes ranging from 66.67 to 91.53 nm. *In vitro* antifungal experiments were performed against three plantpathogenic fungi (i.e., *A. Alternata*, *F. oxysporum* and *R. stolonifer*), the difenoconazole nanoemulsion demonstrated high antifungal activity (EC_{50} = 112.93, 151.73 and 857.22 mg/L for *A. alternata, F. oxysporum* and *R. stolonifer*, respectively). Therefore, difenoconazole nanoemulsion showed high inhibition of spore germination (I (%) = 60.23 and 65.22 %, for *A. alternata* and *F. oxysporum*, respectively). The nanoemulsion has better permeability through fungal spore cells, giving better results compared to the commercially available difenoconazole emulsifiable concentrate.

Keywords*: A. alternata, Antifungal activity, Difenoconazole nanoemulsion, F. oxysporum, R. stolonifer*

Introduction

Fungal infections represent a persistent and growing challenge across various sectors, including agriculture and public health. The impact of these infections is significant, highlighting th urgent need for more effective and sustainable control strategies (Garvey *et al.,* 2022). Difenoconazole, a prominent triazole fungicide, has demonstrated broad-spectrum efficacy against a wide range of fungal pathogens by targeting ergosterol biosynthesis pathway, an essential pathway for maintaining fungal cell membrane integrity (Li *et al*., 2025). Despite its potent intrinsic activity, the practical application of conventional difenoconazole formulations often face limitations. These include poor water solubility, which hinders dissemination and absorption; sensitivity to environmental degradation, which reduces stability; and inefficient

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transport across biological barriers. These factors may require higher application rates, potentially pose a significant environmental impact, and promote the emergence of fungicide resistance (Badawy *et al*., 2024). To understand the importance of developing a difenoconazole nanoemulsion, it is essential to recognize the diverse and widespread nature of fungal pathogens. Among these, *Alternaria alternata*, *Fusarium oxysporum*, and *Rhizopus stolonifer* stand out as prominent examples, each presenting unique challenges in disease management due to their broad host range, pathogenic mechanisms, and environmental adaptability (Asmawi *et al*., 2024). *A. alternata*, a ubiquitous filamentous fungus, is of significant concern in agriculture, causing a variety of diseases such as leaf spots, blights, and fruit rots (Abel-Fernández *et al*., 2023). *F. oxysporum* is a highly diverse and widespread soil fungal pathogen, known to cause devastating vascular wilt diseases in a wide range of plant species (Naguib *et al*., 2021). *R. stolonifer*, commonly known as black bread mold, is a fast-growing, environmentally widespread mold fungus, primarily known for causing soft mold and black mold diseases. The distinct biological properties of these fungi and their widespread impact highlight the ongoing need of these fungi for innovative and highly effective antifungal strategies (Vazhacharikal *et al.,* 2015). In response to these challenges, advanced drug delivery systems, particularly those utilizing nanotechnology, have emerged as a highly promising field. Among these systems, nanoemulsions stand out as a revolutionary platform. Characterized by their transparent or optically translucent appearance and droplet sizes typically ranging from 20 to 200 nm, nanoemulsions offer several distinct advantages. Their ultra-small droplet size significantly increases the absorption surface area and improves the stability of poorly soluble active ingredients such as difenoconazole (Gao *et al*., 2021). For antifungal applications, this implies the potential for improved cellular uptake and enhanced accumulation of the active ingredients at the site of infection.

This study is devoted to the preparation and evaluation of the antifungal efficacy of difenoconazole nanoemulsion on fungal cells of *Alternaria alternata*, *Fusarium oxysporum*, and *Rhizopus stolonifer*. The current study demonstrates that the formulation of difenoconazole as a nanoemulsion will not only overcome the solubility and stability limitations of conventional formulations but also significantly improve its ability to penetrate the complex fungal cell wall and membrane of these diverse pathogens. The research will detail the formulation development, while determining the physicochemical properties of the difenoconazole nanoemulsion. Furthermore, a comprehensive *in vitro* evaluation will be conducted to evaluate enhanced antifungal activity against these major fungal pathogens.

Materials and Methods

Fungicide and Chemicals

Difenoconazole (1-[[2-[2-chloro-4-(4-chlorophenoxy)phenyl]-4-methyl-1,3-dioxolan-2-yl]methyl]-1,2,4-triazole) (Curve 25% EC) was supplied by El-Hoda company (Wadi El Natrun, Behira Governorate, Egypt). Tween 80, catechol, 3,5-Dinitrosalicylic acid and dimethyl sulphoxide (DMSO) were obtained from Sigma-Aldrich, St. Louis, MO, USA. Potato dextrose agar (PDA) was obtained from Oxoid Ltd., Basingstoke, Hampshire, UK. Para-Aminobenzoic acid was obtained from Acros

Organics, NJ, USA. Carboxymethyl cellulose was obtained from Kelong Chemical Agent Factory, Chengdu, China. Bromothymol Blue was obtaine from Merck, Germany. Dife.

Microorganisms

The fungi used as plant pathogens are *Alternaria alternata* (Fries), *Fusarium oxysporium* (Schiech), and *Rhizopus stolonifer* (Ehren). They were obtained from the Microbiology Laboratory, Department of Plant Pathology, Faculty of Agriculture, Damanhour University, Damanhour, Egypt, and stored in PDA medium at 27 ± 2°C.

Preparation of difenoconazole nanoemulsion

The nanoemulsion was prepared using the method of (Sugumar *et al.*, 2014), with some modifications. The nanoemulsion was prepared in two steps. The coarse emulsion was prepared by stirring, followed by further emulsification using a high-power ultrasonic process. First, 0.5% a.i (w/v) difenoconazole was completely dissolved in dimethyl sulfoxide (DMSO). The solution was then mixed with a surfactant (Tween 80) and stirred with water at 4000 rpm. The oil phase was slowly added to the aqueous phase while stirring at 4000 rpm for 30 min. The formed emulsion was then sonicated by ultrasonic probe. The process was carried out (15 min), using power (75 % of sonicator power (20 kHz)) and pulses (9 cycle/sec) to produce the nanoemulsion at 25°C (Figure 1)(Li and Chiang, 2012).

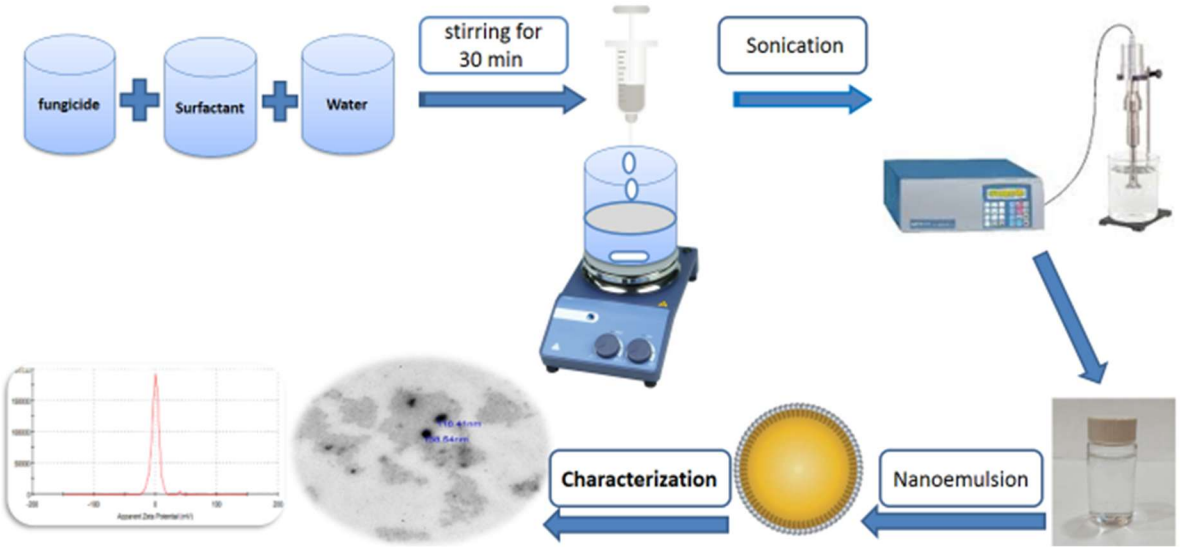

Figure 1. Schematic illustration of the preparation of difenoconazole nanoemulsion.

Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) analysis was performed using a JEOL JSM-5410 electron microscope (Japan) equipped with a W-source and operating at 25 kV. The sample was prepared on a 1×1 cm glass slide after washing with ethanol. A small drop of the nanoemulsion was evenly spread on the glass slideover and allowed to dry in air. To make it conductive, it was coating with gold using a Jeol Quick Auto Coater (JFC-1500). The slides were then subjected to SEM analysis under ambient conditions.

Particle size and Polydispersity DIndex (PDI) test

The average droplet size and dispersityindex (PDI) of the nanoemulsion formulation were measured using dynamic light scattering method (DLS) using a Zetasizer Nano ZS (Malvern Instruments, UK) at room temperature. All nanoemulsion samples were diluted 10% with deionized water before measurements to avoid polydispersity effects. The emulsion droplet size was estimated by the average of three measurements and presented as the average diameter in nanometers. A higher PDI value indicates lower particle size uniformity in the nanoemulsion (Tyagi *et al*., 2012).

Physiochemical stability

Three Physical and chemical aspects of optimal nanoemulsions were determined centrifugation, stability at low temperature, and stability above room temperature. The sample was centrifuged for 30 min at 5000 rpm and phase separation, cracking and breakage were observed**.** A freeze-thaw cycle test was conducted to determine the accelerated stability of the nanoemulsion formulation. The formulation was subjected to two different temperatures (-21°C and 21°C) for at least 24 h. A heating-cooling cycle test was used to demonstrate the irradiation effect of heating and cooling on the stability of the prepared nanoemulsion. The prepared nanoemulsion was stored at 4°C and 40°C with a storage period of 48 h for each temperature test (Kadhim and Abbas, 2015).

Viscosity and pH determination

The dynamic viscosity of the nanoemulsion was determined using a Rotary Myr VR 3000 digital viscometer with L1 and L1 spindles at 100 rpm at 29.5°C. Each reading was taken after the equilibrium of the sample for two min. The sample was repeated three times and the data expressed in mPa.s. The pH value is one of the main parameters of nanoemulsion. The digital pH meter was used to determine the pH values of the prepared nanoemulsion (Junyaprasert *et al*., 2007).

***In vitro* valuation of difenoconazole nanoemulsion on mycelial growth**

The antifungal activity was tested by using the radial growth technique (Badawy *et al*., 2014). Portions of the crude solutions were added to PDA medium and then transferred to Petri dishes. After solidification, the mixtures were injected with a 5 mm diameter mycelium into the centre of Petri dishes and incubated in the dark at 27 ± 2**°**C. Fungal growth was measured when the control reached the edge of the dish. Fungal growth inhibition was calculated as percentage inhibition of radial growth compared to the control. The EC_{50} for each compound was estimated by probit analysis **(**Finney, 1971) using SPSS 26.0 software. The percentage inhibition was calculated according to (Topps and Wain, 1957) as follows:

$$I\% = \left[\left(\frac{A-B}{A}\right)\right] \times 100$$

Where I (%): is the inhibition percent, A: is the diameter of untreated hyphal growth of fungus and B: is the diameter of treated hyphal growth of fungus.

In vitro evaluation of difenoconazole nanoemulsion on spore germination

Spores of *A. alternata, F. oxysporium and R. stolonifer* spores were collected from a 2-week-old PDA culture, grown under fluorescent lights in 9-cm diameter Petri dishes at 26°C. 5 ml of sterile water was added to the Petri dish culture. Spores were gently removed from the surface using a sterile glass rod and the suspension was filtered through three layers of cheesecloth. The suspension was diluted with sterile water until the absorbance coefficient reached 0.25 at 425 nm ($1.0*10^6$ conidia/mL) as determined by a Unico 1200-Spectrophotometer. Small aliquots (50 µl) of the spore suspension were placed in Eppendorf tubes containing 500 µl of potato dextrose broth (PDB) medium with difenoconazole nanoemulsion concentrations. Preliminary screening tests were performed at two concentrations of 500 and 750 mg/L. The tubes were incubated at 26°C for 24 h. Samples were placed on two chambers of a Neubauer hemocyte counter by carefully touching the edges of the cover glass with the pipette tip, allowed capillary action to fill the counting chambers, and then examined under a microscope for spore germination. Spore counting was performed using a light microscope at 40x magnification. All experiments were conducted in three replicates. Spores were considered germinated when the germ tube length was equaled to to or greater tha the spore length. The numbers of germinated and non-germinated spores were recorded, and the percentage inhibition of spore germination (%) was calculated (Griffin, 1994).

Effects of difenoconazole nanoemulsion on polyphenol oxidase (PPO) activity

Polyphenol oxidase activity was determined according to the method described by El-Samra *et al*. (2003). PD medium containing EC_{50}, 1/10 EC_{50}, ¼ EC_{50} and ½ EC_{50} of the tested difenoconazole nanoemulsion was prepared in 100 ml conical flasks. The tested fungi disks were placed on the surface of the medium and incubated until the fungal hyphae were completely grown in untreated flasks (control group). The medium was then filtrated under vacuum. The filtrate was then centrifuged for 15 min at 4000 rpm. The supernatants (PPO source) (1 ml) were added to the reaction mixture (2.0 ml of pH 9.0 buffered borate solution), 1.0 ml of 1% para-aminobenzoic acid (alcoholic solution) and 2.0 ml of 1% catechol). Enzyme activity was measured as absorbance at 575 nm after one-hour of incubation in water bath at 45 °C (Fuerst *et al.*, 2011). The percentage inhibition of PPO enzyme activity was calculated from the following equation:

$$\mathbf{I\ (\%) = [(Ac - At)/Ac] \times 100,}$$

Where Ac is the absorbance in control group and At is the absorbance in the treated group.

Effects of difenoconazole nanoemulsion on cellulase activity

Fungal cultures were grown on potato dextrose (PD) medium amended with 3% of carboxymethyl cellulose for 12 days at 27°C. The medium was filtrated using Whatman No. 1 paper. The filter was used as a source of crude cellulase enzyme. 1 ml of crude enzyme was added to 2 ml of citrate buffer solution pH 4.8, and the mixture was heated in a water bath at 50°C for 30 min. 1 ml of difenoconazole nanoemulsion was then added and incubated at 28°C for 24 hours. The nanoemulsion of

difenoconazole at concentrations of EC_{50}, 1/10 EC_{50}, ¼ EC_{50} and ½ EC_{50}. Then, 3 ml of the reaction mixture [3,5-dinitrosalicylic acid (10 g), sodium hydroxide (10 g), phenol (20 ml), sodium sulphate (0.5 g), and distilled water to 1000 ml] was added. Three replicates of each treatment, a control and blank (without enzyme) were prepared. After incubation for 15 minutes at 50°C in a water bath, the absorbance was measured at a wavelength of 575 nm (Helal *et al.*, 2022). The percentage of cellulase inhibition was calculated from the equation:

$$\mathbf{I\ (\%) = [(Ac - At)/Ac] \times 100}$$

Where: Ac is the absorbance in control sample and At is the absorbance in treatment sample.

Statistical analysis

Statistical analysis was performed using the statistical SPSS version 26.0 (SPSS, Chicago, Illinois, USA). The log dose–response curves allowed the determination of EC_{50} for bioassays according to probit analysis (Finney 1971), and the 50% lethal concentration (EC_{50}). The 95% CL and standard error for the range of EC_{50} values for the compound used in growth inhibition experiments were determined using least-square regression analysis of the relative growth rate (% control) versus the logarithm of the compound concentration. Statistical significance was determined using one-way analysis of variance (ANOVA) comparing means using the SNK method with a probability of 0.05 Steel and Torrie (1980). The IC_{50} value (concentration causing 50% decrease in enzyme activities) for each fungicide was estimated by using probit analysis (LdP Line), and the means and standard error (SE) were obtained from three independent replicates performed for each treatment (Finney, 1971).

Results and Discussions

Characterization of difenoconazole nanoemulsion

Scanning electron microscope (SEM) estimation

The morphology and shape of difenoconazole nanoemusion were studied by scanning electron microscopy (SEM), and the data are presented in Figure 2. The morphology of the nanoemulsion is highly variable, with spherical, and sometimes triangular, nanoemulsion observed in the micrograph, with a size range of 66.67 to 91.53 nm. Elsharkawy *et al.* (2022) revealed that the morphology of Lambda-cyhalothrin nanoemulsions (LCNs) is quasi-spherical with an average diameter of 70.3 nm.

The scanning electron microscope was performed on a JEOL JSM-1200EX II scanning electron microscope operating at an accelerating voltage of 25.0 kV.

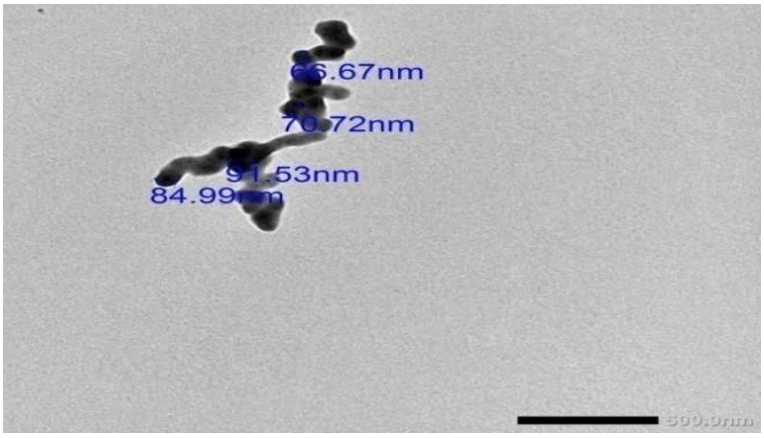

Figure 2. Scanning electron micrograph of the prepared difenoconazole nanoemulsion.

Zeta potential and polydispersity index (PDI)

The prepared nanoemulsion showed a negative zeta potential of -0.0132 mV (Figure 3). The zeta potential is a better way to improve sample stability and save time spent in shelf-life tests. It is a strong indicator of NE stability, resisting flocculation and aggregation for longer periods, and is related to the droplet surface potential. Negative values are essential for droplet repulsion, thus enhancing nanoemulsion stability (Bruxel, 2012). The highest stability of formulations with zeta potential values is associated with repulsive forces that exceed attractive van der Waals forces, resulting in dispersed particles and a deflocculated system (Mahdi *et al*., 2011).

The PDI is defined as a dimensionless measure of the amplitude of the size distribution, calculated from the aggregation analysis and taking into account the ratio of the standard deviation to the mean particle diameter (Gurpreet and Singh, 2018). The PDI value was 0.99±0.03 (Figure 3). The PDI can range from 0 to 1, with a value between 0.7 to 1 indicating a wide range of droplet sizes (polydispersity). Values less than 0.08 indicate a practically monodisperse sample. Therefore, the midpoint region of PDI ranges from 0.08 to 0.70, and distribution algorithms achieve their best performance (Danaei *et al*., 2018).

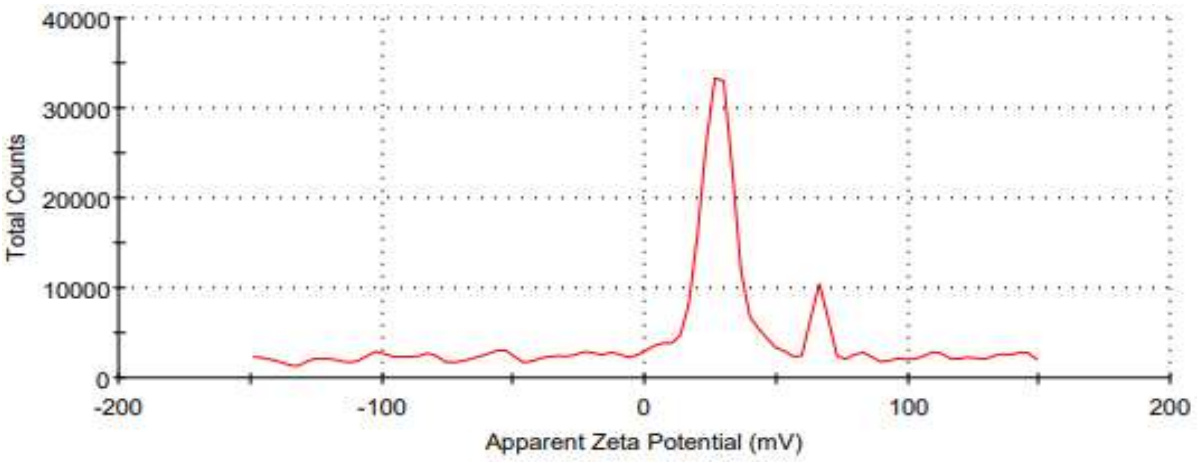

Figure 3. Typical particle size distribution by dynamic light scattering of difenoconazole nanoemulsion

Physiochemical stability

The nanoemulsion was stable upon centrifugation at 5000 rpm, heating, and freezing and thawing for 4 weeks. This structure was not observed in any phase separation. The nanoemulsion was a thermally stable system, forming at a specific composition of oil, surfactants, and water, without any separation, creaming, cracking, or coalescence. Centrifugation could accelerate the sedimentation or burning rate, indicating that the decomposition of the emulsion may be related to the force of the gravity (Tadros *et al*., 2004). These tests were conducted to confirm stability, small surfactant composition, nanoemulsion droplet and stable physical and chemical properties.

Viscosity and pH measurement

Viscosity was measured to ensure optimal dispersion of the formulation, and the viscosity value of the difenoconazole nanoemulsion was recorded at46 MPa. Viscosity is significantly influenced by several factors such as the dispersion phase, volume fraction, colloidal interactions, droplet size, component phases characteristics, and droplet charge (McClements, 2012). The pH of the difenoconazole nanoemulsion was 7.32.

Antifungal activity of difenoconazole nanoemulsion against *A. alternata*, *F. oxysporum* and *R. stolonifer*

Table (1) provides a comprehensive analysis of the antifungal activity of difenoconazole nanoemulsion against three fungal pathogens: *A. alternata, F. oxysporum*, and *R. stolonifer*. For *A. alternata*, the NE exhibited a significantly lower EC_{50} value of 112.93 mg/L compared to the emulsifiable concentration (EC) of 282.25 mg/L and the technical formulation (T) at 2534.83 mg/L, indicating that the nanoemulsion is more effective in inhibiting this pathogen. The confidence limits for NE formulation are also narrower, indicating more reliable efficacy. For *F. oxysporum*, the EC_{50} for NE is 151.73 mg/L, which is also lower than the EC and T formulations, demonstrating the potential of the nanoemulsion in combating this pathogen. The slope values indicate the steepness of the dose-response curve, with NE exhibiting a slope of 2.18, indicating a strong concentration - effect relationship. For *R. stolonifer*, the EC_{50} for NE is significantly higher at 857.22 mg/L compared to the EC at 74.68 mg/L. This suggests that while the nanoemulsion is effective, it may not be as effective against other pathogens as *R. stolonifer*. The varying efficacy across different pathogens highlights the importance of selecting the appropriate formulation to combat the targets fungus. Overall, the data suggest that difenoconazole nanoemulsion may be a promising alternative to convential methods.

Table 1. Antifungal activity of difenoconazole emulsifiable concentrate, technical and nanoemulsion against *A. alternata, F. oxysporum* and *R. stolonifer*

Formulation	EC_{50}[a] (mg/L)	(95%Confidence limits)		Slope[b] ±SE	Intercept[c] ±SE	$(X)^{2}$[d]
		Lower	Upper			
			A. alternata			
EC	282.25	154.28	394.47	1.94±0.32	-4.76±0.95	2.35
NE	112.93	17.12	232.47	1.39±0.33	-2.86±0.99	3.09
T	2534.83	2054.8	3361.0	1.54±0.21	-5.25±0.67	3.29
			F. oxysporum			
EC	387.34	266.68	490.40	2.25±0.32	-5.82±0.94	2.23
NE	151.73	26.30	268.52	2.18±0.59	-4.76±1.68	0.91
T	507.61	434.51	568.78	4.22±0.56	-11.42±1.59	1.00
			R. stolonifer			
EC	74.68	52.09	92.55	2.73±0.43	-5.11±0.94	3.67
NE	857.22	774.77	942.65	4.04±0.36	-11.85±1.08	0.85
T	2610.47	1757.91	4927.43	3.65±0.35	-12.49±1.18	5.13

a: The concentration causing 50% enzyme inhibition. b: Slope of the concentration-inhibition regression line ± standard error. c: Chi square value. T: Technical, NE: Nanoemulsion and EC: Emulsifiable concentrate.

Difenoconazole-loaded) microcapsules (CS-DIF were formated by encapsulating difenoconazole in biocompatible chitosan (Chang *et al*., 2024). The antifungal activity ofCS-DIF capsules against *Curvularia lunata* was confirmed by monitoring colony growth, *in vitro* and *in vivo* inoculation, fungal morphology, and DNA and protein leakage. The antioxidant enzyme activities of superoxide dismutase, peroxidase, and catalase decreased by 65.1%, 84.9%, and 69.7%, respectively. When *Curvularia lunata* was treated with 200μg/mL microcapsules compared to the control group within 24 h. The enzyme activity of polyphenoloxidase decreased by 323.8%. The content of reactive oxygen species (ROS) in hydrogen peroxide and superoxide anions increased by 204.6% and 164%, respectively.

Antifungal activity of difenoconazole and its preparations on spores germinating of *A. alternata*, *F. oxysporium* and *R. stolonifer*

The results in Table (2) indicate varying effects of different difenoconazole treatments on the spore's germination of *A. alternata, F. oxysporum*, and *R. stolonifer*. Among the treatments, the 750 mg/L nanoemulsion (NE) showed the highest inhibition of *A. alternata* spores' germination, achieving 60.23%. This was closely followed by the 750 mg/L EC emulsion, which achived 59.09%. For *F. oxysporum*, the 750 mg/L nanoemulsion again achieved the highest inhibition of 65.22%, while the 750 mg/L EC emulsion showed significant inhibition of 56.52%. The control groups of both *A. alternate* and *F. oxysporum* showed negligible inhibition, highlighting the effectiveness of the treatments. In the case of *R. stolonifer*, an EC concentration of 750 mg/L resulted in 63.27% inhibition, making it the most effective treatment for this specie as well. Overall, the nanoemulsion treatments consistently outperformed EC treatments across different concentrations and species, indicating their potential as a more effective solution for c ntrolling spore germination in these pathogens.

Table 2. Effect of emulsifiable concentration, technical and nanoemulsion of difenoconazole on spore germination of *A. alternata, F. oxysporum* and *R. stolonifer*

Formulation	Concentration (mg/L)	Inhibition of spore germination (%) ± SE
	A. alternata	
Control	0.00	$2.27^{a} \pm 1.31$
EC	500	$44.32^{cd} \pm 4.69$
	750	$59.09^{ef} \pm 3.71$
NE	500	$55.68^{def} \pm 4.69$
	750	$60.23^{ef} \pm 1.14$
T	500	$46.59^{cde} \pm 2.86$
	750	$57.95^{ef} \pm 5.04$
	F. oxysporum	
Control	0.00	$2.17^{a} \pm 1.26$
EC	500	$35.87^{de} \pm 4.48$
	750	$56.52^{fg} \pm 3.55$
NE	500	$55.43^{fg} \pm 1.09$
	750	$65.22^{g} \pm 1.77$
T	500	$20.65^{bc} \pm 4.48$
	750	$28.26^{cd} \pm 2.66$
	R. stolonifer	
Control	0.00	$0.00^{a} \pm 4.30$
EC	500	$58.16^{hi} \pm 2.69$
	750	$63.27^{i} \pm 1.07$
NE	500	$44.90^{fg} \pm 1.07$
	750	$51.53^{gh} \pm 1.61$
T	500	$27.04^{c} \pm 2.69$
	750	$37.24^{def} \pm 0.54$

Different letters in the same column indicate significant differences according to the Student-Newman-Keuls (SNK) test (P≤0.05). T: Technical, NE: Nanoemulsion and EC: Emulsifiable concentrate.

The effects of the polar polyoxin B, the strobilurin fungicides, azoxystrobin and trifloxystrobin, and the sterol inhibitor difenoconazole on spore germination, fungal growth and fruit decay in detached *A. alternate* were reported (Reuveni and Sheglov, 2002). Germination was most sensitive to polyoxin B and trifloxystrobin among the tested compounds. The 50% and 95% effective concentration (EC_{50}, and EC_{95}), values for in vitro fungal germination inhibition were the lowest for polyoxin B and trifloxystrobin, ranging from <0.01 to 0.15 µg/ml and 180 µg/ml, respectively. Germination was least sensitive to difenoconazole and azoxystrobin (EC_{50} and EC_{95}) values ranging from 25 to 72 µg/ml to 720 µg/ml, respectively. *In vitro* growth of *A. alternata* was most sensitive to difenoconazole (EC_{50} and EC_{95} values of 0.8 and 12 µg/ml, respectively) and least sensitive to both fungicides strobilurin (EC_{95} >1000 µg/ml). Solublization formation by *A. alternata* on mature adherent fruits was most suscebtible to trifloxystrobin and azoxystrobin (EC_{50} and EC_{95} values of 0.015–0.087µg /ml and 8 µg/ml, respectively), intermediate in sensitivity to Polyoxin B (EC_{50} and EC_{95} of 1 to 33 µg/ml, respectively), and difenoconazole was the least effective EC_{50} and EC_{95} from 20 to 490 µg/ml, respectively).

Wang *et al*. (2016) reported the effects of the fungicide astrobilurin, azoxystrobin and as terolinhibit or difenoconazole on fungal growth, spore germination, and control of brown spot. The results of bioassay for fungal growth and spore germination showed

that the sensitivity of *A. alternata* to difenoconazole was significantly lower than that of azoxystrobin. Azoxystrobin and the combination of azoxystrobin and difenoconazole provided excellent efficacy in brown spotin control. Thedisease control efficacy of three sprays of azoxystrobin at doses of 0.094, 0.19 and 0.28 Kg a.i./ha, azoxystrobin plus difenoconazole at doses of 0.15, 0.22 and 0.29 Kg a.i./ha, and difenoconazole at doses of 0.12 Kg a.i./ha were 86.00% - 89.67%, 86.14% - 89.23%, and 55.14 - 58.41%, respectively.

Effects of difenoconazole and its formulations on PPO enzyme activity

Table 3 shows the fungicidal activity of the different difenoconazole formulations against the tested pathogens, with a particular focus on the PPO enzyme. The results indicated varying levels of effectiveness among the formulations, with the nanoemulsion (NE) exhibiting the lowest IC_{50} values for *A. alternata* and *F. oxysporum*, demonstrating superior efficacy compared to the emulsifiable concentrate (EC) and technical (T) formulations. For *A. alternata*, the IC_{50} concentration of NE in the *A. alternata* formulation was 0.02 mg/ml, significantly lower than the EC concentration of 0.17 mg/ml. Similarly, for *F. oxysporum*, the nanoemulsion NE also demonstrated superior efficacy with an IC_{50} concentration of 0.09 mg/ml. In contrast, the *R. stolonifer* showed a different trend, with the EC formulation recording the lowest IC_{50} value of 0.03 mg/ml, indicating increased efficacy. Confidence limits and slope values support the reliability of these results, highlighting the importance of selecting the appropriate formulation in managing these pathogens by inhibiting PPO activity.

Table 3. Effect of the emulsifiable concentration, technical and nanoemulsion of difenoconazole on PPO activity in *A. alternata*, *F. oxysporum* and *R. stolonifer*

Formulation	IC_{50}[a] (mg/ml)	95%Confidence limits		Slope[b]±SE	$(X)^2$[c]
		Lower	Upper		
		A. alternata			
EC	0.17	0.16	0.19	3.76±0.53	0.04
NE	0.02	0.01	0.03	1.08±0.32	1.01
T	1.29	1.11	1.47	2.25±0.30	1.5
		F. oxysporum			
EC	0.47	0.33	1.18	1.09±0.30	0.22
NE	0.09	0.07	0.11	2.05±0.34	1.58
T	0.81	0.53	3.31	1.05±0.31	0.86
		R. stolonifer			
EC	0.03	0.02	0.04	1.24±0.28	0.31
NE	0.63	0.51	0.85	1.38±0.29	0.95
T	1.68	1.47	1.95	2.22±0.31	3.21

a: The concentration causing 50% enzyme inhibition. b: Slope of the concentration-inhibition regression line ± standard error. c: Chi square value. T: Technical, NE: Nanoemulsion and EC: Emulsifiable concentrate.

Six fungicides (i.e., copper oxychloride, azoxystrobin-difenoconazole, cyazofamid-cymoxanil, mancozeb-dimethomorph, trifloxystrobin–tebuconazole, and dimethomorph-pyraclostrobin), and four four chemical inducers (i.e., salicylic acid, chitosan, humic acid and ascorbic acid) were tested (Hamed *et al*., 2024). These inducers were combined with the recommended fungicide (azoxystrobin-difenoconazole) at a 1:1 ratio. These fungicides and chemical inducers were evaluated for their effectiveness in reducing onion downy mildew, caused by *Peronospora destructor*, under field conditions. The activities of antioxidant enzymes (polyphenol oxidase and peroxidase

enzymes) in onion were also evaluted. The results showed that all the fungicides and chemical inducers significantly reduced the spread of downy mildew and increased the activities of antioxidant enzymes (POX and PPO). Forethereremore, the combination of azoxystrobin-difenoconazole, ascorbic acid at 0.704 g and chitosan at of 2 g combined with difenoconazol-azoxystrobin had the least severe and the highest efficacy. In contrast, the combination of copper oxychloride, chitosan, and the combination of difenconazol-azoxystrobin with chitosan at concentration of 2 g resulted in the highest increase in polyphenol oxidase and peroxidase activities.

Difenoconazole-loaded (CS-DIF) microcapsules were synthesized by encapsulating difenoconazole into biocompatible chitosan (Chang *et al*., 2024). The antifungal activity of the CS-DIF microcapsules against *C. Lunata* was confirmed through observations of colony growth, in vitro and in vivo inoculation, mycelium morphology, as well as DNA and protein leakage. The antioxidant enzyme activity of superoxide dismutase, peroxidase, and catalase decreased by 65.1%, 84.9%, and 69.7%, respectively, when *C. Lunata* was treated with 200 μg/mL microcapsules, compared with the control in 24 h. The enzymatic activity of polyphenoloxidase decreased by 323.8%. There active oxygen species contents of hydrogenperoxide and superoxide anions increased by 204.6% and 164%, respectively.

Effects of difenoconazole and its formulations on cellulase activity

Table 4 displays the fungicidal activity of different difenoconazole formulations against the cellulase produced by different fungal species. The results indicate that the nanoemulsion (NE) formulation exhibits the lowest IC_{50} values for both *A. alternata* and *F. oxysporum*, indicating a higher inhibition efficacy against cellulase activity compared to the emulsifiable concentrate (EC) and technical (T) formulations. For *A. alternata*, the IC_{50} for NE is 0.05 mg/ml, while that for EC is 0.10 mg/ml. Similarly*, F. Oxysporum* exhibits an IC_{50} of 0.05 mg/ml for the NE formulation, compared to 0.22 mg/ml for the EC and 0.32 mg/ml for the T formulation. Incontrast, *R. stolonifer* exhibits a different trend, with the EC formulation having the lowest IC_{50} value of 0.04 mg/ml, while the NE formulation had a moderate value of 0.40 mg/ml. These results indicate that the efficacy of difenoconazole varies depending on the formulation and fungal species, highlighting the importance of formulation choice in controlling cellulase activity in these pathogens.

In vitro, greenhouse and field experiments were conducted to evaluate the efficacy of propiconazole, difenoconazole and carbendazim in controlling pathogens and their infection rates. Among the fungicides, propiconazole showed the highest inhibition of mycelia growth, biomass production, sporulation and spore germination in vitro at concentrations as low as 0.1 $\mu g ml^{-1}$. The production of Enzymes (PG, PGTE, PTE and cellulases) by *C. capsici* was significantly reduced when the fungicides were incorporate into the growth medium. The highest inhibition of enzyme production was observed with propiconazole, followed by difenoconazole and carbendazim. Greenhouse and field experiments were conducted to study disease control using sprays of propiconazole, difenoconazole and carbendazim. Application of propiconazole at 0.1% resulted in a significant reduction in disease incidence by70% when compared to difenoconazole at 0.05% (58%) and carbendazim at 0.1% (44%) (Filimon *et al*., 2016).

Table 4. Effect of the technical, emulsifiable econcentrate, and nanoemulsion of difenoconazole on cellulase activity in *A. alternata*, *F. oxysporum* and *R. stolonifer*

Formulation	IC_{50}[a] (mg/ml)	95% Confidence limits		Slope[b]±SE	(X)[2c]
		Lower	Upper		
		A. alternata			
EC	**0.10**	0.08	0.12	2.58±0.34	2.14
NE	**0.05**	0.04	0.05	2.98±0.33	3.44
T	**1.30**	1.15	1.46	2.57±0.31	1.29
		F. oxysporum			
EC	**0.22**	0.20	0.25	2.79±0.32	2.80
NE	**0.05**	0.04	0.06	2.56±0.33	3.28
T	**0.32**	0.29	0.36	2.93±0.33	2.49
		R. stolonifer			
EC	**0.04**	0.04	0.05	3.09±0.32	2.35
NE	**0.40**	0.34	0.45	2.52±0.31	2.45
T	**1.54**	1.35	1.75	2.38±0.31	4.31

a: The concentration causing 50% enzyme inhibition. b: Slope of the concentration-inhibition regression line ± standard error. c: Chi square value. T: Technical, NE: Nanoemulsion and EC: Emulsifiable concentrate.

Conclusion

The difenoconazole nanoemulsion was successfully prepared and characterized. Biological activity data showed that all formulations exhibited a significant inhibitory effect on the tested fungi compared to the control group. The current study suggests the potential use of difenoconazole nanoemulsion to combat some plant pathogens that cause damage to crops and vegetables, instead of current harmful fungicides. However, this type of compound warrants further research.

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تحضير وتقييم للمستحلب النانوي للديفينوكونازول المضاد للفطريات في المختبر لتعزيز النقل الفعال إلى الخلايا الفطرية

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الملخص

التقدم الأخير في تكنولوجيا النانو أدى الى تطوير التوصيل الفعال للمبيدات الفطرية إلى مسببات الأمراض المستهدفة، مما يعزز توافرها البيولوجي مع تقليل المخاطر البيئية ومخاطر صحة الإنسان.

تم تحضير المستحلب النانوي من الديفينوكونازول باستخدام طريقة الموجات الفوق صوتية. المستحلب النانوي للمبيد أظهر ثبات على درجة حرارة الغرفة، والتخزين البارد والساخن. تم الحصول على مادة المستحلب النانوي للديفينوكونازول الشفافة ذات التوزيع الضيق لحجم القطيرات، وكانت درجة الحموضة (pH) واللزوجة مطابقة للقيم القياسية. أيضا اظهر تحليل المجهر الإلكتروني الماسح (SEM) عن تكوين قطيرات نانوية منفصلة تتراوح أحجامها من 66.67 إلى 91.53 نانومتر. أجريت التجارب للمستحلب النانوي من الديفينوكونازول ضد ثلاث فطريات مسببة لأمراض النبات (وهي*A. alternata* ، *F. oxysporum*، *R. stolonifer*)، أظهر مستحلب الديفينوكونازول النانوي نشاطًا عاليًا مضادًا للفطريات (EC50=112.93، 151.73، 857.22 ملجم/لتر لـ*A. alternata* ، *F. oxysporum*، و *R. stolonifer*، على التوالي). وكذلك، أظهر مستحلب الديفينوكونازول النانوي تثبيطًا عاليًا لإنبات الجراثيم (I%= 60.23 و65.22، لـ *A. alternata* و*F. oxysporum*) ويمتلك المستحلب النانوي نفاذية أفضل عبر الخلايا الفطرية، مما يعطي نتائج أفضل مقارنة بالمركز القابل للاستحلاب من الديفينوكونازول المتاح تجاريًا.

الكلمات المفتاحية: *النشاط الابادى الفطري، مستحلب الديفينوكونازول النانوي، A. alternata، F. oxysporum, R. stolonifer*