

# BIOLOGICAL POTENTIAL AND MICROENCAPSULATION STABILITY OF D-LIMONENE, GARLIC OIL AND EPOXICONAZOLE FOR CONTROL OF CHILLY LEAF SPOT

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## Abstract

Pesticides based on chemicals are used to protect crops from a wide range of pests, resulting in enhanced agricultural output. Pesticide overuse causes issues such as residues in the environment, pest resistance, pest recurrence and so on. Pesticide overuse is expected to inflict \$100 billion in harm worldwide. As a result, there is an urgent need for environmentally friendly pest management solutions that can minimize pesticide use without compromising agricultural production. Natural pesticides are a fantastic alternative to conventional pesticides since they have a lower impact on the environment and human health. Essential oils include phytochemicals, which are useful owing to their eco-safety, target-specificity, lack of resistance development, decreased number of applications and improved acceptance with minimal or no mammalian toxicity. The essential oil's field applicability, however, is hampered by its limited chemical stability and water solubility. Essential oils have been widely used as an active ingredient in fungicidal formulations. However, essential oils are highly unstable and prone to degradation when exposed to the environment during storage. Microencapsulation techniques help to maintain the stability of molecules in essential oils that are sensitive to environmental stress and therefore improve shelf life. In this study, the physical stability and efficacy of D-limonene 5% + garlic oil 5% + epoxiconazole 5% microencapsulation were evaluated under different storage conditions over a 12-month period by comparing the formulation with a non-encapsulated formulation. The formulations were both stored under two different storage conditions, *i.e.*, heat stability at  $54 \pm 2$  °C for 14 days and cold storage stability at  $0 \pm 2$  °C for 14 days and Room temperature storage stability study done upto 12 months parameters *viz.* ai contents, suspensibility, pH, particle size, pourability, specific gravity and viscosity were studied. Droplet size, zeta potential and pH value were measured after 1, 6 and 12 months of storage to determine their stability. Biological potential or bio-efficacy of microencapsulation essential oil formulation against leaf spot and fruit rot disease of chilly were observed percent disease index (PDI) and percent disease control (PDC) were calculated and showed synergistic and effective in comparison to standard checks used in study as per good agricultural practice (GAP). The purpose of pesticide formulations and recent requirements of pesticide industry are discussed and the importance of research work on pesticide delivery systems, is pointed out. For this purpose, the microcapsules, as one of the typical controlled release formulations, play an important role. Handling safety of the pesticides is improved considerably by microencapsulation due to hazard and exposure reduction.

**Keywords:** Fungicide, Essential oil, Microencapsulation, Stability, Bio-efficacy, Release kinetics, Chilli leaf spot, Chilli fruit rot.

## INTRODUCTION

Non-judicious use of synthetic pesticides in crop protection is a major concern for environmental pollution affecting air, water, soil, human and animal health [1]. Consequently, it is now an utmost necessity to find alternative means for sustainable crop protection with lower impacts on the environment [2,3]. Botanical pesticides can be promising alternatives to synthetic molecules, be cost-effective, IPM compatible and safe for the ecosystem and public health [4]. The bioactive compounds produced in plants during secondary metabolism have been found with good pesticidal potency [5,6]. However, the rapid degradation of botanical extracts caused by volatilization and photo degradation of the active principles and their low solubility towards target surfaces are considered as disadvantages for utilizing these bio-inspired pesticides [7,8].

Essential oils are the secondary metabolites produced and stored in the secretory glands of aromatic plants [9,10]. Independently, they play an important role in a plant's defensive mechanisms against fungi, pathogenic microorganisms, herbivores and insects [11]. Due to these functions, essential oils are widely used in the medicinal, pharmaceutical, cosmetics, as well as pesticide and insecticide industries [12]. In the insecticide industry, in particular, plant extracts are extensively used as active ingredients in repellent products, which are now receiving more attention from consumers than the synthetic repellents.

The essential oil of garlic is obtained by steam distillation of garlic [19]. The sulphur containing constituents diallyl disulfide and diallyl trisulfide are the predominant constituent of the oil, with diallyl disulfide comprising up to 60% of the oil by weight. Garlic is one of many plants that discovered to create secondary metabolites as a defense to pests and disease damage [20].

D-Limonene is a common natural compound which is commonly found in many vegetables, citrus fruits and in some spices with citrus scent and lemon-like flavour. Chemically this monoterpene exhibit toxicity against several insects [21]. It is widely used in pesticides products, cosmetic products (soaps and perfumes) and in food products. It is also used as an insecticide, insect repellent and animals (dogs and cats) repellent and solvent or fragrance in pesticide formulations. It is commonly used in products such as certain food, soaps and perfumes. According to food and drug administration (FDA), D-limonene recognized as safe as a flavouring ingredient. However, research on nanoemulsion is growing, but nanoemulsion based on a plant extract oil are rarely explored due to their weak water solubility.

Epoxiconazole is a fungicide active ingredient from the class of azoles developed to protect crops. In particular, the substance inhibits the metabolism of fungi cells infesting useful plants and thereby prevents the growth of the mycelia (fungal cells). Epoxiconazole also limits the production of conidia (mitospores).

Micro-Encapsulation technique physically entraps the bioactive molecules into a protective matrix at the micro or nano range [13,14] and thereby, improve their delivery at the desired site and time at a specific rate [15]. Wall materials act as barriers to protect the core bioactive principles from degradation, evaporation and control diffusion to ensure sustained release [16]. The usefulness of encapsulation technologies in botanicals in terms of increased solubility, protection against premature degradation and sustained release of bioactive principles have also been reported [17].

## EXPERIMENTAL

### Material and method-stability study

#### Materials

Garlic oil and D-limonene were purchased from local market and epoxiconazole technical were gifted by GSP Crop Science Pvt Ltd. Aromatic hydrocarbon (Solvesso 200), toluene diisocyanate, 4,4'-diphenylmethane diisocyanate (Lupranate series), nonionic surfactant containing alcohol alkoxylates (Atlox series), hexamethylene diamine, acrylic graft copolymer (Atlox series), ethoxylated polyarylphenol (Soprophor series), alkylated naphthalene sulfonate, sodium salt (Morwet series), modified kraft lignin (Reax series), silicone

antifoam (SAG 1572), benzisothiazoline (BIT 20), propylene glycol, polysaccharide (Rhodopol 23), sulphuric acid and sodium hydroxide were received from Solvay, Croda, Stephan, Akzo Nobel, BASF, Clariant and Lamberti. All the chemicals used are of analytical grade. The stirrer, overhead stirrer (IKA Model Euro-ST 20D S000), homogenizer (Silverson) and distilled water, centrifuge, pH meter, beaker, measuring cylinder, particle size analyzer (Malvern 3000), SEM (Zeiss EVO-18 scanning electron microscope), Mettler Toledo, weighing balance *etc.* were used for the preparation of microencapsulation and further studies.

## Method

### Preparation of Primary Microencapsulation of D-limonene 5% + garlic oil 5% + epoxiconazole 5%

**Step-1:** Aqueous phase-charge water to a stainless-steel vessel equipped with a high-speed stirrer. Under agitation, add the alkylated naphthalene sulfonate, sodium salt, modified kraft lignin, ethoxylated polyarylphenol and acrylic graft copolymer into the vessel. Now add 50% quantity of silicone antifoam to avoid foam generation in this vessel.

**Step-2:** Organic phase-charge aromatic hydrocarbons solvent into second stainless-steel reactor. Then, slowly add D-limonene, garlic oil and epoxiconazole active ingredient into the reactor. Afterwards, charge 4,4'-diphenylmethane diisocyanate and toluene diisocyanate (*i.e.* wall forming material) to the reactor. Continue mixing. Cool the reactor contents to room temperature.

**Step-3:** Start the high shear disperser of aqueous phase and charge the 'organic solution' into the 'aqueous phase solution' under gravity in specific rate so that required particle size can be achieved and continue to shear for 30 min. Then, start heating the reactor to around 55 °C and stir the aqueous phase. Now add Hexamethylene diamine as catalyst in this formulation and stir the formulation under slow rpm for 3-4 h so that polymerization reaction gets completed.

**Step-5:** After the wall polymerization reaction, increase the agitator to high speed. Add the rest half quantity of antifoam to the formulation. Under slight vacuum, allow the mixture to de-gas for approximately 30 min to remove CO<sub>2</sub> from the solution. Add the polysaccharide, preservative and in last propylene glycol and mix for some minutes. Add buffering agent (sulphuric acid or sodium hydroxide as 50% solution) for pH adjustment.

### Capsule suspension (CS) formulation of D-limonene 5% + garlic oil 5% + epoxiconazole 5%

Chemical composition	Contents (% w/w)
D-Limonene a.i.	5.00
Garlic oil a.i.	5.00
Epoxiconazole a.i.	5.00
Aromatic hydrocarbon	15.00
Toulene diisocyanate	0.80
4,4'-Diphenylmethane diisocyanate	2.40
Nonionic surfactant containing alcohol alkoxyates	2.00
Hexamethylene diamine (25% HMDA solution)	0.75
Acrylic graft copolymer	2.50
Ethoxylated polyarylphenol	1.50

Alkylated naphthalene sulfonate, sodium salt	0.75
Modified kraft lignin	0.50
Silicone antifoam	0.30
Benzisothiazoline	0.10
Propylene glycol	5.00
Polysaccharide	0.20
Sulphuric acid	0.10
Sodium hydroxide	0.10
Water	53.00
Total	100.00

Photograph of formulation of D-limonene 5% + garlic oil 5% + epoxiconazole 5% prepared in lab and its dispersion pattern

Fig. 1. Photograph of CS formulation at ambient and after 14 days HST along with dispersion in water



## Result

Table-1: Storage stability study-capsule suspension (CS) formulation of D-limonene 5% + garlic oil 5% + epoxiconazole 5%

Parameters	Specification (In house)	Initial	Heat stability at 54±2 °C for 14 days	Cold storage stability at 0±2 °C for 14 days

Description	Off white colour flowable liquid	Complies	Complies	Complies
D-Limonene content percent by mass	4.75-5.5	5.22	5.19	5.21
Garlic oil content percent by mass	4.75-5.5	5.25	5.22	5.21
Epoxiconazole content percent by mass	4.75-5.5	5.21	5.20	5.20
D-Limonene suspensibility percent mini.	80	98.75	97.43	98.75
garlic oil suspensibility percent mini.	80	98.79	97.23	98.73
Epoxiconazole suspensibility percent mini,	80	95.77	95.52	95.75
pH range (1% aq. suspension)	6.0 to 8.5	7.00	7.10	7.15
Particle size (micron)	D50 <5, D90 <20	4.81, 17.0	4.94, 16.9	4.81, 17.0
Pourability	95 % min	97	96	97
Specific gravity	1.05 – 1.15	1.07	1.07	1.07
viscosity at spindle no. 62, 20 rpm	350 -800 cps	615	645	635

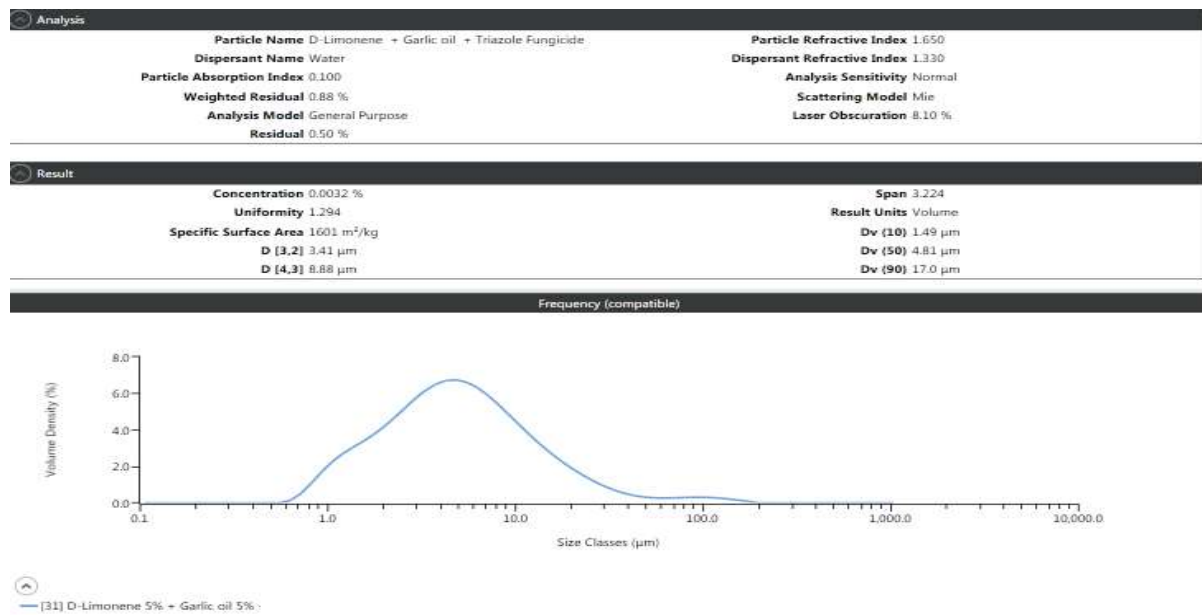
#### Room temperature storage data

Parameters	Specification	Study duration		
	In House	1 month	6 months	12 months
Description	Off White colour flowable liquid	Complies	Complies	Complies
D-Limonene content percent by mass	4.75-5.5	5.22	5.22	5.21
Garlic oil content percent by mass	4.75-5.5	5.25	5.25	5.23
Epoxiconazole content percent by mass	4.75-5.5	5.21	5.21	5.20
D-Limonene suspensibility percent mini.	80	98.75	98.11	97.9
Garlic oil suspensibility percent mini.	80	98.75	93.95	89.88
Epoxiconazole suspensibility percent mini,	80	95.77	95.7	95.65
pH range (1% aq. Suspension)	6.0 to 8.5	7.00	7.10	7.15
Particle size (micron)	D50 <5, D90 <20	4.81, 17.0	4.82, 17.0	4.83, 17.0
Pourability	95 % min	97	97	97
Specific gravity	1.05 – 1.15	1.07	1.07	1.07
viscosity at spindle no. 62, 20 rpm	350 -800 cps	615	645	635

## Stability of D-limonene 5% + garlic oil 5% + epoxiconazole 5% CS

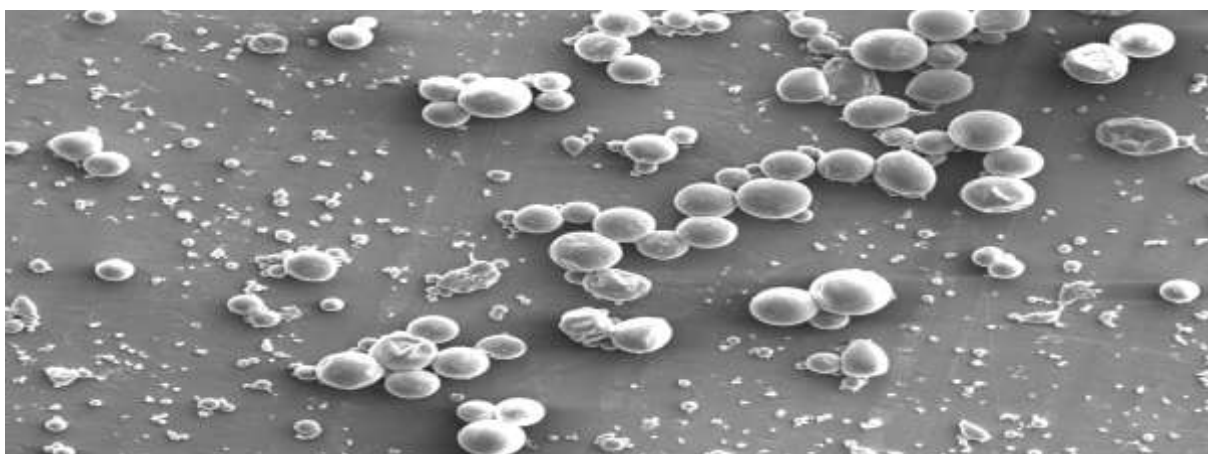
The stability of microencapsulation depends on the depends on the physicochemical characteristics of its ingredients including surfactants, oil phase and water phase [19,29]. This study was carried out by keeping sample at 54 degree for 14 days (D-limonene, garlic oil and epoxiconazole). This microencapsulated formulation is physically stable at room temperature and after stability study at 54 °C for 14 days (Table-1). The stability of microencapsulated formulation droplets of the oil was due to the surfactant which reduced the interfacial free-energy providing the mechanical barrier to calescence [20,28].

Particle size data of Ambient sample of D-limonene 5% + garlic oil 5% + epoxiconazole 5% CS



SEM images-photograph of capsule of CS formulation of D-limonene 5% + garlic oil 5% + epoxiconazole 5% CS

Fig. 5. Photograph of capsule of D-limonene 5% + garlic oil 5% + epoxiconazole 5% CS



## Material and method-biological potential/bioefficacy

Bio-efficacy study of D-limonene 5% + garlic oil 5% + epoxiconazole 5% CS at field using chilli crop

A synergistic effect exists wherever the action of a combination of active ingredient is greater than the sum of the action of each of the components alone. Therefore, a synergistically effective amount or an effective amount of a synergistic composition or combination is an amount that exhibits greater pesticidal activity than the sum of the pesticidal activities of the individual components.

In the field of agriculture, it is often understood that the term “synergy” is as defined by Colby S.R. in an article entitled “ Calculation of the synergistic and antagonistic responses of herbicide combinations” published in the journal Weeds, 1967, 15, p. 20-22, incorporated herein by reference in its entirety. The action expected for a given combination of two or three active components can be calculated as follows:

Colby's formula for calculating synergism between two active ingredients

$$E = (X+Y) - \frac{(X \times Y)}{100}$$

Where, E=Expected control by mixture or combination of Compound A and Compound B in a defined dose

X=Observed control by Compound A

Y=Observed control by Compound B

Colby's formula for calculating synergism between three active ingredients

$$E = (X+Y+Z) - \frac{(XY+XZ+YZ)}{100} + \frac{(X \times Y \times Z)}{10000}$$

Where, E=Expected control by mixture or combination of Compound A, Compound B and Compound C in a defined dose

X=Observed control by Compound A

Y=Observed control by Compound B

Z=Observed control by Compound C

$$\text{Colby's Ratio} = \frac{\text{Observed control}}{\text{Expected control}}$$

If Colby's ratio of O/E >1 means synergism observed

O/E <1 means antagonism observed

O/E =1 means simple additive effect

Higher the ratio means stronger the synergism, Lower ration means weak synergism.

The novel CS (capsule suspension) formulation of D-limonene 5% + garlic oil 5% + epoxiconazole 5% has been developed in the laboratory and evaluated for its efficacy and residual control.

Experiment design for synergistic control of leaf spot and fruit rot disease in chilly

Crop & Variety : Chilly, Rani

Location : Umreth, Dist. Anand, Gujarat

Treatments : 8

Plot size : 30 sq.m.

Crop age : 75 days

Spray water volume : 500 liter per hectare

Method of application: Foliar spray with battery operated knapsack sprayer fitted with hollow cone nozzle.

## Methodology:

After 7 days of spray observation on disease incidence and severity was recorded. Incidence of dieback was taken on plot basis by given formula.

$$\text{Percent Disease Incidence} = \frac{\text{Number of disease plants}}{\text{Total number of plants}} \times 100$$

For disease severity ten randomly selected plants were tagged in each plot and subsequent disease rating was done on scale of 0-5 and percent disease index (PDI) was calculated as given below.

Scale	Area covered by disease
0	No disease
1	Up to 5% of area infected
2	>5-10% of area infected
3	>10-25% of area infected
4	>25-50% of area infected
5	Above 50% of area infected

$$\% \text{ Disease Index (PDI)} = \frac{\text{Sum of total rating} \times 100}{\text{Total plant observed} \times \text{Maximum disease rating}}$$

$$\% \text{ Disease Control (PDI)} = \frac{\text{PDI (\%)} \text{ in untreated} - \text{PDI (\%)} \text{ in treatment}}{\text{PDI (\%)} \text{ in untreated}} \times 100$$

Fruit rot incidence (%) = The total number of healthy and infested fruits were recorded on ten tagged plants and percent fruit rot was calculated.

$$\text{Fruit rot (\%)} = \frac{\text{Number of disease plants}}{\text{Total number of plants}} \times 100$$

Table-1: Treatment details

Treatment Number	Treatment details	gram active per hectare
T1	Epoxiconazole 5% + garlic oil 5% + D-limonene 5% CS, 2000 mL/h	100 + 100 + 100
T2	Epoxiconazole 5% + garlic oil 5% CS, 2000 mL/h	100 + 100
T3	Epoxiconazole 5% + D-limonene 5% CS, 2000 mL/h	100 + 100
T4	Garlic oil 5% SC + D-limonene 5% SC, 2000 mL/h	100 + 100
T5	Epoxiconazole 5% CS, 2000 mL/h	100
T6	Garlic oil 5% SC, 2000 mL/h	100
T7	D-Limonene 5% SC, 2000 mL/h	100
T8	Untreated Check (UTC)	-

## Result

Table-2: Bio-efficacy against Chilly Leaf spot and fruit rot disease

Treatment number	Leaf spot disease control (%)				Leaf spot incidence (%)	Fruit rot (%)
	Observed control	Expected control	Colby's ratio	Synergism (Y/N)		
T1	94.8	82.26	1.15	Yes	0.92	1.12
T2	71.5	76.87	0.93	No	4.74	2.86
T3	70.3	75.76	0.93	No	4.52	2.72
T4	34.8	43.86	0.79	No	8.64	8.84
T5	68.4				3.62	6.28
T6	26.8				7.92	11.47
T7	23.3				8.16	12.36
T8	0.0				16.86	0.00

The field trial results shows that D-limonene 5% + garlic oil 5% + epoxiconazole 5% CS provides synergistic control of leaf spot disease as compared to all conventional treatments.

The synergistic combination of D-limonene 5% + garlic oil 5% + epoxiconazole 5% CS also gives lowest incidence of leaf spot disease and provides excellent control of chilli fruit rot disease.

**Photograph showing infection and no infection after treatment with D-limonene 5% + garlic oil 5% + epoxiconazole 5%**

No treatment on Chilli plant showing fungal infection	Treatment with D-Limonene 5% + Garlic oil 5%+ Epoxiconazole 5% CS (showing no infection)
	
Brownish leaves with fungal infection	Green leaves with no fungal infection

## Discussion

Controlled release are alternatives to ECs or EWs if a slow-release system appears as a promising approach to improve one of the following properties [18]. Normally, encapsulation of toxic AIs is most successful to reduce oral and dermal toxicity or encapsulation of volatile AIs to reduce evaporation losses and thus extending the duration of activity [19,27].

Controlled release is a technique or method whereby active are made available to a specific target at a certain concentration and duration to produce an intended effect. In CRF, the level of the initial applications is chosen in order to maintain the pesticidal concentration above the minimum inhibitory concentration (CMC) [20,30].

Microencapsulation is a technique used in variety of industries including crop protection chemical industries. The technique of microencapsulation generally involves the formation of a dispersion or emulsion of a relatively water-immiscible liquid in an aqueous medium to form an oil phase [21,22]. The oil phase contains the material to be encapsulated and as well as one or more monomers which form a polymeric microcapsules wall surrounding the oil-phase droplets when polymerization is initiated (by heating *etc.*).

Liquid water-immiscible agrochemical/crop protection chemicals which forms the material to be encapsulated may be a low melting point active which is emulsified as a melt or the liquid. Water –immiscible agrochemicals may be solution of solid agrochemicals in an appropriate water immiscible solvent [23,24].

By packaging the agrochemicals/crop protection chemicals into microscopic capsules, liquid can become powder, flammable solvents can be removed, unpleasant smell can magically vanish and product effectiveness can be extended [25,26].

Capsule suspension (CS) is the most environment-friendly formulation with the world's highest technical content and the best development prospects.

## Conclusion

Results exhibited that the microencapsulated formulation of D-limonene 5% + garlic oil 5% + epoxiconazole 5% having essential oil contents and azole fungicide is very stable during the study period of 12 months and at the same time the formulation is found very effective against leaf spot disease and provides excellent control of chilli fruit rot disease. So, it may concluded that right essential oils with azole fungicide in right ratio with microencapsulation technique can bring down the usage of chemical based pesticide and thus will be effective in answering the growing problem of pesticide exposure of human *via* residual exposure in food commodities. This system has the potential for agriculture application.

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