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Development and Evaluation of Median Lethal Concentration (LC50) of Wettable Powder and Oil Based Formulations of *Lecanicillium lecanii* (Zimmermann) IOF1 Strain (KM215209) under *in vitro* Conditions

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A B S T R A C T

The present *in vitro* studies on bio-efficacy of granular, oil based and wettable powder formulations on various sucking pests were carried out at Entomology laboratory, Institute of Organic Farming (IOF), University of Agricultural Sciences, Dharwad. Among different formulations evaluated *viz.*, rice bran oil (60 %) + corn oil (40%) formulation found least LC_{50} value against corn aphids (0.182 x10^6 cfu / ml), grape vine mealy bug (0.560 x 10^6 cfu / ml), cotton thrips (0.591 x 10^6 cfu / ml), and guava whitefly (0.942 x 10^6 cfu / ml). The olive oil formulation recorded least LC_{50} value 0.674 x 10^6 cfu / ml was against soybean mite. The wettable powder formulation found inferior by recording highest LC_{50} value against corn aphid (0.261 x10^8cfu / g), grape vine mealybug (0.740 x 10^8 cfu / g), cotton thrips (1.019 x 10^8 cfu / g), guava whitefly (1.757 x 10^8 cfu / g) and soybean mite (0.917 x 10^8 cfu / g) at 120 h. Oil formulations are compatible with other integrated pest management approaches. These formulations provide scope for the application of entomopathogens in arid climate where the temperature and relative humidity are major constraints.

Introduction

In recent past, increased environmental awareness, failure of conventional chemical insecticides and pesticides, increased number of insecticide resistant species and food safety and concerns, the application of biological control is amplifying abundantly (Digvijay Singh *et al.*, 2017). According to Baker and Cook (1974) and Boyetchko (1999) biological control is "decreasing the density of inoculums or disease fabricating actions of pathogen or parasite in its dynamic or static state, by one or more organisms, accomplished naturally or through alteration of surroundings, host or antagonist ".

Entomopathogenic fungi are potential biological control agents with a wide host range comprising over 100 genera with approximately 750 species (Hasan, 2014). Out of 31 insect orders, 20 are infected by entomopathogenic fungi in all the developmental stages (Araujo and Hughes,

2016). *L. lecanii* is one of several Deuteromycetes species and a potential biocontrol agent of insect order Homoptera, most commonly aphids, scale insects and whiteflies in tropical and subtropical regions. Infected insects develop white mycelial growth all over the body, hence the fungus is commonly called as "white-halo" fungus. The effectiveness of *L. lecanii* was studied and demonstrated first in India by Easwaramoorthi and Jayaraj (1978). Temperature and relative humidity are the major environmental factors, which affect the epizootics of *L. lecanii* under field conditions (Shinde *et al.*, 2010). Entomopathogenic fungi perform well under optimum temperature (25±1^{o}C) and high relative humidity (>70%). Extreme temperatures and poor relative humidity limits the use of these entompathogens in *rabi* and summer seasons and arid climate. To overcome this, there is a need to develop a suitable formulation for the successful utilization of mycoinsecticides. A good formulation helps in preserving organisms, delivering them to their target insect and to improve their activities.

Biological and physical properties of the formulation must remain stable for at least one year, but preferably for more than 18 months for commercialization to take place (Couch and Ignoffo, 1981). Keeping this in view the following study was carried out to evaluate wettable powder and oil based formulations of *Lecanicillium lecanii (*Zimmermann) IOF_1 strain (KM215209) under *invitro* conditions.

Materials and Methods

A laboratory experiment was carried out to prepare and evaluate the wettable powder formulation and different combinations of oil based formulations of *L. lecanii* at the Institute of Organic Farming (IOF), University of Agricultural Sciences, Dharwad.

Isolation and maintenance of pure cultures of *L. lecanii*

The pure culture of *L. lecanii* was isolated from infected spiralling whiteflies collected from the guava orchard. The infected whiteflies have white mycelial growth on the surface of the body. The mycelial growth was taken with the help of inoculation loop, the inoculums was transferred in to a sterile culture petri plates containing SMAY media. The plates were incubated at room temperature 26 ± 1°C at 80% RH for three days and the colonies that came up were further purified by repeated subculture on SMAY media. The isolates that came up on the SMAY medium were identified as *L. lecanii* by microscopic examination according to the outlines given by Samson *et al.*, (1988) and maintained as pure culture.

Mass production procedure for *L. lecanii* and *M. anisopliae*

Mass production procedure for *L. lecanii* and *M. anisopliae* is similar but only the culture is different as per method developed by Lingappa and Patil (2002).

Flow chart for mass production of entomopathogens

Broken rice (250 g) was taken in 1 kg capacity polypropanyle bag

⇩

Added 250 ml of 1% yeast extract solution prepared in distilled water

⇩

Soaked overnight

⇩

Sterilized under autoclave at 15 PSI for 30 min

⇩

After cooling to room temperature inoculated with 2 ml suspension (10^6 conidia/ml) under laminar air flow

⇩

Incubated at room temperature (26 ± 1°C) condition for 20 days at high RH (>80%) harvested and air dried digested material

⇩

Ground the digested material and dried once again to bring down moisture to below 8 %

⇩

Then sieved the digested material through 344 sieve meshes in order to get pure spore for further preparation of different formulations.

Preparation of oil based formulation

The oil based formulation of *L. lecanii* were prepared by using freshly harvested four grams of *L. lecanii* dry conidia (10^9 spores/ g) obtained from broken rice for which 20 ml of oils + 20 ml glycerol, were mixed and homogenized by using vertical mixture for five minutes for proper encapsulation of spores and required quantity of distilled water was added + 0.1% of tween-80 as spreading agent of spores.

Then stored both under ambient temperature and refrigerated conditions in a plastic container (50 ml capacity) for further study (Table 1). The different combination of oil based formulations of *L. lecanii* are as detailed below.

1) **Rice bran oil formulation:** 4 g of dry conidia (10^9 spores/ g) + 20 ml Rice bran oil + 20 ml glycerol + 956 ml distilled water + 0.1% tween 80.

2) **Olive oil formulation:** 4 g of dry conidia (10^9 spores/ g) + 20 ml olive oil + 20 ml glycerol + 956 ml distilled water + 0.1% tween 80.

3) **Rice bran (60%) + Corn oil (40%) formulation:** 4 g of dry conidia (10^9 spores/ g) + 20 ml Rice bran + corn oil + 20 ml glycerol + 956 ml distilled water + 0.1% tween 80.

Preparation of wettable powder formulation

Ten grams of dried conidia of *L. lecanii* cultured on broken rice grains (10^9 cfu / g) mixed with 90 g of carrier material (talc) to get formulated 10^8 cfu / g of product.

Before mixing the carrier material sieved through 355 mesh size sieves to maintain uniformity in particle size of conidial powder.

The carrier material sterilized in an autoclave at 121^0C and 15 Psi for 30 min and mixed with conidial powder after two days. After that 50 g of this formulation was packed in small polyethylene bags.

One set of bags stored in ambient room temperature (26 + 1^0C ART) and another set under refrigerated (4^0C; RC) condition.

Spore assessment

One gram of fungal spores developed on broken rice and sieved under 344 mesh were taken and diluted with 9 ml of sterile distilled water. To the 1-2 drops of Tween-80 was added for uniform distribution of spores in the water. Then the suspension was serially diluted up to dilution of 10^{-6} and 10^{-7}. From which 1 ml of suspension was drawn and the number of conidia per ml were determined by using Neubaeur's haemocytometer under phase contrast microscope (Plate 2).

The number of spores / g was calculated by using the following formula

Number of spores / g =

$$\frac{\text{Number of spores Present}}{\text{Number of cells}} \text{X } 400 \text{ x } 0.1 \text{ x } 1000 \text{ x DF}$$

Where, DF: Dilution factor, 0.1: Depth factor, 1000: Conversion factor

Efficacy of oil based formulations of *L. lecanii* against sucking insect pests under laboratory conditions

Different sucking pests *viz.*, corn aphid, cotton thrips, mealybug, spiralling whitefly and soybean mite were used for assessment of bio efficacy of different oil based formulations and wettable powder formulation of *L. lecanii* under laboratory condition.

The field collected sucking pest's *viz.*,corn aphids, cotton thrips, mealybugs, spiralling whitefly and soybean mites are maintained in field cage containing host plants (maize for aphid, soybean for mite, cotton for thrips, pumpkin for mealybug and flemingia for spiralling whitefly) for multiplication. After multiplication of these pests, the uniform sized aphid, thrips, mealybugs, spiralling whitefly and mites were released in petriplate containing different host leaves placed on water soaked blotting paper and each treatment was replicated three times in each replication 25 aphids were released, similarly in case of cotton thrips, mealybugs, soybean mites and whiteflies 25 individuals were placed in each petriplate for each replicated thrice. After that different concentration of oil based formulations (1.00 ml, 1.50 ml, 2.00 ml, 2.50 ml and 3.00 ml of stock solution containing 10^6 cfu / ml added to 1 litre of water and wettable powder formulation (1.00 g, 1.50 g, 2.00 g, 2.50 g and 3.00 g / litre of water) form that 1 ml of spray solution was sprayed on the test insect by using potter spray tower (15 lbs per square cm) to get uniform distribution of conidia on test insects and kept them in the environmental chamber (26 ± 1^o C temperature and 80 ± 5% RH) for sporulation. For the control distilled water spray was used, the mortality of test insects was recorded daily (1, 2, 3, 4, and 5th day) till the death of all test insects. The data on per cent corrected mortality was finding out by using Abbots formula.

Per cent corrected mortality =

$$\frac{\text{Y Number of grubs dead in control – X Number of grubs dead in treatment}}{\text{X Total number of grubs used in control – Number of grubs dead in control}} \text{X } 100$$

Results and Discussion

The different *L. lecanii* oil based formulations such as rice bran oil, rice bran (60%) + corn oil (40%) and olive oil formulations were evaluated against sucking pests under *in vitro* conditions (Table 2-7). The results of the present findings revealed that the all the sucking pests *viz.*, corn aphid, grapevine mealybug, cotton thrips and spiralling

whitefly showed more susceptibility to the oil based formulation, rice bran oil (60 %) + corn oil (40%) which recorded lower LC_{50} value to the corn aphid (0.182 x10^6 cfu / ml), grapevine mealybug (0.560 x 10^6 cfu / ml), cotton thrips (0.591 x 10^6 cfu / ml) and guava whitefly (0.942 x 10^6 cfu / ml) which was followed by other two oil based formulations such as olive oil and rice bran oil formulation. However, the olive oil based formulation was found best to soybean mite recorded least LC_{50} value 0.674 x 10^6 cfu / ml. The wettable powder formulation recorded highest LC_{50} value against corn aphid (0.261 x10^8cfu / g), grapevine mealy bug (0.740 x 10^8 cfu / g), cotton thrips (1.019 x 10^8 cfu / g), guava whitefly (1.757 x 10^8 cfu / g) and soy bean mite (0.917 x 10^8 cfu / g) at 120 h.

The present finding regarding the superiority of oil based formulation of *L. lecanii* are in agreement with the findings of Kim *et al.*, (2001) who demonstrated that *L. lecanii* (VL10 isolate) oil based formulation was highly pathogenic against *Myzus persicae*. Similar results reported by Yokomi and Gottwald, 1988, observed LC_{50} value of 1.65 × 10^6 cfu / ml against *Myzus persicae.* Asi *et al.*, (2009) also reported that the fungal isolate *Verticillium lecanii* (V17) with LC_{50} of 1.88 × 10^6 cfu / ml was considered the most effective against the aphids. Similarly, Sarnaya *et al.*, (2010), recorded that the lowest LC_{50} value of *L. lecanii* isolate against cowpea aphid, *A. craccivora* (2.5 × 10^4 cfu / ml), *B. brassicae* (1.2 × 10^4 cfu / ml), *A. gossypii* (2.7 × 10^4 cfu / ml).

According to Halyer (1993) who reported that addition of rape seed oil to the fungus *V. lecanii* at 1 x 10^8 cfu / ml increased efficacy up to 90 per cent when tested on aphid, *Aphis gossypii* (Glover) and thrips, *Frankliniella occidentalis* (Pergande), and also in comparison with Ramarethinam *et al.*,(2000) who reported that the Bio power, a commercial formulation of *V. lecanii* cause 43.56 per cent mortality on thrips, *Scirtothrips dorsalis* (Hood) on chilli.

The present findings are in line with Harischandra and Shekharappa (2008) reported that the oil based formulation of *V. lecanii* at 1 x 10^8 cfu / ml, observed 98 per cent mortality of okra aphid at 10^{th} day after treatment followed by wettable powder formulation (96.67%). Similarly, Mote *et al.*, (2003) reported that higher mortality of gerbera aphid was observed in oil based formulation of *V. lecanii* at 0.3% (93.44%) than wettable powder formulation (91.67%).

Table.1 Treatment details of different entomopathogenic fungi formulations of L. lecanii IOF1 strain (KM215209)

Treatments	*Dosage (g or ml/ lit of water)*				
Oil based and wettable powder formulations of *L. lecanii*					
T_1 - Rice bran oil formulation (10^6 cfu/ml)	1.00	1.50	2.00	2.50	3.00
T_2 - Rice bran (60%) + corn oil (40%) formulation (10^6 cfu/ml)	1.00	1.50	2.00	2.50	3.00
T_3 - ***Olive oil*** formulation (10^6 cfu/ml)	1.00	1.50	2.00	2.50	3.00
T_4 - Wettable powder formulation (10^8 cfu/g)	1.00	1.50	2.00	2.50	3.00
T_5 – ***Control***	Distilled water spray				

Table.2 Median lethal concentration (LC50) of oil based formulations of *L. lecanii* IOF1 strain (KM215209) against corn aphid, *Rhopalsiphum maidis* (Fitch)

Formulation	LC_{50} (cfu/ml)	Fiducial limits of LC_{50} (cfu/ml)		Regression equation(Y=a+bx)	LC_{95} (cfu/ml)	χ^2
		Lower limit	Upper limit			
Rice bran oil (60%) + corn oil (40%) formulation	0.182 $x10^6$ (cfu/ml)	0.044 $x10^6$ (cfu/ml)	0.347 $x10^6$ (cfu/ml)	Y= 1.015 + 0.082x	2.883 x 10^6 (cfu/ml)	0.379
Olive oil formulation	0.266 $x10^6$ (cfu/ml)	0.077 $x10^6$ (cfu/ml)	0.461 $x10^6$ (cfu/ml)	Y= 0.700 + 0.074x	5.981 x 10^6 (cfu/ml)	1.689
Rice bran oil formulation	0.316 $x10^6$ (cfu/ml)	0.147 $x10^6$ (cfu/ml)	0.481 $x10^6$ (cfu/ml)	Y= 0.769 + 0.074x	6.114 x 10^6 (cfu/ml)	0.582
Wettable powder formulation	0.261 $x10^8$ (cfu/g)	0.060 $x10^8$ (cfu/g)	0.475 $x10^8$ (cfu/g)	Y= 0.718 + 0.083x	5.674 x 10^8 (cfu/g)	0.523

Table.3 Median lethal concentration (LC50) of oil based formulations of *L. lecanii* IOF1 strain (KM215209) against grape vine mealybug, *Maconellicoccus hirsutus* (Green)

Formulation	LC_{50} (cfu/ml)	Fiducial limits of LC_{50} (cfu/ml)		Regression equation(Y=a+bx)	LC_{95} (cfu/ml)	χ^2
		Lower limit	Upper limit			
Rice bran oil (60 %) + corn oil (40%) formulation	0.560 x 10^6 (cfu/ml)	0.073 x 10^6 (cfu/ml)	1.034 x 10^6 (cfu/ml)	Y= 0.361 + 0.203x	7.845 $x10^6$ (cfu/ml)	0.360
Olive oil formulation	0.903 x 10^6 (cfu/ml)	0.024 x 10^6 (cfu/ml)	1.376 x 10^6 (cfu/ml)	Y= 0.062 + 0.195x	9.401 $x10^6$ (cfu/ml)	0.095
Rice bran oil formulation	1.287 x 10^6 (cfu/ml)	0.764 x 10^6 (cfu/ml)	1.608 x 10^6 (cfu/ml)	Y= 0.249 + 0.196x	13.827 $x10^6$ (cfu/ml)	2.486
Wettable powder formulation	0.740 x 10^8 (cfu/g)	0.131 x 10^8 (cfu/g)	1.207 x 10^8 (cfu/g)	Y= 0.189 + 0.198x	10.206 $x10^8$ (cfu/g)	0.219

Table.4 Median lethal concentration (LC50) of oil based formulations of *L. lecanii* IOF1 strain (KM215209) against cotton thrips, *Thrips tabaci* (Linde)

Formulation	LC_{50} (cfu/ml)	Fiducial limits of LC_{50} (cfu/ml)		Regression equation(Y=a+bx)	LC_{95} (cfu/ml)	χ^2
		Lower limit	Upper limit			
Rice bran oil (60 %) + corn oil (40%) formulation	0.591 x 10^6 (cfu/ml)	0.129 x 10^6 (cfu/ml)	0.921 x 10^6 (cfu/ml)	Y= 0.457 + 0.179x	3.924 x 10^6 (cfu/ml)	0.486
Olive oil formulation	0.751 x 10^6 (cfu/ml)	0.188 x 10^6 (cfu/ml)	1.098 x 10^6 (cfu/ml)	Y= 0.239 + 0.182x	5.378 x 10^6 (cfu/ml)	1.176
Rice bran oil formulation	1.068 x 10^6 (cfu/ml)	0.686 x 10^6 (cfu/ml)	1.313 x 10^6 (cfu/ml)	Y= 0.077 + 0.176x	4.361 x 10^6 (cfu/ml)	1.684
Wettable powder formulation	1.019 x 10^8 (cfu/g)	0.409 x 10^8 (cfu/g)	1.355 x 10^8 (cfu/g)	Y= 0.017 + 0.197x	6.238 x 10^8 (cfu/g)	0.685

Table.5 Median lethal concentration (LC50) of oil based formulations of *L. lecanii* IOF1 strain (KM215209) against spiralling whitefly, *Trialeurodes vaporariorum* (Westwood)

Formulation	LC_{50} (cfu/ml)	Fiducial limits of LC_{50} (cfu/ml)		Regression equation(Y=a+bx)	LC_{95} (cfu/ml)	χ^2
		Lower limit	Upper limit			
Rice bran oil (60 %) + corn oil (40%) formulation	0.942 x 10^6 (cfu/ml)	0.517 x 10^6 (cfu/ml)	1.213 x 10^6 (cfu/ml)	Y= 0.067 + 0.183x	4.137 x 10^6 (cfu/ml)	1.101
Olive oil formulation	1.283 x 10^6 (cfu/ml)	0.840 x 10^6 (cfu/ml)	1.571 x 10^6 (cfu/ml)	Y= 0.221 + 0.158x	7.204 x 10^6 (cfu/ml)	1.195
Rice bran oil formulation	1.530 x 10^6 (cfu/ml)	1.209 x 10^6 (cfu/ml)	1.788 x 10^6 (cfu/ml)	Y= 0.483 + 0.173x	7.516 x 10^6 (cfu/ml)	1.754
Wettable powder formulation	1.757 x 10^8 (cfu/g)	1.464 x 10^8 (cfu/g)	2.052 x 10^8 (cfu/g)	Y= 0.651 + 0.176x	7.299 x 10^8 (cfu/g)	5.075

Table.6 Median lethal concentration (LC50) of oil based formulations of *L. lecanii* IOF1 strain (KM215209) against soybean mite, *Tetranychus urticae* (Koch)

Formulation	LC_{50} (cfu/ml)	Fiducial limits of LC_{50} (cfu/ml)		Regression equation(Y=a+bx)	LC_{95} (cfu/ml)	χ^2
		Lower limit	Upper limit			
Olive oil formulation	0.674 x 10^6 (cfu/ml)	0.210 x 10^6 (cfu/ml)	1.174 x 10^6 (cfu/ml)	Y= 0.220 + 0.192x	4.746 x 10^6 (cfu/ml)	0.378
Rice bran oil formulation	0.744 x 10^6 (cfu/ml)	0.036 x 10^6 (cfu/ml)	1.172 x 10^6 (cfu/ml)	Y= 0.210 + 0.200x	6.542 x 10^6 (cfu/ml)	0.708
Rice bran oil (60 %) + corn oil (40%) formulation	0.901 x 10^6 (cfu/ml)	0.409 x 10^6 (cfu/ml)	1.207 x 10^6 (cfu/ml)	Y= 0.096 + 0.173x	7.377 x 10^6 (cfu/ml)	0.875
Wettable powder formulation	0.917 x 10^8 (cfu/g)	0.080 x 10^8 (cfu/g)	1.342 x 10^8 (cfu/g)	Y= 0.059 + 0.193x	9.194 x 10^8 (cfu/g)	0.354

Table.7 Comparisons of median lethal concentration (LC50) different oil based formulations of *L. lecanii* IOF1 strains (KM215209) against different sucking pests

Formulations	Corn aphid	Grape vine mealybug	Cotton thrips	Gauva whitefly	Soybean mite
Rice bran oil (60 %) + corn oil (40%) formulation	0.182 x10^6 (cfu/ml)	0.560 x 10^6 (cfu/ml)	0.591 x 10^6 (cfu/ml)	0.942 x 10^6 (cfu/ml)	0.901 x 10^6 (cfu/ml)
Olive oil formulation	0.266 x10^6 (cfu/ml)	0.903 x 10^6 (cfu/ml)	0.751 x 10^6 (cfu/ml)	1.283 x 10^6 (cfu/ml)	0.674 x 10^6 (cfu/ml)
Rice bran oil formulation	0.316 x10^6 (cfu/ml)	1.287 x 10^6 (cfu/ml)	1.068 x 10^6 (cfu/ml)	1.530 x 10^6 (cfu/ml)	0.744 x 10^6 (cfu/ml)
Wettable powder formulation	0.261 x10^8 (cfu/g)	0.740 x 10^8 (cfu/g)	1.019 x 10^8 (cfu/g)	1.757 x 10^8 (cfu/g)	0.917 x 10^8 (cfu/g)

In the present study, the superiority of oil based formulation of *L. lecanii* to the cotton thrips were more susceptible to oil based formulation which shows the early morality to the oil based formulation. These findings are conformity with the results of Mote *et al.,* (2003) who reported that the oil based formulation of *V. lecanii* @ 0.3 % recorded more than 91.67 per cent mortality of Gerbera thrips in polyhouse at 14 days after treatment compared to wettable powder (WP) @ 0.3% which causes less than 88.33 per cent mortality.

The efficacy results of three oil based formulations of *L. lecanii* against soybean mite, *T. urticae* revealed that the olive oil based formulation with least LC_{50} value (0.674 x 10^6 cfu / ml) compared to other oil based formulations which proved to be the best used for mite control. These findings corroborated with the report of Amjad *et al.,* (2012) who reported that the oil based formulation of *V. lecanii* (V17) isolate recorded lower LC_{50} (5.7 × 10^6 cfu / ml) after inoculation which showed the most virulent strain against mite, *T. urticae*. The *V. lecanii* at 0.3% of oil based formulation recorded 82.40 per cent mortality of *Tetranychus urticae* infesting gerbera at 14^{th} day after treatment in green house (Mote *et al.,* 2003).

According to Harischandra and Shekharappa (2008) reported that the oil based formulation of *V. lecanii* 1 x10^8 cfu / ml recorded the highest per cent mortality (97.00%) against okra thrips, followed by wettable powder formulation at 10^{th} day after spray. The present study also in agreement with earlier report of Nier *et al.,* (1993) who reported that pathogencity of *V. lecanii* against spiralling whitefly, *T. vaporariorum* and *Bemisia tabaci* (Gennadius), at the concentration of 3.2 x 10^6 cfu/ ml resulting in 92 and 100 percent mortality, respectively after 7 days after treatment. The results of the present investigation indicated more virulence of oil based formulation found more effective at lower concentration compared to wettable powder formulation, It is due to the oil based formulation prevented the desiccation of the conidia and helps in longer survival period and better penetration of peg into the integuments as per the report of (Burges, 1998).

From the present study it is evident that oil based formulations of entomopathogenic fungi are more effective than wettable powder formulation under laboratory condition. This efficacy can be attributed to oil based formulations which prevented that spores from desiccation and increased viability. Oil formulations are compatible with other integrated pest management approaches. These formulations provide scope for the application of entomopathogens in arid climate where the temperature and relative humidity are major constraints.

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