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Lethal and sub-lethal toxicity of *Verticillium lecanii* on the biology of Mealybug (Pseudococcidae; Homoptera) under laboratory conditions

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Abstract: ***The Mealybug, Planococcus citri (Risso) (Hemiptera: Pseudococcidae) is a polyphagous pest of Citrus, grapevine, coffee and ornamental plants. The entomopathogenic fungus Beauveria bassiana, Metarhizium anisopliae, Verticillium lecanii and Isaria fumosorosea are very effective and commonly used as a biopesticide against various insect pests of different crops. The present study was conducted to check the lethal and sub-lethal effects of V. lecanii on mortality and life period of adult mealybug. Firstly LC_{50} value for nymph and adult was calculated by applying four different concentrations 1×10^5, 1×10^6, 1×10^7 and 1×10^8 CFU/ml. For nymphs, the median lethal concentration was 5.57×10^6 CFU/ ml, and for adults, 2.66×10^7 CFU/ml. After this, two lethal, 3.99×10^7 and 2.66×10^7 CFU/ml, and two sub-lethal concentrations, 1.33×10^7 and 6.65×10^6 CFU/ml, was prepared and tested against adult mealybug. Dose-dependent mortality was observed, and the highest mortality was due to the highest concentration of V. lecanii. The percentage mortality was 72.23, 52.78, 27.78 and 19.45% due to 3.99×10^7, 2.66×10^7, 1.33×10^7 and 6.65×10^6 CFU/ml, respectively, after 10 days of application. The weight of adult females was highest in the control treatment while lowest in high concentration, and it was gradually decreased after every day in the first three concentrations. The fecundity of mealybug also had an inverse relation with concentrations of V. lecanii, and the numbers of eggs were more in the control treatment than the other four treatments. The fecundity in lethal concentrations was reduced after the 8^{th} day of application. The life duration was most extended due to control treatment, 27 days, while the lowest period was due to the highest concentration, 13 days.***

Keywords: Verticillium lecanii, mealybug, mortality, weight, fecundity, life duration

I. ABBREVIATIONS

Vl: *Verticillium lecanii*, CFU: Colony Forming Units

II. INTRODUCTION

Citrus is the world's most important fruit crop as a significant component of the human diet. Among the world fruit production, Citrus has crucial importance. The genus of Citrus is "*Citrus*", and the family is *Rutaceae*. The well-known citrus fruits are grapes, lime, lemon, sweet orange, kinnow and tangerines. Around the world, in about 140 countries, all of these citrus varieties are grown. Each year citrus Annual production increases enormously, up to 122 million metric tons (FAO, 2008). Citrus is a crucial fruit crop in Pakistan. Annually the manufacturing capability is about 1.7 million tons, while the total growing area for citrus fruit is about 160,000 hectares. Pakistan is ranked among the 10 top citruses (kinnow, hybrid mandarin and orange) producing and exporting countries. Citrus crop contributes its share about 45% among all fruit crops in Pakistan. In Pakistan, 80% of citrus crop production contains Feuterel's early and mandarins (Kinnow) (Naz et al., 2014).

Mealybug *Planococcus citri* belongs to the Class Insecta, order Hemiptera, family Pseudococcidae, Genus *Planococcus* and Species *citri* (Cox, 1989). It belongs to the second largest family of scale insects (Downie & Gullan, 2004). Mealybug has to pierce and suck mouthparts with the help of which they suck plant cell sap and cause severe infestation, which results in reduced plant vigour, premature leaf drop and production of honeydew which offer a suitable quality medium for the growth of black sooty mould fungus (Serrano et al., 2001). If the black sooty mould is not washed off on time, it would decrease fruit quality and create objection to fruit export in foreign countries (Demirci et al., 2011; Hattingh et al., 1998). Mealybug attacks on different host plants such as banana, mango, Citrus, coffee, cotton, annona, arabica and carambola (Rao et al., 2006).

Veticillium. lecanii (Zimmerman) is an effective bio-control agent used for various destructive insect pests in fruit, vegetables and crops. (Ferron, 1978). *V. lecanii* proved effective against the members of Homoptera like coccids, whiteflies, mealybugs and aphids as causing infection in them (Ekbom, 1979; Horne, 1915). *Verticillium lecanii* is eco-friendly and performs like hyperparasites of phytopathogenic fungi like rusts and powdery mildews (Ramarethinam et al., 2005). Fungi penetrate the host body via the host alimentary canal or cuticle (Broome et al., 1976). Mostly, the fungus gets its insertion into the host body via cuticle (Akbar et al., 2005) due to particular long-chain hydrocarbons in the cuticle that facilitate conidial attachment by hydrophobic interaction in the conidial cell wall (Boucias & Pendland, 1991). The growth of *V. lecanii* is mainly influenced by two climatic factors, humidity and temperature (Schuler, 1991). As a result of different greenhouse and laboratory experiments, two commercial products "Vertalec®" and "Mycotal®" based on strains, were developed. These products are used against mealybugs, aphids, and whiteflies (Gardner et al., 1984). *Verticillium lecanii* has been experienced practically in various countries of the world against many insect pests with encouraging results (Helyer & Wardlow, 1987; Kitazawa et al., 1984). In recent days due to the endless use of insecticides is possessing severe threats not only to the environment but also affect human health to a great extent. This way of using pesticides indiscriminately can cause severe damages. Therefore, there is a need to have some alternates of chemicals which are toxic to insects but safe for humans, the environment and other vertebrates. So, the entomopathogenic fungi will be evaluated for this purpose. The present research can be a source to identify entomopathogenic fungi as an alternative to these toxic chemicals against mealybug.

III. MATERIALS AND METHODS

A. Collection of Mealybug for culturing

For the experiment, mealybug, adults, and 2nd instar nymphs were collected from the citrus orchard at their early life cycle stages. For the mealybug collection, the different branches of the citrus plant containing mealybugs were cut off and brought to the Department of Entomology, College of Agriculture, and the University of Sargodha. Mass culturing of mealybugs were done on potato sprouts treated with 5% solution of sodium hypochlorite solution. The temperature was maintained between ranges from 21 to 25^0C in the rearing room.

B. Fungus Source

The commercial product of *V. lecanii* (Mealikil VL®) was used in this experiment imported from Agri Life (pvt) limited, India. This product was soluble homogenous powder with pH 6-8 and 6% moisture contents. The colony-forming units (CFU) were 1×10^9 in 1g powder.

C. Dose Preparation

This procedure consists of two sections. Firstly, four concentrations of *Vl* were made to calculate the LC_{50} value against the 2nd instar nymph and adult of mealybug. These concentrations were prepared by making stock solution, mixing 1g powder in 10 ml of distilled powder. This stock solution prepared four concentrations according to the spores requirement, and distilled water was mixed in them to make a 1ml solution. These concentrations were 1×10^5 CFU/ml, 1×10^6 CFU/ml, 1×10^7 CFU/ml and 1×10^8 CFU/ml, and bioassay was done to calculate the lethal value. When LC_{50} was calculated, again, four concentrations were prepared by the same method discussed above depending upon the lethal value. These concentrations were two lethal 3.99×10^7 CFU, 2.66×10^7CFU and two sub-lethal 1.33×10^7 CFU and 6.65×10^6 CFU. Bioassay was the same for both experiments.

D. Bioassay Test

An experiment was performed in the entomology laboratory of the College of Agriculture to calculate the LC50 of *V. lecanii* for both nymphs and adult mealybug. Adult mealybugs and 2nd instars nymphs were collected from the rearing chamber and released on citrus leaves and twigs in Petri dishes. Each treatment was replicated six times for nymphs as well as for an adult. In each Petri dish, six individuals were placed. Each Petri dish contained filter paper. The concentration was the same for both nymph and adult. The solution was measured by pipette.1 ml solution of different concentrations was sprayed directly using a mini sprayer. Fresh stem and leaves were changed every day as a diet. After applying entomopathogenic fungus, the dead number of insects was recorded every 24 hours till 10 days, and mortality was corrected through Abbot's formula (1-alive/total)*100. The second experiment was conducted to check the effect of lethal and sub-lethal concentrations on mortality and survival rate of adult mealybug. Bioassay was the same, and mortality data were recorded every 24 hours till 10 days, and after 10 days, alive mealybugs were left to check their life period. The number of eggs laid by females was calculated daily, and weight was also done after every three days interval on weighing balance. This experiment was done on only adults.

E. Statistical Analysis

Data of mortality, weight and fecundity were analyzed by using ANOVA, and factorial design was used. Means were compared by using Tukey HSD Test. LC_{50} value was calculated through probit analysis by making the line graph between log10 concentrations and probit mortality. The survival graph was made through the Kaplan Meier survival test. All data were analyzed by using Statistics 8.1® software.

IV. RESULTS

The commercial product used in this experiment gave significant results about the mortality of nymph and adults of mealybug. Dose-dependent mortality was observed in the whole experiment, and high concentrations showed high mortality than lower concentrations. The mortality data was collected till 10 days of application. The mean mortality of nymph was 21.11±4.11 after 10 days at the highest concentration of *Vl*, 1×10^8 CFU/ml, while the percentage mortality was 91.67±5.69. The mean mortality of nymph was 3.61±0.9, 9.76±1.55, 10.28±1.82, 17.78±2.77, 21.11±4.11, and the percentage mortality was 16.67, 25, 33.33, 58.33 and 91.67 due to control, 1×10^5, 1×10^6, 1×10^7 and 1×10^8 CFU/ml, respectively after 10 days of application. The effect of the first three treatments was the same, while the two highest concentrations had a different and significant impact.

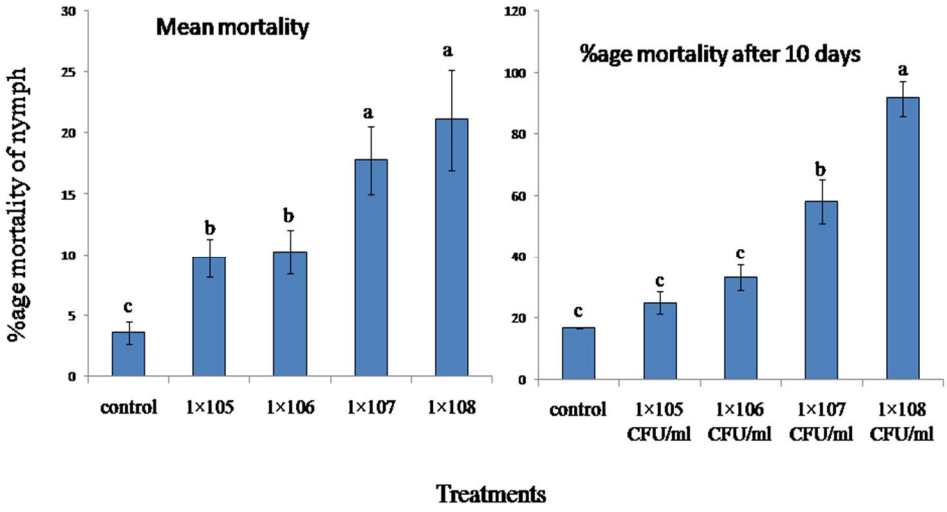

Fig. 1 Mortality of 2nd instar nymph of mealybug due to different concentrations of *V. lecanii* after 10 days

The mortality of adult mealybug was lower than 2nd instar nymph on the same concentrations of *V. Lecanii*. The mean mortality of adults was 0, 5.28±1.15, 9.45±1.45, 11.95±1.86, 20.28±3.15 and percentage mortality was 0, 16.67±4.3, 25±3.72, 33.33±6.08, 66.67±6.08 after 10 days of treatment due to control, 1×10^5, 1×10^6, 1×10^7 and 1×10^8 CFU/ml, respectively. Here only the last and highest concentration gave significant results while the effect of the other three concentrations of Vl and control treatment was the same, and similarly again, dose-dependent mortality was observed (Fig. 2).

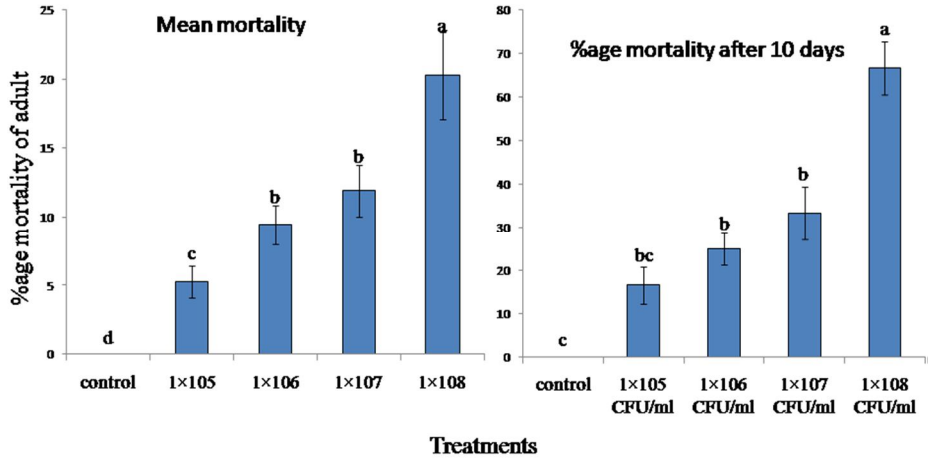

Fig. 2 Mortality of adult mealybug due to the application of different concentrations of *V. lecanii* after 10 days

The median lethal concentration (LC_{50}) for both nymph and adult was calculated through probit analysis by making the line graph between log10 doses and mortality in probits. The LC_{50} value was 5.57×10^6 and 2.66×10^7 CFU/ml for nymph and adult after 10 days.

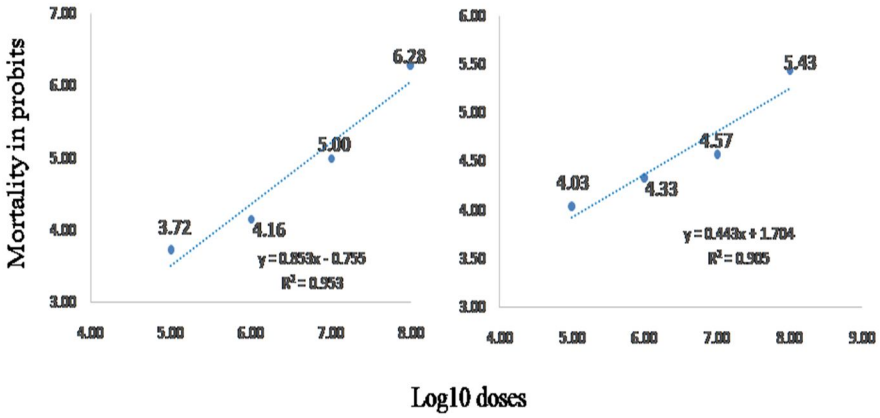

Fig. 3 Graph between log10 doses and mortality in probits to calculate LC_{50} value for 2nd instar nymph and adult mealybug

Table 1- LC_{50} value of *V. lecanii* for 2nd instar nymph and adult of mealybug

Mealy bug	slope± S.E(Log10 dose)	LC_{50}(CFU/ml)(95%FL)
Nymph	0.853±0.272	$5.57\times10^6(1.63\times10^6\text{-}1.89\times10^7)$
Adult	0.444±0.479	$2.66\times10^7(3.06\times10^6\text{-}2.30\times10^8)$

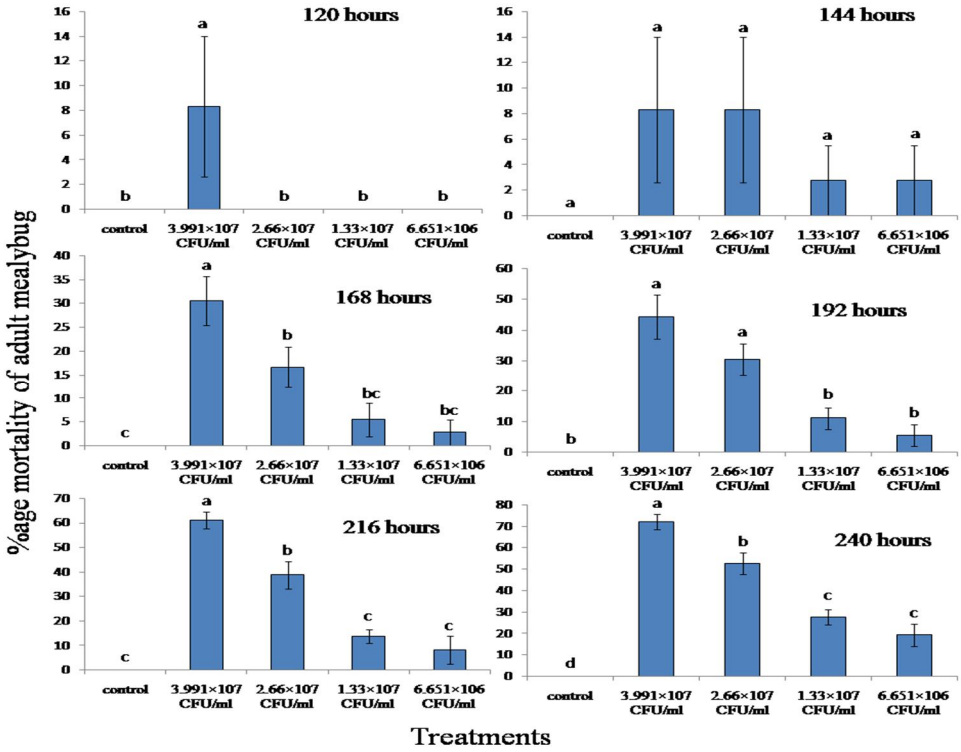

Fig.4 Mortality of adult mealybug due to lethal and sub-lethal concentrations of *V. lecanii*

After calculating the LC_{50} value, two lethal and two sub-lethal concentrations were tested against mealybug adults, and results were again significant for mortality and survival. The data for mortality was recorded till 10 days of application. Fig (4) shows that the death of insects was started on the 5th day of treatment, and on that day, mortality occurred only in the highest concentration. Here also dose-dependent death of insects was examined every day. High concentrations gave high mortality, while low concentrations gave less mortality of adult mealybug. After 144 days, the death due to the first two concentrations (3.99×10^7, 2.66×10^7 CFU/ml) was 8.33%, and after this, the highest mortality due to the highest concentration. After 10 days of application, the percentage mortality recorded due to different concentrations of *Vl* was 72.22, 52.78, 27.78 and 19.45% due to control, 3.99×10^7, 2.66×10^7, 1.33×10^7 and 6.65×10^6 CFU/ml, respectively.

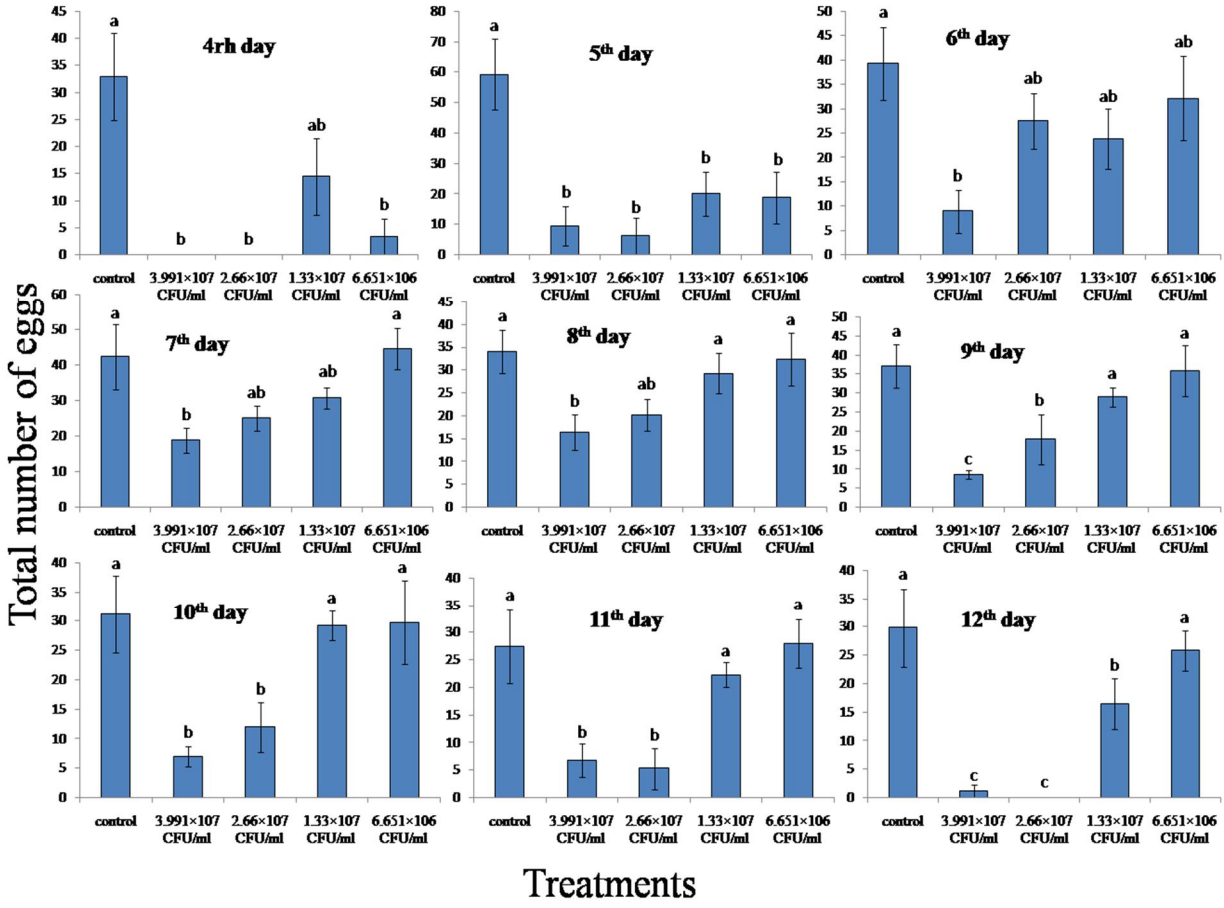

Fig. 5 Fecundity of adult female Mealybug after application of lethal and sub-lethal concentrations of *V. lecanii*

Fig (5) shows the average number of eggs due to lethal and sub-lethal concentrations. The results were significant, and the number of eggs was higher in the control treatment while lowest in the highest treated concentration of *V. lecanii*. It shows that the fecundity of females had an inverse relation to concentrations treated in the experiment. The effect of the first two higher concentrations on fecundity was similar, while the impact of the last and control treatments was the same. The fecundity in the first two concentrations was gradually decreased after every 24 hours interval, while in the control treatment, it remained constant. It means that these concentrations had a significant effect on the fecundity of mealybug. After the 11th day, the number of eggs was nearly zero in the highest concentration.

Similarly, the weight of adult females had also inverse relation to treated concentrations of *V. lecanii*. The weight was gradually reduced every day, but in the control treatment, it remained constant. The lowest weight of adult females was recorded in the highest concentration, and in the control treatment, the weight was highest, followed by the other three treatments. After 10 days of mortality, the surviving insects were left to check their life duration. The graph shows that the maximum life period was 27 days due to control treatment, while the lowest days were due to the highest concentration of *Vl*. The life period was recorded as 13, 15, 19 and 23 days for 3.99×10^7, 2.66×10^7, 1.33×10^7 and 6.65×10^6 CFU/ml, respectively.

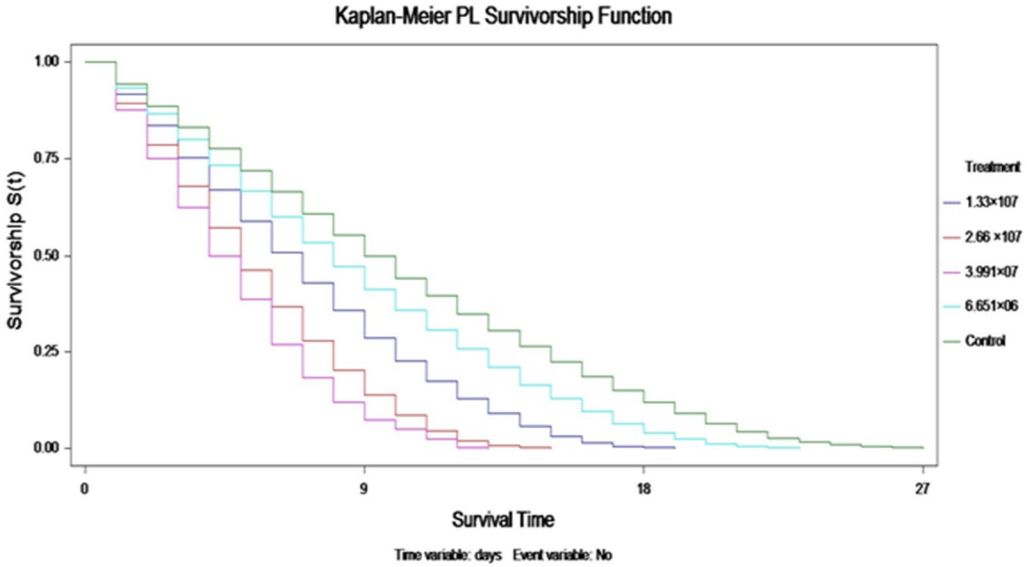

Fig. 6 Survivorship graph of adult mealybug due to the application of lethal and sub-lethal concentrations of *V. lecani*

Table 2- Lethal and sub-lethal effects of *V.lecani* on fecundity and weight loss of adult mealybug

Treatments (CFU/ml)	Number of eggs laid by a single female									Weight of single mealybug		
	3rd day	4th day	5th day	6th day	7th day	8th day	9th day	11th day	12th day	1st day	2nd day	3rd day
Control	33	59.33	39.33	42.33	34.12	37.23	31.24	27.5	29.83	0.063	0.057	0.051
	±8.07	±11.6	±7.49	±9.05	±4.7	±5.67	±6.53	±6.76	±6.9	±0.005	±0.006	±0.006
3.99×10^7	0±0	9.5	9	18.83	16.5	8.67	7	6.83	1.17	0.037	0.034	0.031
		±6.39	±4.41	±3.49	±3.91	±1.14	±1.67	±3.11	±1.17	±0.002	±0.002	±0.002
2.66×10^7	0±0	6.17	27.5	25.17	20.17	17.83	12	5.33	0±0	0.047	0.043	0.04
		±6.16	±5.69	±3.48	±3.45	±6.64	±4.28	±3.71		±0.005	±0.005	±0.005
1.33×10^7	14.5	20	23.83	30.83	29.33	29	29.33	22.33	16.5	0.052	0.047	0.043
	±7.13	±7.18	±6.24	±2.98	±4.4	±2.54	±2.52	±2.23	±4.4	±0.003	±0.003	±0.003
6.651×10^6	3.33	18.83	32.17	44.67	32.33	35.83	29.83	28	25.83	0.058	0.053	0.048
	±3.33	±8.43	±8.66	±5.73	±5.76	±6.69	±7.17	±4.48	±3.54	±0.006	±0.006	±0.005

V. DISCUSSION

Nowadays, farmers are excessively using insecticides and pesticides to control agricultural insect pests, so in this situation, there is a need to keep these chemicals to specific limits for environmental and health concerns. The primary purpose of this research study was to evaluate the effectiveness of different concentrations of *V. lecanii* along with control treatment against mealybug. This study was conducted to check the toxicity of the commercial product of *V. lecanii* against mealybug. In the case of mortality of mealybug, 2.66×10^7 CFU/ml (LC_{50}) is found to be more effective for control. While in the 2nd experiment, the highest concentration, 3.991×10^7 CFU/ml (LC_{75}), was found to be more effective after 240 hours. In the whole experiment, dose-dependent mortality was observed. Kumar et al. (2017) compared phytotoxicity and efficacy of *V. lecanii*, 1.15% WP, $1x10^8$ CFU/g) against mealybugs on citrus plants. Total seven treatments were applied, and the dose, 1.15% WP used @ 2500 g/ha, was optimum to control mealybugs on Citrus (acid lime). All the treatments were non-toxic to natural enemies and non-phytotoxic to citrus plants. LC_{50} value of *V. lecanii* was calculated, and it was determined that a low concentration of that fungus could give a better result than chemical insecticides. Chavan & Kadam (2010) checked the virulence of *V. lecanii* against *Maconellicoccus hirsutus*. After 14 days of treatment, the highest (100%) mortality was observed on the highest concentration (16×10^5 CFU/ml), and the lowest concentration (2×10^5 CFU/ml) caused the most negligible mortality (53.30%).

Similarly, Avalos & Wilson (2015) evaluated the efficacy of *L. lecanii* and *B. bassiana* against *P. citri*. Mortality of nymphs occurred after 72 hours of application. For *B. bassiana,* the mortality was 78.3 and 85.0%, and for *L. lecanii,* the mortality was 81.5 and 80.0% at concentrations of 10^6 and 10^7 conidia/ml, respectively. This study examined that the highest concentration of Vl gave the most increased mortality of nymphs and adults while the mortality of adults was lower than nymphs. The colour of insects was changed into dark black, and the size was reduced than the original.

For the life duration of adult mealybug, the most extended period was due to control treatment, and the smallest was due to the highest concentration. The adults exposed to spores of the *Vl* diet after different time intervals and infect highest concentration gave 100% mortality after 13 days. Ghaffari et al. (2017) used *L. longisporum* and *L. lecanii* against the 2nd instar nymphal stage and adult stage of citrus mealybug and found that *L. lecanii* is more efficient in their control. Banu & Gopalakrishanan (2012) calculated the time mortality (LT_{50}) for different formulations and reported that with the increases in storage time, the LT_{50} was found to be increased. LT_{50} values of two formulations, talc and oil, was 10.21 and 10.31, respectively. Tefera & Pringle (2003) recorded that the considerable decrease in utilization has been due to entomopathogenic fungi toxic substances production inside the host body, which caused a mechanical interruption in the insect structural integrity.

The weight of females was lower in high concentrations. The reason is that the body of the mealybug was shrieked due to the fungal effect, and the size was reduced than the original size. Similarly, the number of eggs laid by a female was also calculated lower in high concentrations than in the control treatment, and the fecundity was gradually decreased after every 24 hours interval, but it remained constant in the control treatment. Xu et al. (2009) checked the effect of *V. lecanii* on whitefly predator, *Axinoscymnus cardilobus* biological characteristics by using different concentrations. The most extended development period was observed due to the highest concentration. The lowest fecundity was observed due to the lowest concentration with an average of 126.2±34.85 eggs/female, while the maximum fecundity rate (133.2±32.18 eggs) was recorded in the control treatment. Tefera & Pringle (2003) recorded that the considerable decrease in utilization has been due to entomopathogenic fungi toxic substances production inside the host body, which caused the mechanical interruption in the insect structural integrity. Sahayaraj & Tomson (2010) observed a 33.34 % decline in body weight of *Dysdercus cingulatus* treated with metabolites of *B. bassiana* fraction 2 (BBF2). Malarvannan et al. (2008) found a decrease in the larval weight of *S. litura* treated with the fractions of *Argemone mexicana* during development and noticed the formation of a shrivelled pupa. Thangavel et al. (2013) performed a study by using different entomopathogenic fungi against whitefly. Entomopathogenic fungi were investigated for pathogenicity and ovicidal effect against spiralling whitefly, *Aleurodicus disperses*. They recorded that *L. lecanii* (L1strain) caused the lowest egg hatchability (23.2%) as compared to control treatment (92.6%).

So it is concluded from the present study and above discussion that all entomopathogenic fungi, especially V. lecanii, can reduce the population of mealybug. Using sub-lethal concentrations can reduce or control the different biological parameters like weight and fecundity. It is reported that concentration, 3.991×0^7CFU/ml, (LC_{75}) was more effective than the other concentrations. Dose-dependent mortality was observed in this study.

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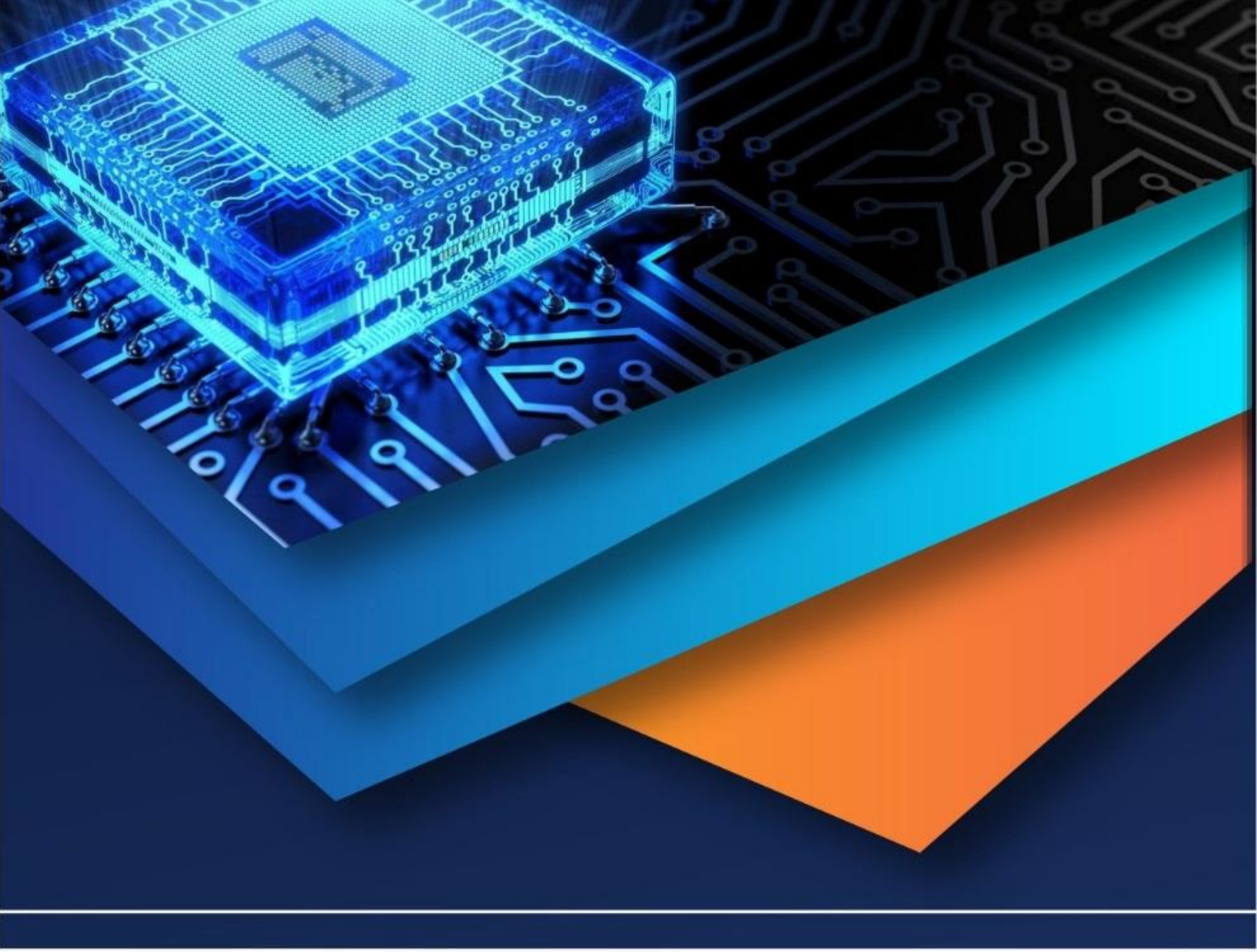

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