CHAPTER 4

RESULTS AND DISCUSSION

4. RESULTS AND DISCUSSIONS

4.1. Pathogenicity of the biocontrol agent *Verticillium lecanii* against the mealybug *Icerya seychellarum* during October 2012

4.1.1. Effect of *Verticillium lecaii* against *Icerya seychellarum* on citrus

The biological performance of *Verticillium lecanii* as a biocontrol agent, during 2012 for the control of the mealybug *Icerya seychellarum* located on young citrus trees is shown in Table 2.

The impact of the entomopathogenic fungi spores' suspension (*Verticillium lecanii*) on seychellus scale insect (*Icerya seychellarum*) located on citrus young trees showed that the proportion of mortality percentage increases as the concentration of spore suspension increases. Also, the mortality percentage increase as the exposure time post-application increases. The mortality of *Icerya seychellarum* reached 95.4% after 14 days of treatment with the concentration of 1.3×10^5 spores/ml and it was 97.7% at the concentration of 1.3×10^6spores/ml. The mortality percentage reached 100% after 11 days post-treatment at the concentration of 1.3×10^7 spores/ml and 1.3×10^8 spores/ml.

Table (3) and Fig. (1 and 2) show the LT_{50} values of the four tested concentrations of the biological agent 1.3×10^5, 1.3×10^6, 1.3×10^7 and 1.3×10^8 spores/ml. The LT_{50} values (days) decreases as they were calculated for the tested insect. The effect of the tested biocontrol agent against the insect individuals was similar since the slopes of the LT_{50} lines are more or less the same and they were parallel to each other. The LT_{50} values of the different

tested concentrations of *Verticillium lecanii* (1.3×10^8 - 1.3×10^5 spores/ml) tested against *Icerya seychellarum* ranged between 3.7- 6.4 days. Also, it could be said that the entomopathogenic fungi, *Verticillium lecanii* was effective against *Icerya seychellarum* located on young citrus trees.

The represented results are in agreement with those reported by **Fadayivata *et al.* (2014),** who evaluated the susceptibility of two cereal aphids, *Sipha maydis* (Passerini) and *Metopolophium dirhodum* (Walker), to the entomopathogenic fungus, *Lecanicillium longisporum* (Zimm.) Zare and Gams strain LRC 190, under controlled conditions.The conidial suspension of the fungus was administered using a sprayer on the whole plant over apterous adult aphids. The results indicated that both aphid species were susceptible to *L. longisporum* and that aphid populations were significantly reduced, compared to the control. Nine days after treatment, the LT_{50} value of the fungus at the tested concentration of 10^8 conidia / ml was obtained as 2.9 and 4.4 days for *S. maydis* and *M. dirhodum*, respectively. The results demonstrated that there was a varying susceptibility to the fungus between both two aphid species tested.

Also, **Gindin *et al.* (2000)** determined the pathogenicity of *Verticillium lecanii* against different developmental stages of the silverleaf whitefly, *Bemisia argentifolii.* They found that LT_{50} of *V. lecanii* against *Bemisia argentifolii* (Hemiptera) ranged between 3.2-3.8 days at the biological agent concentration of 10^7 conidia/ml.

Table (2): Bioassay of the biocontrol agent *Verticillium lecaii* used at different concentrations against *Icerya seychellarum* (On citrus) in the greenhouse during October 2012.

Conc. Spores/ml	Mortality%													
	Pre-spray	Days after treatment												
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
Untreated check	0.00	1.1* (±1.9)	1.1 (±1.9)	2.2 (±1.9)	2.2 (±1.9)	2.2 (±1.9)	2.2 (±1.9)	2.2 (±1.9)	3.3 (±0.0)	3.3 (±0.0)	3.3 (±0.0)	3.3 (±0.0)	3.3 (±0.0)	3.3 (±0.0)	3.3 (±0.0)
1.3X 10^5	0.00	4.4 (±5.1)	7.8 (±5.0)	17.0 (±8.8)	19.2 (±6.7)	28.4 (±3.7)	39.7 (±3.1)	45.4 (±4.3)	49.4 (±2.0)	60.0 (±2.0)	72.4 (±3.4)	77.0 (±2.0)	92.0 (±2.0)	94.3 (±2.0)	95.4 (±2.0)
1.3 X 10^6	0.00	7.8 (±8.4)	12.3 (±6.8)	25.0 (±3.7)	38.6 (±4.5)	42.0 (±3.6)	48.9 (±2.6)	56.8 (±1.7)	61.0 (±4.0)	64.4 (±2.0)	73.6 (±2.0)	87.4 (±5.3)	95.4 (±2.0)	96.6 (±0.0)	97.7 (±2.0)
1.3 X 10^7	0.00	12.3 (±3.8)	19.1 (±3.7)	33.0 (±1.8)	45.4 (±6.5)	53.4 (±5.2)	60.3 (±4.7)	71.6 (±3.7)	79.3 (±7.0)	84.0 (±2.0)	95.4 (±2.0)	100.0 (±0.0)	100.0 (±0.0)	100.0 (±0.0)	100.0 (±0.0)
1.3 X 10^8	0.00	12.3 (±3.8)	22.5 (±3.7)	34.0 (±2.8)	47.7 (±6.0)	54.6 (±3.6)	64.8 (±4.5)	72.8 (±3.0)	82.8 (±3.5)	93.1 (±3.4)	98.9 (±2.0)	100.0 (±0.0)	100.0 (±0.0)	100.0 (±0.0)	100.0 (±0.0)

*Results are shown as mean ± SD

Table (3): LT_{50} values (days) of the biocontrol agent *Verticillium lecaii* used at different concentrations against *Icerya seychellarum* (on citrus) in the greenhouse.

Tested Conc. Spores/ml	LT_{50} (days)	Fiducial limits (Lower limit -Upper limit)	Slope (± SE)
1.3 X 10^5	**6.4**	**(5.4 – 7.6)**	**3.3 (± 0.2)**
1.3 X 10^6	**5.2**	**(4.3 - 6.0)**	**2.9 (± 0.2)**
1.3 X 10^7	**3.9**	**(3.2 – 4.5)**	**3.1 (± 0.2)**
1.3 X 10^8	**3.7**	**(3.1 - 4.3)**	**3.0 (± 0.2)**

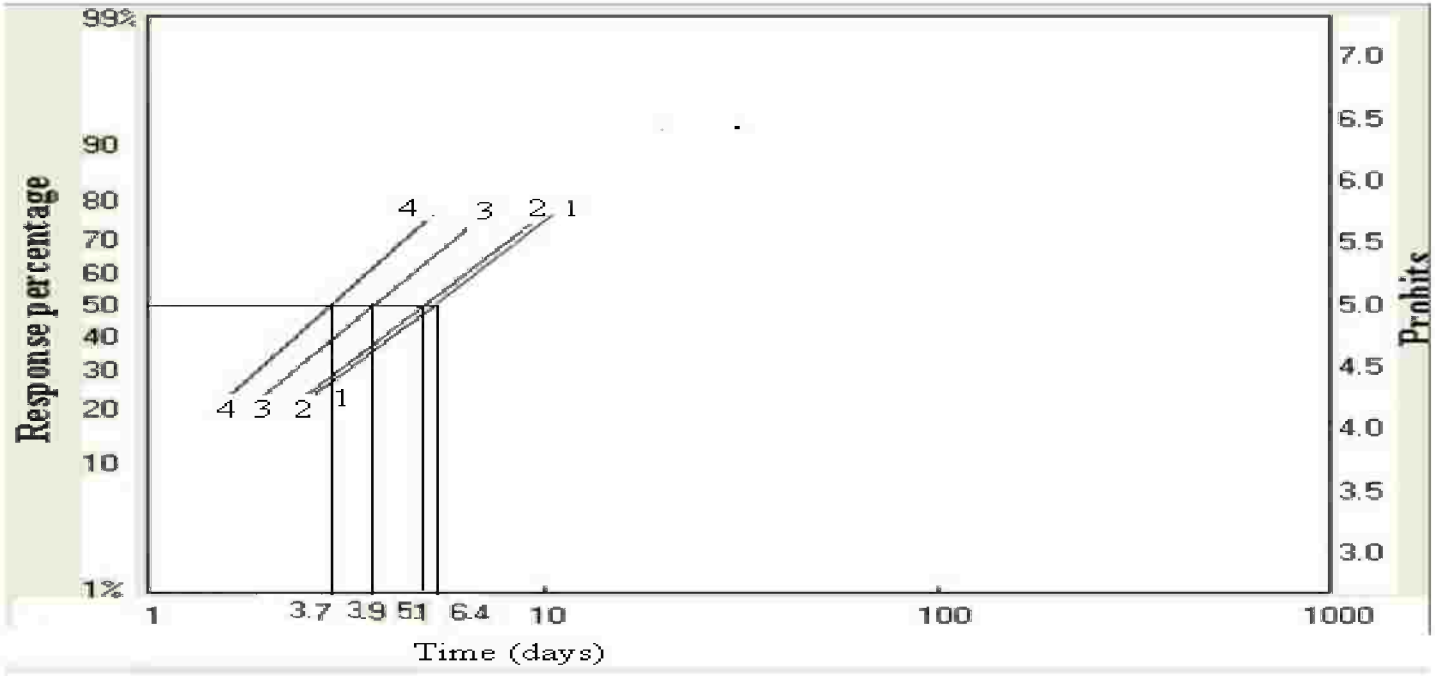

Fig. 1: LT $_{50\,s}$ lines of the four tested concentrations of the fungus *Verticillium lecanii* against the mealy bugs *Icerya seychellarum* located on young citrus trees

***[Conc. 1 = $1.3X10^5$ spores /ml , Conc. 2 = $1.3X10^6$ spores /ml , Conc.3 = $1.3X10^7$ spores /ml and Conc.4 = $1.3X10^8$ spores /ml]**

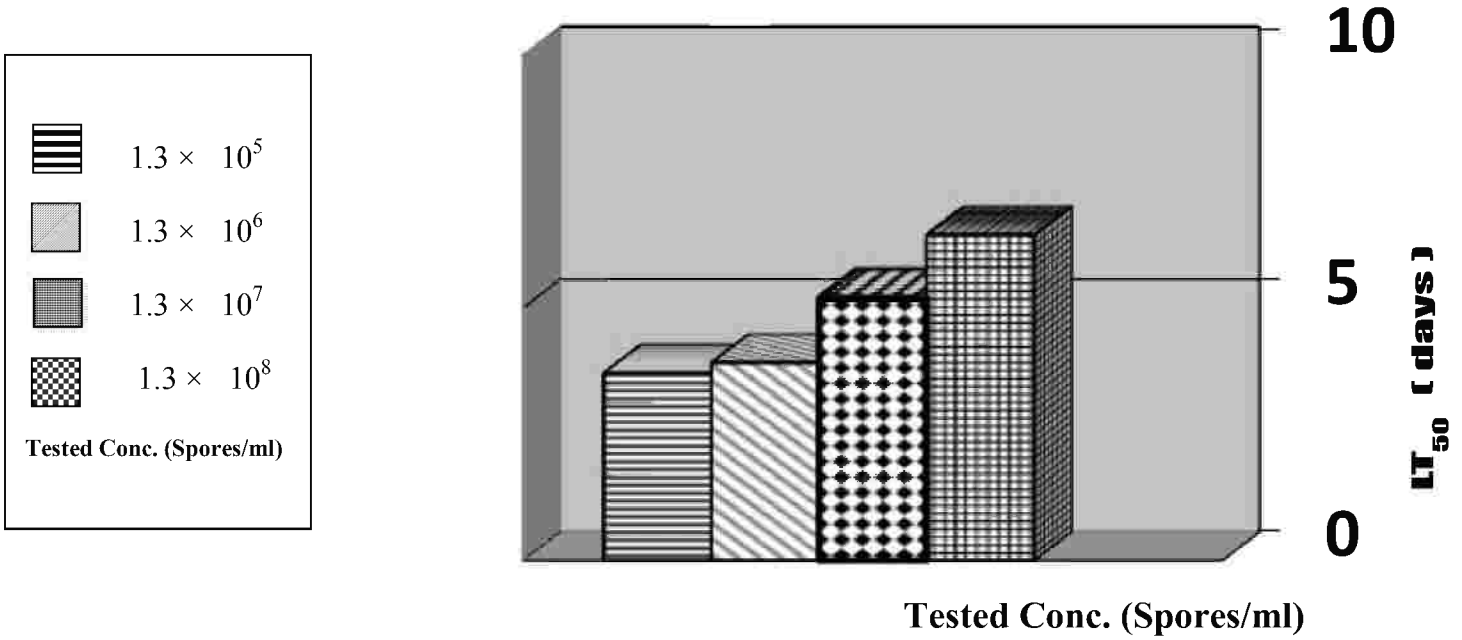

Fig. 2: LT_{50} values (days) of the strain biocontrol agent *Verticillium lecaii* used at different concentrations against *Icerya seychellarum* (on citrus) in the greenhouse

Jeong and Kim (2008) found that the LT_{50} values for *V.lecanii* against *Aphis gossypii* (Hemiptera) ranged between 2.7and 3.3 days after treatment by the concentration of 10^7 conidia / ml.

De-Souza *et al.* (2004) evaluated the pathogenicity of the entomopathogenic fungus *Verticillium lecanii* (isolate IBCB 473) against the aphid *Cinara atlantica* (Hemiptera: Aphididae), using suspension concentrations of 10^6, 0.5×10^7, 10^7, 0.5×10^8 and 10^8 conidia/ml. The evaluations were carried out daily, recording the individual mortality of each treatment. The most effective concentration was 1.0×10^8 conidia/ml with mortality percentage of 86% after five days. LT_{50} of each of the tested concentration was 5.82, 5.24, 4.60, 4.34 and 3.83 days, respectively.

4.2. Virulence of *Verticillium lecanii* (Zimm.) Viegas passages through artificial media and an insect host *Icerya seychellarum* (Hemiptera: Monophlebidae)

The entomopathogenic fungi are known to lose their desirable features after repeated sub-culturing declining their virulence through successive passages (subcultures). *Verticillium lecanii*, which lose virulence, may sometimes be restored to their former potency by passing them through their natural insect host. The results of the effect on virulence of *V.lecanii* (Zimm.) Viegas passage through an artificial medium and an insect host *I. seychellarum* are recorded in Tables (4 and 5) and illustrated in Fig. 3, from which it could be deduced that. The mortality percentages and LT_{50} values of the adult individuals of the mealy bug *I. seychellarum* exposed to mother and its obtained passages was varied according to differed tested subcultures, time of exposure and the used medium at a single concentration of 1.7×10^8 spores/ml. Daily recorded mortality percentages proved that the

lethal effects of the tested cultures were systematically arranged with increasing the time of exposure in all cases (subcultures) reaching 94.4, 79.8, 51.7, 44.9 and 36% mortality in treatment with 1^{st}, 2^{nd},3^{rd},4^{th} and 5^{th} sub cultures, respectively when passaged through the MYB artificial medium compared with complete death (100% mortality) of the insect population treated with the mother culture after 13 days. These results proved that the virulence of *V. lecanii* against *I. seychellarum* was decreased with increasing its passage's number through the MYB artificial medium. This was clear when the LT_{50} values of the different passages through the artificial medium (MYB) were compared. The lowest value of LT_{50} for the zero passage is 4.6 days followed by 5.8, 7.4, 11.3, 13.9 and 17.7 days for the five passages cultures, respectively (Table 5).

Moreover, these results also confirmed that the passages of *V. lecanii* through a suitable insect-host, *I. seychellarum* had an increase of their virulence, showing the lowest value of LT_{50} (4.6 days) for the zero passage followed by 5.3, 6.6, 8.4, 9.0 and 9.47 days for the five derived passages (subcultures), respectively . Comparing the five passages through the natural insect-host, *I. seychellarum* with the used artificial medium, it was found that virulence of subcultures obtained form the natural insect host was increased by 1.9 fold relative to that of subcultures obtained from the artificial medium based on their LT_{50} value.

The obtained results are in agreement with **Nagaich (1973)** who found loss of virulence of *V.lecanii* (Zimm.) after two or three subculturing. Meanwhile, some strains need to be successively subcultured 10 to 12 times before a significant decline in virulence is occurred.

On the other hand, **Asghar (2013)** investigated the effects of repeated subculturing of *Beauveria bassiana* and *Metarhizium anisopliae in vitro* and their passages through insects and their virulence against *Uvarovistia zebra*. The virulence of both fungi was reduced after four subcultures in Potato Dextrose Agar media (PDA), but this reduction was not quite significant for *B. bassiana*. Attenuated fungi obtained from the fourth subculturing were passaged through 3rd effects instar nymphs of *U. zebra*. The insect passage was repeated 2 times and virulence of the fungi was evaluated by its lethal effect. Following passage, there was a small, but non-significant increase in the virulence of the fungi. The same trend as that presented in the present study was reported by **Adames *et al.* (2011)** as they assayed the virulence of strain M379 of the fungus, *Metarhizium anisopliae* (Metchnikoff) Sorokin (Hypocreales: Clavicipitaceae) after different passages through a suitable host using different concentrations for controlling of both acaricide-susceptible and resistant strains of the tick, *Rhipicephalus* (formerly *Boophilus*) *microplus* Canestrini (Ixodida: Ixodidae) *in vitro*. The highest value of LC_{50} for the susceptible strain corresponded to zero passage was 7.68×10^7 conidia/ml followed by the fourth passage with LC_{50} of 2.68×10^7 and hence the lethal concentration was reduced to 2.87-fold. Comparing LC_{50} values of the fourth vs. the seventh passage (2.59×10^5 conidia/ml), it was found that the lethal concentration was reduced by 103.47-fold for the seventh passage when the fungus *Metarhizium anisopliae* was maintained on ticks (natural host).

Table 4: Mortality of *Icerya seychellarum* treated with 1.7×10^8 spores/ml of *Verticillium lecanii* derived from different passages maintained on MYB medium and its insect-host *I.seychellarum*

strain (passage)	Medium type	Pre-spray	Mortality percentages at different times (days)													
			1	2	3	4	5	6	7	8	9	10	11	12	13	14
Untreated check		0.0	0.0	0.0	1.1*	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1
		0.0	±0.0	±0.0	±1.9	±1.9	±1.9	±1.9	±1.9	±1.9	±1.9	±1.9	±1.9	±1.9	±1.9	±1.9
Mother culture	MYB	0.0	2.2	6.7	29.1	53.9	57.3	60.7	64.1	65.2	75.3	88.7	93.3	95.5	100.0	100.0
		0.0	±1.9	±2.7	±8.1	±1.1	±2.5	±1.2	±1.3	±1.7	±1.7	±1.8	±0.1	±1.9	±0.0	±0.0
1st passage	MYB	0.0	1.1	4.4	16.9	35.9	44.9	46.0	57.3	61.8	66.3	76.4	87.6	89.9	94.4	94.4
			±1.9	±1.6	±0.3	±1.3	±3.1	±1.0	±2.5	±1.7	±0.7	±0.5	±2.1	±0.2	±1.9	±1.9
	I.seychellarum	0.0	1.1	4.4	25.8	50.5	51.7	55.1	56.2	57.3	64.1	71.8	82.0	89.0	95.5	98.9
			±1.9	±1.6	±1.5	±2.6	±1.7	±1.7	±0.9	±2.5	±1.3	±1.6	±1.8	±0.2	±1.9	±1.9
2nd passage	MYB	0.0	1.1	3.3	6.7	25.9	38.2	39.3	44.9	47.2	51.7	65.2	73.0	74.1	79.8	83.1
			±1.9	±4.4	±0.1	±2.2	±1.7	±1.2	±3.1	±0.9	±1.7	±1.7	±0.5	±2.2	±0.4	±0.3
	I.seychellarum	0.0	1.1	5.6	17.9	25.9	35.9	41.6	53.9	57.3	59.5	62.9	67.4	83.1	89.9	92.1
			±1.9	±1.6	±1.8	±2.2	±1.3	±1.7	±1.1	±2.5	±0.8	±0.7	±2.3	±0.3	±0.2	±2.1
3rd passage	MYB	0.0	1.1	2.2	2.2	13.5	23.6	31.5	34.8	37.1	40.5	47.2	49.4	51.7	51.7	55.0
			±1.9	±1.6	±1.9	±0.3	±0.5	±1.7	±1.7	±0.7	±0.8	±0.2	±0.9	±1.7	±1.7	±1.7
	I.seychellarum	0.0	1.1	1.1	4.5	6.7	22.4	31.5	43.8	49.4	51.7	57.3	62.9	70.8	73.0	83.1
			±1.9	±1.6	±1.9	±0.1	±3.7	±1.7	±2.7	±1.0	±1.7	±2.5	±0.7	±1.4	±0.5	±0.3
4th passage	MYB	0.0	1.1	1.1	1.1	6.7	10.1	20.2	28.1	32.6	33.7	35.9	38.2	43.8	44.9	46.0
			±1.9	±1.6	±1.9	±0.1	±0.2	±0.4	±1.7	±1.3	±0.7	±1.3	±1.7	±0.9	±1.7	±1.1
	I.seychellarum	0.0	1.1	1.1	5.6	10.1	21.3	24.7	30.3	42.7	53.9	59.6	60.7	65.2	67.4	76.4
			±1.9	±1.6	±2.0	±0.2	±1.8	±1.7	±0.6	±1.1	±1.1	±2.7	±1.2	±3.3	±1.3	±0.5
5th passage	MYB		1.1	1.1	1.1	3.4	4.5	7.9	11.2	15.7	20.2	26.9	32.6	33.7	35.9	38.2
			±1.9	±1.6	±1.9	±0.1	±2.1	±1.9	±1.8	±1.7	±0.4	±0.5	±1.3	±0.7	±1.3	±1.7
	I.seychellarum	0.0	1.1	2.2	6.7	12.3	22.4	23.6	31.5	43.8	48.3	51.7	52.8	62.9	68.5	73.0
			±1.9	±1.6	± 0.1	±1.7	±3.7	±2.9	±1.7	±2.7	±1.7	±1.7	±0.9	±0.7	±1.7	±0.5

*Results are shown as mean ± SD

Table (5): LT_{50} values on *Icerya seychellarum* exposed to *Verticillium lecanii* (Zimm.) derived from different passages maintained through artificial media and an insect host (*I. seychellarum*)

Tested culture of *Verticillium lecanii* (passage)	Medium type	LT_{50} (days)	Fiducially limits (Lower limit -Upper limit)	Slope (± SE)
Mother culture	MYB	4.7	4.0 – 5.2	3.2 ± 0.2
1st passage	MYB	5.8	5.5 – 6.1	3.4 ± 0.2
	I.seychellarum	5.3	4.6 – 6.0	3.0 ± 0.1
2nd passage	MYB	7.4	7.0 – 7.9	3.1 ± 0.2
	I.seychellarum	6.6	6.1 – 7.1	3.0 ± 0.1
3rd passage	MYB	11.3	10.4 – 12.4	2.4 ± 0.2
	I.seychellarum	8.4	7.7 – 9.2	3.5 ± 0.2
4th passage	MYB	13.9	12.6 – 15.6	2.6± 0.2
	I.seychellarum	9.0	8.5 – 9.5	3.3 ± 0.2
5th passage	MYB	17.7	15.7 – 24.1	2.7 ± 0.3
	I.seychellarum	9.5	8.9 – 10.1	3.0 ± 0.2

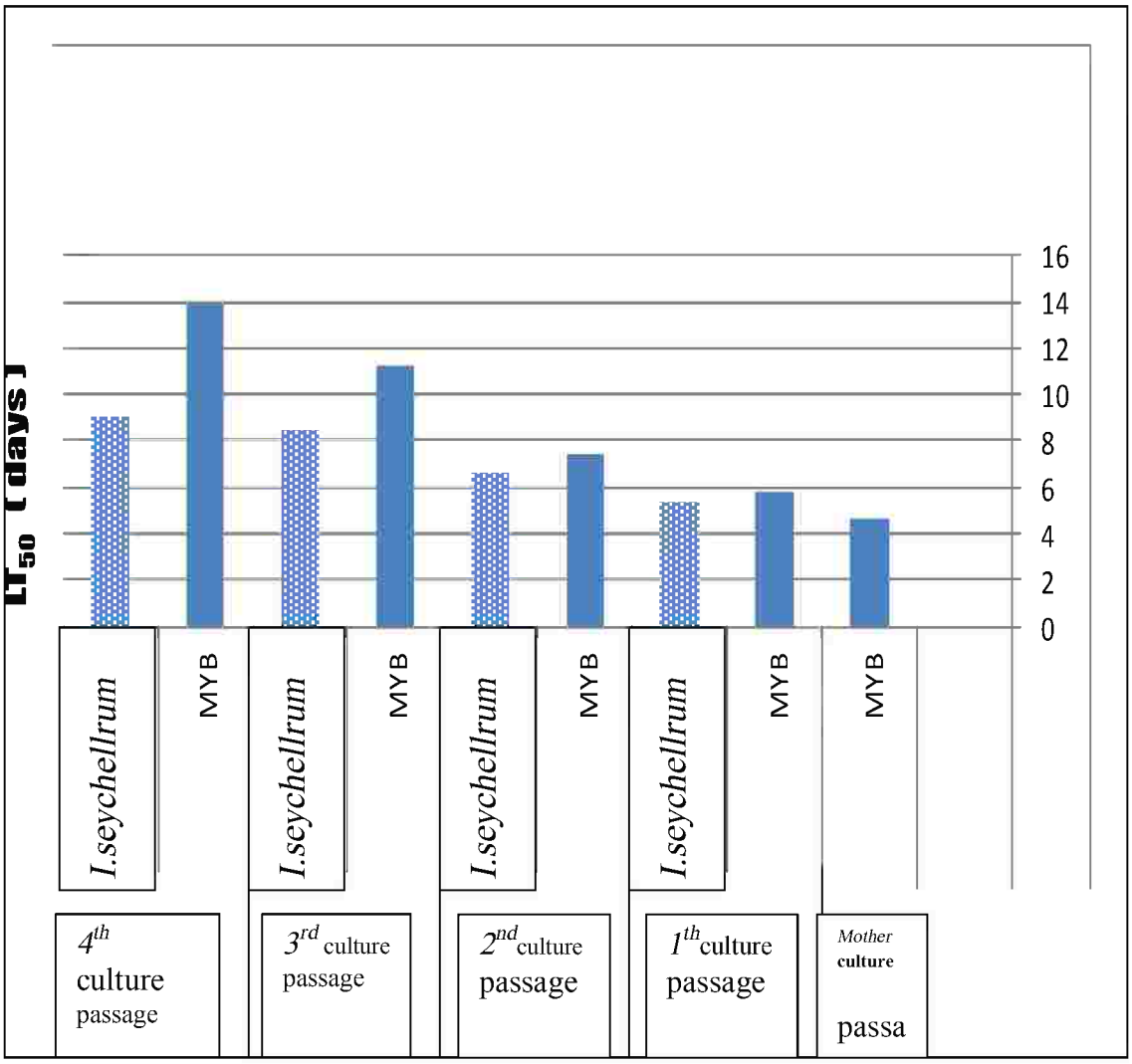

Fig.3: LT_{50}valus of the *Verticillium lecanii* (Zimm.) Viegas passage through artificial media and an insect host *Icerya seychellarum*

4.3. Mass-production of *Verticillium lecanii* on different media types

For mass production of the entomopathogenic fungus *Verticillium lecanii* (Zimmerman) viegas which originally isolated from Alaska, USA and EMCC Number: 919™ and obtained from the Egyptian microbial culture collection, ain-shams univ., three methods were evaluated (liquid state, solid state and diphasic system). In the liquid state of production, five media were tested such as PDB, PSB, MYB, SYB and PCB. Molasses Yeast Broth (MYB) supported maximum spore production of 9.380x 10^8 spores /ml) and biomass production of 0.89 g/100 ml) with the liquid nitrogen drying technique. The spore production of 8.70x 10^8 spores / ml) and biomass production of 0.69 g/100 ml was obtained with the oven 40-45 °C drying technique. Mean while, the drying technique by liquid nitrogen supported maximum spores germination (96.44%), while the spores germination was (93.44%) in case of using the oven at 40-45 °C drying technique. In the solid state the production on six substrates were tested and these substrates were rice, wheat, corn, barley, rice husk and rice straw. Rice grains supported maximum sporulation (1.97×10^9 spores/g) and the highest biomass production (0.96 g/100g). In diphasic state of production, combination of MYB and rice grains produced the greatest amount of spores, (2.5g/100g) and the highest sporulation (2.17×10^9 spores/g) (Table 6).

4.3.1. Liquid media

Biomass production count is significantly affected by type of media (Table 6) and among five media tested, it was found that MYB produced maximum biomass of 0.69g/100 ml and PSB gave 0.63g/100 ml, followed by PCB, SYB and PDB which produced 0.53, 0.46 and 0.44 g/100 ml, in case of using respectively it was oven – dried at 40-45 °C technique and the

same trend was obtained in the case of the liquid nitrogen-dried technique supporting the highest produced biomass in this media MYB (0.89 g/100 ml while the media of PSB gave 0.69 g/100ml, followed by PCB, SYB and PDB which produced 0.68, 0.61 and 0.59 g/100 ml, respectively. Sporulation is an important factor in mass-production, and therefore, it was found that MYB supported maximum sporulation of 8.7×10^8 spores / ml and 9.38×10^8 spores / ml in the two cases of drying, respectively. The spore's germination percentages indicated that viability of spores had not significant effect on mass- production in all different media tested, although MYB media was higher in its spore germination (96.44%) than other media.

The presented findings are in accordance with those of **Mehta *et al.* (2012)** who reported that among different organic media for mass production of *V. lecanii*, Yeast extract medium had the highest rates of dry weight of biomass of 30.82 g / 250 ml.

Table (6): Effect of different liquid media on biomass, spore production and spore germination

Media	Drying method	Dry weight of biomass (g./100ml)	Spores production (spores/ml)	Spores germination %
PDB	40-45 °C	0.44^{b}	$6.37\text{x } 10^{8\,d}$	93.11^{a}
PSB		0.63^{a}	$6.70\text{x } 10^{8\,cd}$	92.44^{a}
MYB		0.69^{a}	$8.70\text{x } 10^{8\,a}$	93.44^{a}
SYB		0.46^{b}	$7.08\text{x } 10^{8\,c}$	92.11^{ab}
PCB		0.53^{b}	$7.83\text{x } 10^{8\,b}$	90.11^{b}
$LSD_{0.05}$		0.08875	0.52254	2.00456
PDB	Liquid nitrogen	0.59^{d}	$7.130\text{x } 10^{8\,c}$	96.99^{a}
PSB		0.69^{b}	$8.050\text{x } 10^{8}b^{c}$	94.99^{ab}
MYB		0.89^{a}	$9.380\text{x } 10^{8\,a}$	96.44^{ab}
SYB		0.61^{cd}	$8.170\text{x } 10^{8\,bc}$	96.44^{ab}
PCB		0.68^{bc}	$8.620\text{x } 10^{8\,ab}$	94.66^{b}
$LSD_{0.05}$		0.08204	1.04772	1.91294

Derakhshan *et al.* (2008a) evaluated different media for mass production and viability of *V.lecanii* and found the maximum biomass and spore production was observed at MYB media (0.746 g/100 ml and 8.33×10^8 spores/ml). **Farsi *et al.* (2005)** found that among different media tested for mass production of *V.lecanii*, sugar beet molasses had the highest sporulation.

4.3.2. Solid media

In solid state production, four cereal grains (rice, wheat, corn and barley) and two solid agricultural wastes (rice husk and rice straw) were evaluated. Cereal grain was significantly higher than rice husk and rice straw. The maximum spore dust was harvested from rice grain (0.96 g/100g) followed by barley, corn, wheat, rice husk and rice straw (0.93, 0.9, 0.87, 0.5 and 0.48, respectively). Sporulation among different media was significantly affected by the type of the media and it was found that rice supporting of maximum sporulation 1.9×10^9 spores/g. The viability of spores in rice media was higher than other media since the viability reached 96.99% (Table 7).

The afore-mentianed finding is in agreement with those of **Mamta *et al.* (2011)** who evaluated a mass scale cultivation of the entomopathogenic fungus *Nomuraea rileyi.* Among the evaluated grains rice supported the maximum spore production of fungus (5.53 x 10^7spore/g). **Derakhshan *et al.* (2008a)** evaluated different media for mass production and viability of *V.lecanii* and found the maximum biomass and spore production was observed using rice media (1.25 g/100g rice media and 1.97×10^9 spores/g). **Nelson *et al.* (1996)** found that *Beauveria* and *Metarhizium* fungi produced more spores on rice over other substrates. **Amala *et al.* (2012)** also evaluated various solid substrates for the mass multiplication of the fungus,

Paecilomyces lilacinus. Among the evaluated substrates, rice bran recorded the maximum spore count of $4.32x10^8$ spores/ml followed by wheat bran ($3.19x10^8$ spores/ml)

Table (7): Effect of different solid media on biomass, spore production and spore germination of the entomopathogenic fungus *V. lecanii*

Media	Dry weight of biomass (g/100g media)	Spores production (spores/g)	Spores germination %
Rice	0.96[a]	1.97×10^{9} [a]	96.99[a]
Wheat	0.87[a]	1.66×10^{9} [c]	93.66[b]
Corn	0.90[a]	1.92×10^{9} [b]	96.44[a]
Barley	0.93[a]	1.71×10^{9} [c]	90.77[c]
Rice husk	0.48[b]	0.87×10^{9} [d]	87.44[d]
Rice straw	0.50[b]	0.72×10^{9} [e]	81.22[e]
$LSD_{0.05}$	0.140333	0.0614833	2.643966

4.3.3. Diphasic media system

In diphasic system of production, five liquid media and four solid substrates were tested. Combination of MYB + rice, PSB + rice and SYB +rice significantly supported higher biomass production than the other evaluated combined media yielding 2.5, 2.2 and 2.2 g/100g. In a similar trend, the combination of MYB + rice supporting maximum spores production of 2.17×10^9 spores/g and a viability of spores reached 98.22% (Table 8).These finding are in agreement with those of **Santoro *et al.* (2005)** who obtained greatest sporulation when rice grain was combined with liquid media made with crysalid flour or with crysalid flour + potato + dextrose, reaching a yield of 2.7 and 2.8 x 10^{12} /g, respectively.

Different methods were evaluated for the production of *V. lecanii* using liquid and solid substrates and a combination of them (**Derakhshan *et al.*, 2008a and Machado *et al.*, 2010).** These authors observed that the production of conidia using the biphasic culture system with a combination of 4% sugar cane molasses and grain rice provided an increase of 2.43 and

1.16 times of that obtained in either the liquid or solid media, respectively. Present results of mass production of *Verticillium lecanii* revealed that the combination of MYB+ rice supported highest spore production and this was 3.6 and 2.6 times more than that of MYB and rice used alone as liquid and solid state, respectively.

Table (8): Effect of different combined diphase media (liquid + solid) on biomass, spore production and spore germination of *V. lecanii*

Liquid media	Solid Media	Dry weight of biomass (g/100g)	Spores production (spores/g)	Spores germination %
PDB+	R*	2.03^{bcd}	$2.04\times10^{9\ c}$	96.44^{bcde}
	W	1.6^{g}	$1.78\times10^{9\ h}$	91.77^{k}
	C	1.83^{defg}	$1.96\times10^{9\ e}$	96.55^{bcd}
	B	1.66^{g}	$1.93\times10^{9\ de}$	95.22^{efgh}
PSB+	R	2.2^{b}	$2.14\times10^{9\ ab}$	97.66^{ab}
	W	1.73^{fg}	$1.84\times10^{9\ g}$	97.10^{abc}
	C	2.1^{bc}	$2.13\times10^{9\ ab}$	96.11^{cdef}
	B	1.8^{efg}	$1.94\times10^{9\ de}$	94.22^{hi}
MYB+	R	2.5^{a}	$2.17\times10^{9\ a}$	98.22^{a}
	W	1.83^{defg}	$1.95\times10^{9\ d}$	97.33^{abc}
	C	2.2^{b}	$2.10\times10^{9\ b}$	97.33^{abc}
	B	2.06^{bc}	$2.02\times10^{9\ c}$	98.33^{a}
SYB+	R	2.2^{b}	$2.13\times10^{9\ ab}$	97.22^{abc}
	W	1.76^{fg}	$1.90\times10^{9\ ef}$	95.44^{defgh}
	C	2^{bcde}	$2.05\times10^{9\ c}$	96.22^{cdef}
	B	1.9^{cdef}	$1.88\times10^{9\ f}$	93.88^{ij}
PCB+	R	2.03^{bcd}	$2.12\times10^{9\ b}$	95.55^{defg}
	W	1.66^{g}	$1.93\times10^{9\ de}$	92.88^{jk}
	C	1.8^{efg}	$2.12\times10^{9\ b}$	95.11^{fghi}
	B	1.63^{g}	$2.04\times10^{9\ c}$	94.44^{ghi}
$LSD_{0.05}$		0.199849	0.0405895	1.14420

R= rice, W= wheat, C= corn and B= barley

4.5.1. Shelf life and viability of laboratory prepared wettable powder formulation of the entomopathogenic fungi *Verticillium lecanii* (Zimm.) Viegas

The results of shelf life and viability of laboratory prepared wettable powder formulation of the entomopathogenic fungi *Verticillium lecanii* (Zimm.) Viegas are shown in Tables 9 and10. The fungal viability was affected by several factors, including moisture content, method of mass production, culture media, as well as the filler types and the storage temperature of the formulation. Among three levels of moisture content (5.2% 9.7 % 13.4%) evaluated, the results showed that the vitality of fungi was higher at the lower level moisture, where the percentages of germination were 77%, 70.66% and 60.66%, respectively after 180 days of storage on the MYB media and starch as a filler under refrigerator temperature (4-6 °C), while the results of storage under the same afore-mentianed conditions, but at ambient room temperature (30 -35 °C) were 71.33%, 54% and 46.33% , respectively which demonstrates that the low-temperature increases the vitality of fungi and also increases the percentage of germination after long-term storage. When talc powder was used as a filler, the germination percentage of stored spores were 77.33%, 69% and 58.66% under refrigerator conditions (4-6 °C) and 67%,47% and 45% at ambient room temperature (30 -35 °C).

These findings are in accordance with those of Derakhshan *et al.* (2008b) who studied the effect of culture media, storage temperature and moisture content on the viability and virulence of *Verticillium lecanii* to *Brevicryne brassicae.* Among nine media evaluated, Molasses Yeast Broth (MYB) plus2% polyethylene Glycol (PEG) maintained the fungus viability

Table (9): Shelf life and viability of laboratory prepared wettable powder formulation of the entomopathogenic fungi *Verticillium lecanii* (Zimm.) Viegas at ambient room temperature (30 -35 °C)

Initial moisture content	Mass-production media		carrier	Filler	Viability of *Verticillium lecanii* as a W.P formulation (% germination)						
					Days after storage						
					Pre storage	30	60	90	120	150	180
5.2%	Liquid	MYB	Silica gel	Starch	97.00^{abcd}*	88.33^{a}	86.33^{a}	83.33^{a}	74.66^{ab}	72.33^{b}	71.33^{a}
				Talc	95.33^{bcd}	83.66^{bc}	87.33^{a}	79.33^{b}	71.33^{bc}	69.33^{c}	67.00^{b}
	Solid	Rice		Starch	96.33^{abcd}	83.66^{bc}	82.00^{bc}	75.33^{bcd}	70.33^{c}	66.00^{d}	63.00^{c}
				Talc	94.66^{d}	83.00^{bcd}	80.33^{cd}	76.33^{bc}	68.33^{cd}	65.00^{d}	60.00^{d}
	Diphasic	MYB+Rice		Starch	98.66^{a}	88.00^{a}	83.00^{b}	78.66^{b}	77.33^{a}	75.66^{a}	65.00^{bc}
				Talc	98.33^{a}	81.00^{de}	78.33^{de}	76.00^{bc}	72.00^{bc}	71.66^{bc}	63.00^{c}
9.7%	Liquid	MYB		Starch	98.66^{a}	81.66^{cde}	78.00^{e}	69.33^{efg}	62.00^{e}	60.00^{e}	54.00e
				Talc	96.33^{abcd}	79.33^{ef}	73.33^{fg}	64.33^{h}	61.33^{e}	52.33^{hi}	47.00^{fgh}
	Solid	Rice		Starch	97.33^{abcd}	80.66^{de}	76.66^{e}	73.00^{cde}	66.33^{d}	55.33^{fg}	52.33^{e}
				Talc	97.33^{abcd}	77.00^{fg}	74.33^{f}	68.66^{efg}	60.66^{ef}	53.66^{fgh}	48.33^{fg}
	Diphasic	MYB+Rice		Starch	97.66^{abc}	84.33^{b}	81.00^{bc}	71.66^{def}	61.66^{e}	56.00^{f}	54.66^{e}
				Talc	95.33^{bcd}	81.00^{de}	78.66^{de}	72.33^{cde}	62.33^{e}	53.33^{gh}	49.33^{f}
13.4%	Liquid	MYB		Starch	98.66^{a}	84.00^{bc}	76.66^{e}	70.00^{ef}	62.33^{e}	51.66^{hij}	46.33^{gh}
				Talc	97.66^{abc}	78.00^{f}	72.33^{fg}	69.33^{efg}	59.00^{ef}	50.66^{ijk}	45.00^{hi}
	Solid	Rice		Starch	98.00^{ab}	75.33^{g}	74.33^{f}	67.33^{fgh}	57.00^{fg}	48.00^{lm}	45.00^{hi}
				Talc	96.00^{abcd}	75.00^{g}	71.00^{g}	63.33^{h}	59.00^{ef}	46.66^{m}	44.66^{hi}
	Diphasic	MYB+Rice		Starch	96.33^{abcd}	75.33^{g}	71.33^{g}	67.33^{fgh}	55.00^{gf}	49.33^{jki}	44.66^{hi}
				Talc	95.00^{cd}	72.30^{h}	71.33^{g}	65.33^{gh}	52.33^{h}	48.33^{klm}	41.00^{j}
L.S.D					2.4765	2.2299	2.1203	3.8143	3.4645	2.4121	2.6418

* Means fallowed with the same letter(s) are not significantly different.

Table (10): Shelf life and viability of laboratory prepared wettable powder formulation of the entomopathogenic fungi *Verticillium lecanii* (Zimm.) Viegas at refrigerated condition (4-6 °C)

Initial moisture content	Mass-production media		carrier	Filler	Viability of *Verticillium lecanii* as a W.P formulation (% germination)						
					Days after storage						
					Pre storage	30	60	90	120	150	180
5.2%	liquid	MYB	Silica gel	Starch	99.00^{a} *	95.00^{a}	87.66^{a}	83.66^{a}	80.00^{bc}	78.00^{cd}	77.00^{a}
				Talc	94.00^{b}	91.66^{bc}	83.00^{bc}	82.33^{abc}	79.33^{c}	78.00^{cd}	77.33^{a}
	solid	Rice		Starch	96.66^{ab}	89.66^{bcd}	84.66^{b}	84.33^{a}	82.33^{ab}	81.00^{a}	77.00^{a}
				Talc	96.33^{ab}	86.33^{ef}	83.33^{bc}	82.00^{abc}	82.66^{a}	80.33^{ab}	77.66^{a}
	diphasic	MYB+Rice		Starch	94.66^{b}	92.33^{b}	81.00^{cd}	83.33^{ab}	82.66^{a}	81.00^{a}	74.00^{b}
				Talc	97.33^{ab}	89.33^{cd}	85.00^{b}	81.66^{abcd}	80.00^{bc}	78.66^{bc}	72.33^{bc}
9.7%	liquid	MYB		Starch	96.00^{ab}	89.66^{bcd}	81.33^{cd}	80.00^{cde}	80.00^{bc}	76.33^{d}	70.66^{cd}
				Talc	96.66^{ab}	86.00^{efg}	80.00^{de}	80.66^{bcde}	76.00^{de}	73.66^{e}	69.00^{de}
	solid	Rice		Starch	95.33^{ab}	87.33^{de}	97.66^{de}	80.00^{cde}	76.33^{d}	70.00^{f}	67.33^{ef}
				Talc	95.33^{ab}	85.33^{efg}	80.00^{de}	79.00^{de}	74.00^{ef}	69.66^{fg}	66.00^{fg}
	diphasic	MYB+Rice		Starch	94.66^{b}	85.00^{efg}	78.66^{de}	78.00^{ef}	73.33^{f}	70.33^{f}	66.00^{fg}
				Talc	96.00^{ab}	85.33^{efg}	77.33^{e}	76.00^{fg}	71.00^{g}	67.66^{gh}	64.66^{g}
13.4%	liquid	MYB		Starch	94.00^{b}	85.66^{efg}	79.00^{de}	74.00^{g}	68.00^{h}	67.33^{h}	60.66^{h}
				Talc	96.66^{ab}	84.33^{efg}	78.00^{e}	70.00^{h}	63.33^{i}	62.33^{j}	58.66^{i}
	solid	Rice		Starch	97.33^{ab}	83.66^{fgh}	79.33^{de}	71.33^{h}	67.33^{h}	65.00^{i}	59.33^{hi}
				Talc	94.66^{b}	81.33^{hi}	74.33^{f}	68.66^{h}	63.66^{i}	60.00^{k}	56.00^{j}
	diphasic	MYB+Rice		Starch	96.33^{ab}	83.00^{ghi}	74.66^{f}	66.00^{i}	62.66^{i}	58.66^{kl}	58.00^{i}
				Talc	95.33^{ab}	80.33^{i}	74.33^{f}	63.33^{j}	59.00^{j}	56.66^{l}	54.66^{j}
L.S.D					3.2511	2.6520	2.5393	2.6638	2.1954	2.1203	1.7989

* Means fallowed with the same letter(s) are not significantly different.

higher than other media followed by MYB plus 1% PEG and rice powder. Viability in refrigerator temperature was significantly higher than that in room temperature. Among three moisture levels tested, viability at 5 and 10% were on par and were significantly higher than that occurred at 15%. Viability over time decreased and the differences in viability among the three storage times were significant. Storage time and media had significant effect on aphid mortality. Mortality decreased over storage time but the rate of decrease in aphid mortality was less than the rate of decrease of the fungal viability. Concerning the storage temperature and moisture content as main factores affecting the spores viability.

Moore *et al.* (1996) reported that the conidia moisture content needs to be reduced to be around 5% for optimal storage. **Stathers *et al.* (1993)** found that long-term conidial viability is maintained better at low storage temperatures. Also **Hedgecock *et al.* (1995)** found that the conidial viability declined due to high temperatures and high moisture contents.

4.6. Biological performance of bio-pesticide, a mineral oils, botanical and chemical insecticides aganist the mealy bug *Icerya seychellarum* in Citrus trees during the summer season of 2013 at Alexandria governorate as compared with the prepared formulation of *V. lecanii*

In fact the use of biopesticides within the biological control as an element of (IPM) program must prove to be effective and selective against the insect-pests. Therefore, the bioinsecticide must meet the demand of controlling the pest and increasing the yield via the reduction of the pest population.

The different applied groups of control agents were evaluated to show the possibility of involving them within an IPM program for controlling that horticultural main pest: *Icerya seychellarum* (Hemiptera) that attack orange

trees. The evaluated biopesticides were Bio-Catch® 1.15 % (*Verticillium lecanii*) and laboratory prepared wettable powder formulation containing *V. lecanii.* Also, natural products as a Nimbecidine® 0.03% (azadirachtin), chemical insecticides Malathion® 57% E.C and Actara® 25 W.G ([thiamethoxam]) and a mineral oil (Kz oil® 95% E.C) were evaluated.

The performances of these compounds were evaluated under field conditions during the summer season of 2013. The tested insecticides were evaluated on orange *Citrue sinensis* (Family: Rutaceae) to show their effect on *I. seychellarum* which considered as more important, economic and serious insect-pest that effecting yield and yield quality. The demonstrated results in Table (11) and Fig. (4) show the effect of the applied treatments against the mealybug *Icerya seychellarum* population on orange trees. The effect of the tested compounds against *Icerya seychellarum* (Westwood) was determined under field conditions. The statistical analysis showed that there were significant differences among the running treatments.

Table (11): Biological performance of a prepared formulation of *V. lecanii* a bio- pesticide, a mineral oils, botanical and chemical insecticides aganist the mealy bug *Icerya seychellarum* in Citrus trees during the summer season of 2013 at Alexandria governorate: at sunset

Treatments	infestation reduction%			
	Inspection period (weeks after application)			
	1	2	3	4
Bio-catch® *(Verticillium lecanii)* 1.15% W.P	53.00^{b}*	57.61^{d}	63.55^{c}	70.13^{c}
***(Verticillium lecanii)* 2. 5% W.P (prepared formulation)**	54.69^{b}	61.19^{c}	65.41^{c}	71.68^{c}
Actara® 25 W.G (thiamethoxam)	87.74^{a}	83.50^{b}	81.39^{b}	82.02^{b}
Malathion® 57%E.C	89.80^{a}	88.66^{a}	87.47^{a}	87.11^{a}
Nimpecidine® 0.03% E.C (azadirachtin)	41.60^{c}	46.51^{e}	53.99^{d}	61.49^{d}
Kz oil® 95% E.C	90.71^{a}	89.54^{a}	88.37^{a}	88.70^{a}
L S D $_{0.05}$	3.8202	2.6550	2.4416	2.9544

* Means fallowed with the same letter (s) are not significantly different.

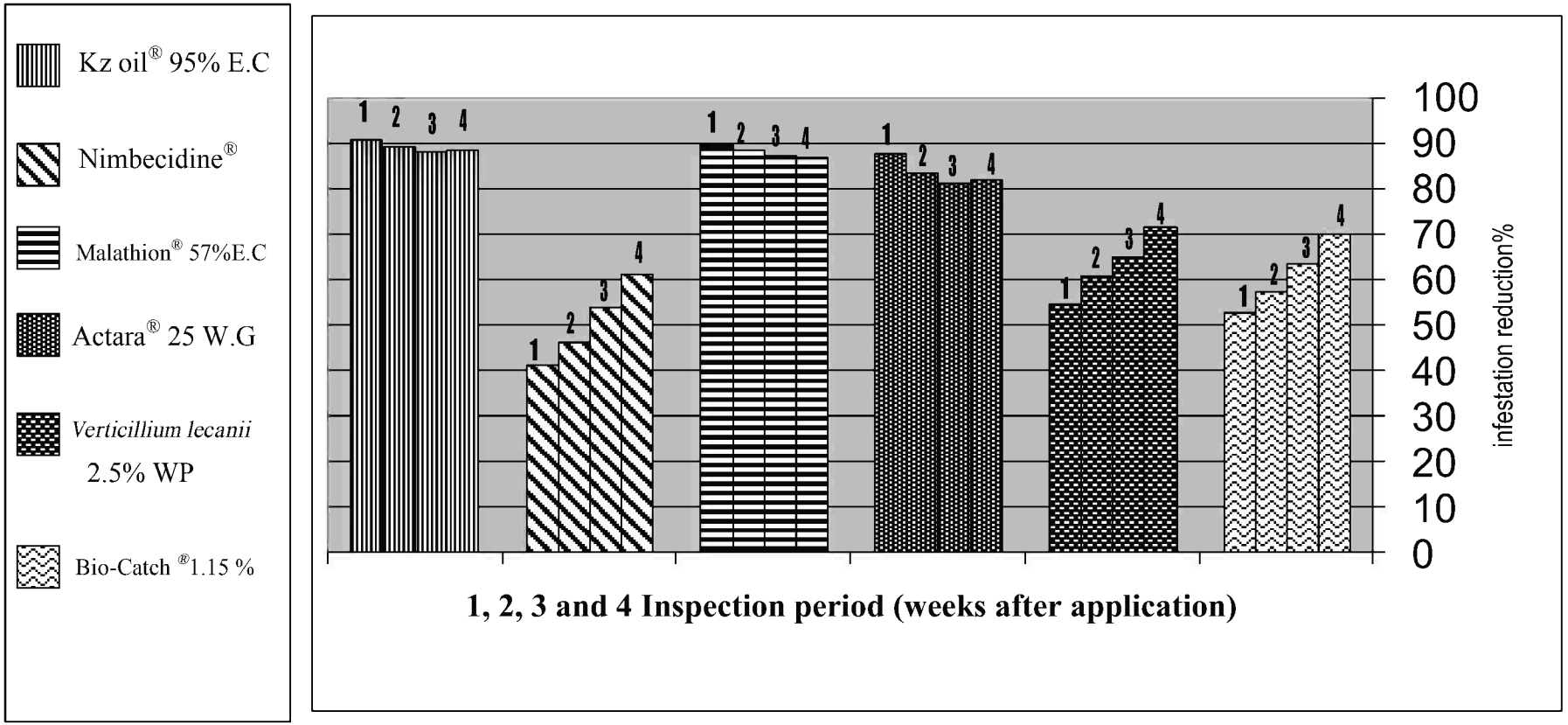

Fig.4: Biological performance of a prepared formulation of *V. lecanii* a bio- pesticide, a mineral oils, botanical and chemical insecticides aganist the mealy bug *Icerya seychellarum* in Citrus trees during the summer season of 2013 at Alexandria governorate: at sunset

4.6.1. Application at sunset

It is obvious that the mineral oil Kz oil® 95% E.C was the most effective insecticide evaluated against the mealybug *Icerya seychellarum (*Westwood) followed by Malathion® 57%E.C, actara® 25 W.G (thiamethoxam), a laboratory prepared wettable powder formulation, Bio-Catch® 1.15 % (*Verticillium lecanii*) and Nimbecidine® giving initial insect reduction of 90.71%, 89.80%, 87.74%, 54.69%, 53% and 41.60%, respectively, after one week post-application at sunset.

Regarding the biological performance of the evaluated treatments on infestation reduction percentages after two weeks post treatment, the same trend was observed. Kz oil® 95% E.C exhibited high reduction percentage estimated by 89.54% followed by Malathion® 57%E.C (88.66%), Actara® 25 W.G (83.5%), laboratory prepared wettable powder formulation (61.19%), Bio-Catch®1.15 % (57.61%) and Nimbecidine® (46.51%).

Also, after three weeks post-treatment, the reduction percentage of *I. seychellarum* showed that the superior treatment was Kz oil® 95% E.C recording the highest reduction of infestation of 88.37%, followed by followed by Malathion® 57%E.C (87.47%), Actara® 25 W.G (81.39%), laboratory prepared wettable powder formulation (65.41%), Bio-Catch® 1.15 % (63.55%) and Nimbecidine® (53.99%) which was the least effective compound.

. It is found that Kz oil® 95% E.C was always in the 1 st rank in reducing the insect population all over the period of inspection that lasted for 4 week post-application. The data showed that Kz oil® 95% E.C and Malathion®

57%E.C are significantly more effective than the other evaluated insecticides as they caused higher reduction percentages of 88.70 and 87.11%, respectively after 4 weeks post- treatment followed by Actara® 25 W.G which caused 82.02% reduction. There were no significant differences between Bio-catch® and the laboratory prepared wettable powder formulation while both showing the same value of reduction 70.13 and 71.68%, respectively recorded at four weeks after application but the lowest reduction of 61.49% was achieved by Nimpecidine® (Azadirachtin). These results showed that the microbial evaluated insecticides had high residual effect that lasts for four weeks after application.

4.6.2. Application in the morning:

It is obvious from the results presented in Table (12) that the mineral oil Kz oil® 95% E.C was the most effective evaluated insecticide against the mealybug *Icerya seychellarum* (Westwood) followed by Malathion® 57%E.C and Actara® 25 W.G (thiamethoxam), laboratory prepared wettable powder formulation, Nimbecidine® and Bio-Catch® 1.15 % (*Verticillium lecanii*) giving initial reduction of target insect of 91.58%, 91.41%, 85.05%, 47.25%, 40.73% and 39.43%, respectively after one week post-application (in the morning).

Regarding the biological performance of the tested materials on infestation reduction percentages after two weeks post treatment, the same trend was observed. Kz oil® 95% E.C exhibited high reduction percentage estimated by 90.41% followed by Malathion® 57%E.C (90.18%), Actara® 25 W.G (83.39%), Nimbecidine® (51.13%), laboratory prepared wettable powder formulation (44.67%) and Bio-Catch® 1.15 % (43.98%).

Also, after three weeks post-treatments, the reduction percentage of *I. seychellarum* showed that the superior treatment was Kz oil® 95% E.C recording a reduction of infestation of 89.14%, followed by Malathion® 57%E.C (88.51%), Actara® 25 W.G (82.31%), laboratory prepared wettable powder formulation (54.67%), Bio-Catch® 1.15 % (52.97%) and Nimbecidine® (51.76%).

It is also found that Kz oil® 95% E.C was always in the 1 st rank in reducing the insect population all over the period of inspection that lasted for 4 week post-application.The data showed that Kz oil® 95% E.C and Malathion® 57%E.C are significantly more effective than the other tested insecticides as they caused reduction percentages of 88.61 and 88.25%, respectively, followed by Actara® 25 W.G which caused 80.78% reduction. There were significant differences between Bio-catch® and laboratory prepared wettable powder formulation where caused reduction of 60.88 and 66.86%, respectively four weeks after application, but the lowest reduction of 57.06% was recorded by Nimpecidine® (Azadirachtin). These results showed that the microbial insecticides had high residual effects and lasted for four weeks after application showing high effect.

As a comparison between the two times of application (at sunsed and in the morning), the data presented in Tables (11and12) and Fig. (4 and 5) showed that the commercial microbial insecticide and prepared formulation of *V. lecanii* applied at sunset [Bio-catch® *(Verticillium lecanii)*] were more effected than other applications in the morning and they caused insect reduction percentage of 70.13 and 60.88%, respectively. In the same trend, the reduction percentage caused by laboratory prepared wettable powder

formulation applied either at sunset or in the morning were 71.68 and 66.86%, respectively after four weeks post-treatment.

These findings are in accordance with those of **Mangoud and Abd El-Gawad (2003)** who tested two spraying methods (oriented and whole tree spraying) to control the Egyptian fluted mealybug, *Icerya aegyptiaca* (Douglas) (Homoptera : Margarodidae) in August 2002. Biofly® and oil gave poor average reduction in the infestation against adult females (41.6% and 49.1%, respectively), while NeemAzal® and Sulphur gave moderate effect (64.1% and 60.7%, respectively). Malathion and Malathion + Oil gave good reduction (85.1% and 91.4%, respectively). Biofly®, NeemAzal®, oil and Sulphur gave moderate average reduction against nymphs (67.5%, 69.3%, 71.5% and 71.0%, respectively), while Malathion and Malathion + oil gave good average reduction (93.9% and 91.9%, respectively).

Also, **Mohammad *et al.* (2010)** reported that the pink hibiscus mealybug *Maconellicoccus hirsutus* is a serious pest on the ornamental plant *Hibiscus rosa sinensis* at Ismailia governorate. So, the comparative effects of six commonly used insecticides [four nonconventional pesticides; Biofly®, Biovar®, Bioranza® and Orange oil® and two conventional pesticides Admiral® (pyriproxyfen) and Cidial® (phenthoate)] were evaluated against that mealybug. They were applied under field conditions on the population(s) of the pink hibiscus mealybug, *Maconellicoccs hirsutus* (Green). Results showed that the lower reduction values were recorded by Biovar®, Bioranza®, Admiral® and Orange oil® on the mealybug population(s). The highest effect was occurred by Cidial treatment.

Table (12): Biological performance of a prepared formulation of V. lecanii a bio- pesticide, a mineral oil, botanical and chemical insecticides aganist the mealy bug *Icerya seychellarum* in Citrus trees during the summer season of 2013 at Alexandria governorate in the morning

Treatments	infestation reduction%			
	Inspection period (weeks after application)			
	1	2	3	4
Bio-catch® *(Verticillium lecanii)* 1.15% W.P	39.43^{d}*	43.98^{d}	52.97^{c}	60.88^{d}
***(Verticillium lecanii)* 2. 5% W.P (prepared formulation)**	40.73^{d}	44.67^{d}	54.67^{c}	66.86^{c}
Actara® 25 W.G (thiamethoxam)	85.05^{b}	83.39^{b}	82.31^{b}	80.78^{b}
Malathion® 57%E.C	91.41^{a}	90.18^{a}	88.51^{a}	88.25^{a}
Nimpecidine® 0.03% E.C (azadirachtin)	47.25^{c}	51.13^{c}	51.76^{c}	57.06^{d}
Kz oil® 95% E.C	91.58^{a}	90.41^{a}	89.14^{a}	88.61^{a}
L S D $_{0.05}$	3.1635	2.6602	3.8845	4.2139

* **Means fallowed with the same letter (s) are not significantly different.**

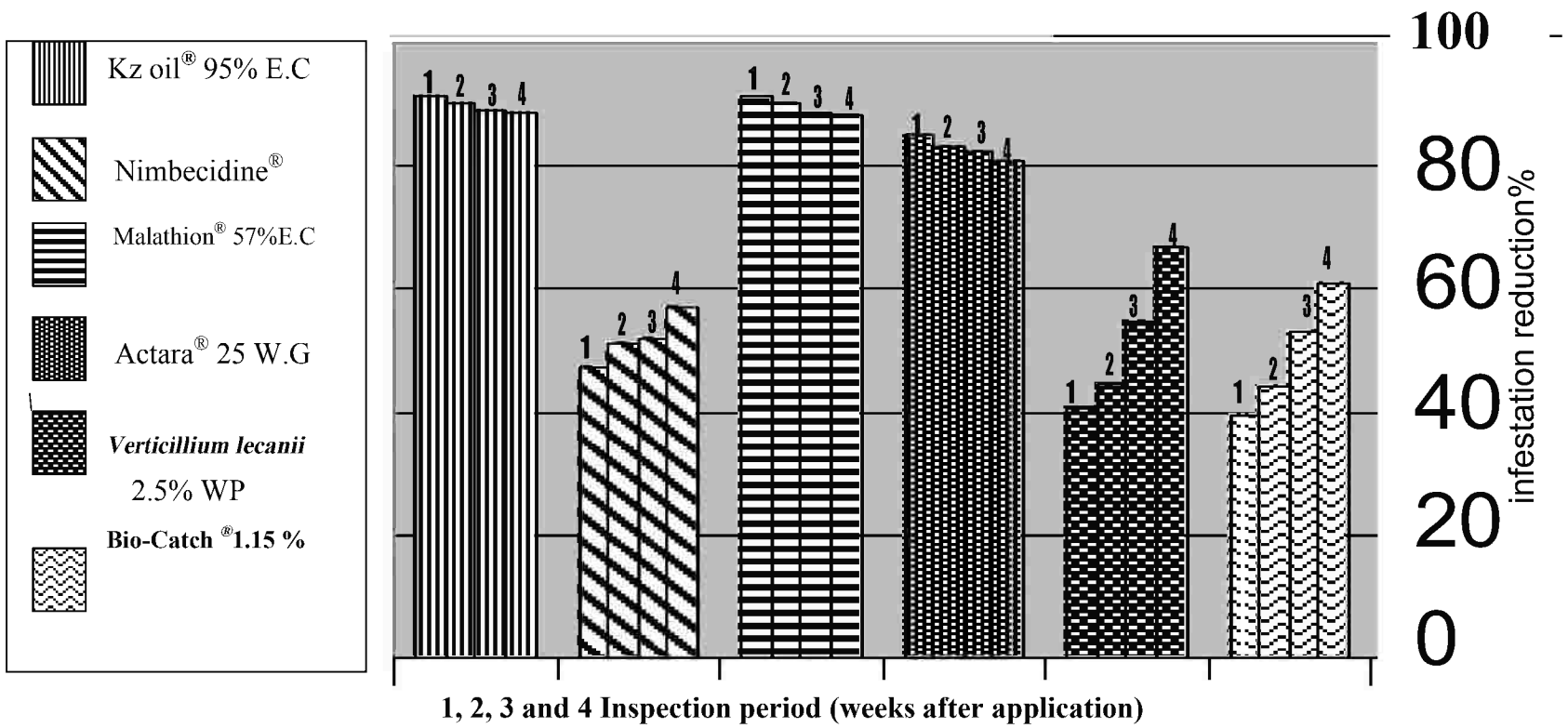

Fig.5: Biological performance of a prepared formulation of V. lecanii a bio- pesticide, a mineral oil, botanical and chemical insecticides aganist the mealy bug *Icerya seychellarum* in Citrus trees during the summer season of 2013 at Alexandria governorate in the morning

Mangoud *et al.* (2012) studied the relative toxicity of different compounds against the seychellarum mealybug, *Icerya seychellarum* (Westwood) (Homoptera: Margarodidae) and the vedalia beetle, *Rodalia cardinalis* (Mulsant) (Coleoptera: Coccinellidae) on mango leaves under laboratory conditions. The obtained data indicated that different compounds (Biofly®, NeemAzal® and Super Mesrona oil®) gave medium effects on nymphs, and adult females of *I. seychellarum* and immature and mature stages of their predator *R.cardinalis*, compared with Malathion which gave highly effects on the mealybug and its predator using direct exposure technique.