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Entomopathogenic fungus, *Lecanicillium lecanii* R. Z are & W. Gams anchored into MCM-41: A new and effective bio-insecticide against *Brevicoryne brassicae* (Linnaeus, 1758) (Hom: Aphididae) to protect cabbages

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Entomopathogenic fungus, *Lecanicillium lecanii* R. Z are & W. Gams anchored into MCM-41: A new and effective bio-insecticide against *Brevicoryne brassicae* (Linnaeus, 1758) (Hom: Aphididae) to protect cabbages

Abstract: *Brevicoryne brassicae* is a significant pest of cultivated cabbages and vegetable crops in the world. The present study was carried out to examine a potential strategy to enhance the insecticidal activity of *Lecanicillium lecanii* for cost-effective management of *B. brassicae*. The insecticidal efficacy of pure entomopathogenic fungus (PEF) and MCM-41 (Mobil Composition of Matter) *L. lecanii* were assessed against the cabbage aphid under laboratory and greenhouse conditions. The fungus was supported on MCM-41 and was completely characterized by Scanning Electron Microscope (SEM), thermogravimetric analysis (TGA) and Fourier transform infrared (FT-IR) techniques. LC_{50} values of PEF and MCM-41@fungus were 1.9×10^6 and 2.5×10^4 and 2.0×10^7 and 2.0×10^5 conidia/ml on adults of *B. brassicae* under laboratory and greenhouse conditions, respectively. Bioassays demonstrated that MCM-41@fungus significantly decreased LC_{50} values of entomopathogenic fungus and it was more toxic than *L. lecanii* at adult stage of the pest. The results showed that pure *L. lecanii* and its nano-formulation could play key roles as bio-pesticides in *B. brassicae* management programs.

Key words: *Brevicoryne brassicae*; *Lecanicillium lecanii*; MCM-41@fungus; virulence

Entomopatogena gliva, *Lecanicillium lecanii* R. Zare & W. Gams, vključena v MCM-41: Novi učinkoviti bio-insekticid za zatiranje mokaste kapusove uši (*Brevicoryne brassicae* (Linnaeus, 1758) (Hom: Aphididae)) pri zaščiti zelja

Izvleček: Mokasta kapusova uš (*Brevicoryne brassicae*) je pomemben škodljivec zelja in drugih zelenjadnic širom po svetu. Raziskava je bila izvedena za preučitev potencialne strategije povečanja insekticidne aktivnosti glive *Lecanicillium lecanii* za učinkovito in poceni zatiranje mokaste kapusove uši. Insekticidna učinkovitost čistega pripravka entomopatogene glive (PEF) in njene vključitve v MCM-41 (Mobil Composition of Matter)@*L. lecanii*) je bila ocenjena na kapusovi mokasti uši v laboratoriju in v rastlinjaku. Gliva, ki je bila vključena v MCM-41, je bila podrobno opisana z vrstičnim elektronskim mikroskopom (SEM), termogravimetrično analizo (TGA) in Fourierjevo transformacijsko unfrardečo tehniko (FT-IR). LC_{50} vrednosti za odrasle osebke mokaste kapusove uši so bile za PEF in MCM-41@gliva 1,9 × 106 in 2,5 × 104 ter 2,0 × 107 in 2,0 × 105 konidijev/ml v laboratoriju, oziroma rastlinjaku. Biotest je pokazal, da je kombinacija MCM-41@gliva značilno zmanjšala LC_{50} vrednosti entomopatogene glive in, da je bila bolj toksična za odrasle uši kot gliva sama. Rezultati so pokazali, da lahko imajo čiste kulture glive *L. lecanii* in njeni nano pripravki ključno vlogo kot biopesticidi v programih biološkega uravnavanja mokaste kapusove uši.

Ključne besede: *Brevicoryne brassicae*; *Lecanicillium lecanii*; MCM-41@gliva; virulenca

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1 INTRODUCTION

Cabbage (*Brassica oleracea* L. var. *capitata*) is one of the significant vegetables throughout the world such as Iran. Brassica crops are attacked by many species of insect pests that reduce the quantity and quality of them (Neupane, 1999). *Brevicoryne brassicae* (Lineus, 1758), (Hom: Aphididae) is one of the serious pests of crucifer crops throughout the world such as Iran (Mousavi Anzabi et al., 2013). The pest causes severe damage by direct feeding or indirect via transfer of viral diseases that could be seriously led to plant destruction (Abdu-Allah, 2012). Heavily infested plants become covered with a mass of aphids that can finally lead to leaf decay and plant death (Griffin & Williamson, 2012). Today, applying chemical insecticides are still considered the foremost and the most important action to manage insect pests. Nevertheless, relying on chemical insecticides has been resulted in adverse effects on environmental and human health. Abuse of non-selective chemical insecticides can destroy the natural enemies and beneficial organisms and induce problems such as development of pest resistance (Sharma & Gupta, 2009). Therefore, these adverse effects supported the development of alternative pest management tactics in which microbial controls may play principle roles (Collantes et al., 1986; Llanderal-Cázares et al., 1996). Entomopathogenic fungi are efficient microbial control agents of Homopteran pests (Goettel et al., 2008). *Lecanicillium lecanii* R. Zare & W. Gams is an entomopathogenic fungus that its mycelium produces a cyclodepsipeptide toxin called bassianolide (Suzuki et al., 1977; Kanaoka et al., 1978). Insect mortality is caused by secrete of mycotoxins and extreme fungal growth (Burges, 1981). In spite of the potential of entomopathogenic fungi in the pest control, these bio-control agents have some defects (including sensitivity to environmental factors such as moisture, light, and temperature) that have limited their applications in storage, greenhouse and field conditions. So, these problems will be overcome through recent technological advances such as nanotechnology that will permit future use of entomopathogenic fungi in crop production systems and the nano-formulation approaches can improve their efficacy and pathogenicity. The hexagonal array of uniform mesoporous of MCM-41 with particular properties such as exceptionally high surface areas and high pore volume (Rath & Parida, 2011) attracted our interest in applying it in plant protection and *B. brassicae* management. Since it has 1D uniform mesopores, the entomopathogenic fungi can be grafted into the MCM-41. Consequently, we selected MCM-41 as a support which belongs to the M41S family that mainly made up of silica, SiO_2 (Rath & Parida, 2011; Shylesh & Singh, 2005). Silica has unique benefits as a support like extraordinary thermal and chemical stability, ease of handling, and abundance of exposed silanol (Si-OH) groups (Abdollahi-Alibeik & Pouriayevali, 2012).

2 MATERIALS AND METHODS

2.1 INSECT REARING

Adults of *B. brassicae* were collected from the cabbage fields in West Azarbaijan Province, Urmia, Iran and then transferred to cabbage plants var. capitata growing on a mixture of soil, sand and manure in plastic plant pots in the greenhouse at 22 ± 2 °C, 70 ± 5 % RH and a photoperiod of 12 L: 12 D. These colonies were rearing on cabbages for 3-4 generations. Cabbages were replaced with new 4-7 leaf stage cabbage plants at every eight days. Trials were carried out with cohorts of apterous adults. Seedlings used for producing leaf disks for entomopathogenic fungus bioassays in Petri dishes.

2.2 MICROORGANISM AND CULTURE MEDIA

The entomopathogenic fungus, *Lecanicillium lecanii* (IRAN229) was provided from Mycology collections of Urmia University, Urmia, Iran. Fungal species was cultured in potato dextrose agar (PDA) at 25 ± 1 °C. After 2 weeks (enough growth and sporulation of fungus), conidia were scrapped to make an aqueous suspension with 0.02 % Tween-80. The conidia suspension was filtered through three layers of sterile cheesecloth to remove mycelium and was enumerated with an improved Neubauer haemacytometer (Weber Scientific International Ltd., United Kingdom). Before the bioassay tests, spore viability percentage was determined by inoculating plates of PDA with the suspension. After 24 h, the germination rate was observed under a light microscope. Germination was considered positive when the length of germ tube was as long as the width of the conidia (Hall, 1981). The germination rate of *L. lecanii* conidia was 97 %.

2.3 NANO STRUCTURE ANALYSIS

The particle morphology was surveyed by a scanning electron microscope (SEM) (Day Petronic Company, Tehran, Iran), using FESEM-TESCAN MIRA3. The thermo gravimetric analysis (TGA) curves were recorded on a Shimadzu DTG-60 instrument (University of Kurdistan, Sanandaj, Iran). Fourier transform infra-

red (FT-IR) spectra were recorded with KBr pellets on a Nexus 670 FT-IR spectrometer (Medical Sciences of Urmia University, Urmia, Iran).

2.4 PREPARATION OF SILICEOUS MCM-41

Mesoporous Si-MCM-41 was synthesized through Sol-gel tactic according to Cai et al., (2001). The synthesis procedure of Si-MCM-41 was as follow: In a typical procedure, to a solution containing 480 ml deionized water and 3.5 ml NaOH (2M) which was stirred at 80 ˚C, 1.0 g (2.74 mmol) of surfactant cetyltrimethylammonium bromide (CTAB) was added, when the solution became homogeneous, 5 ml of TEOS was slowly added dropwise into the solution, giving rise to a white slurry. The resulting mixture was refluxed for 2h at the same temperature under continuous stirring. The collected product was filtered, washed with deionized water and dried in an oven at 70˚C followed by calcination at 550˚C for 5 h with a ramp 2˚C min^{-1} to remove the residual surfactants. Finally, we obtained the mesoporous Si-MCM-41.

2.5 PREPARATION OF MCM-41-ENTOMOPATHOGENIC FUNGUS

In a 100 ml round bottom flask, a mixture of MCM-41-Cl (1 g), entomopathogenic fungus (10 ml of determined concentrations) and Et_3N (1.5 ml) in H_2O (50 ml) were stirred under room temperature for 24 h. Then, the final product was dried at room temperature (Abdollahi-Alibeik & Pouriayevali, 2012).

2.6 BIOASSAYS

2.6.1 Aphid-dipping in the laboratory and greenhouse conditions

The fresh leaves used in aphid-dipping under laboratory conditions came from cabbages grown in the research field of Urmia University, Urmia, Iran. For greenhouse trials, cabbages were grown in pots under greenhouse conditions.

To investigate adult sensitivity, each one-day-old aphid was dipped in spore suspensions of pure *L. lecanii* and MCM-41@fungus treatments separately under laboratory and greenhouse conditions that determined by preliminary dose setting experiments. The concentration ranges for PEF and MCM-41@fungus in laboratory and greenhouse conditions were 10^4-10^8 and 10^2-10^6 and 10^5-10^9 and 10^3-10^7conidia/ml, respectively. For each trial, adults were dipped in the spore suspensions and MCM-41@fungus treatments separately. For controls, the aphids were immersed in 0.02 % Tween-80 aqueous solution. In the laboratory, when the water had evaporated and the aphids were dry, they were transferred into Petri dishes with fresh cabbage leaves kept at 22 ± 2 °C, 70 ± 5 % RH and a photoperiod of 12 L: 12 D. In the greenhouse, aphids that were immersed in the spore suspensions of PEF and MCM-41@fungus treatments separately were placed on fresh cabbage leaves. Mortality was recorded after 7 days. There were three replicates of 20 adults per fungal isolate, MCM-41@fungus and control. In MCM-41@fungus experiments, 0.1 g of each nanocomposite was dispersed in 100 ml distilled water containing 0.02 % Tween-80 until water absorbance was stabilized. After shaking and product dispersion, adult

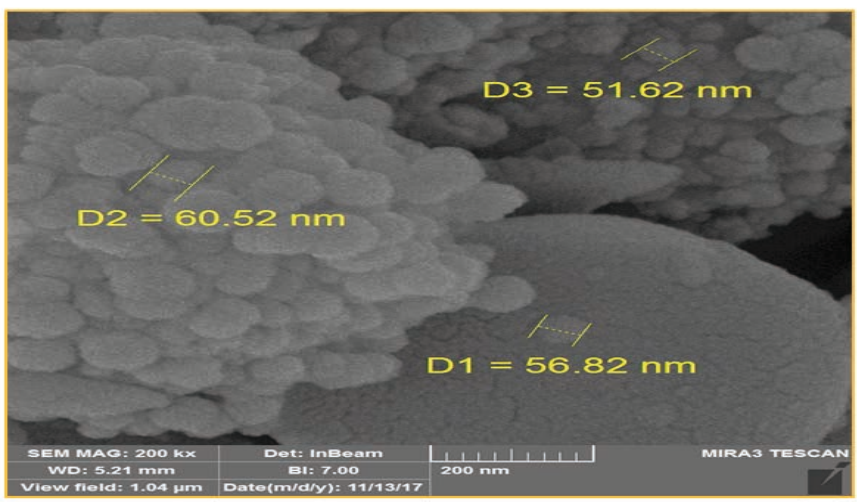

Figure 1: SEM image of MCM-41@fungus

aphids were dipped in the corresponding solutions for 10 s.

2.7 DATA ANALYSIS

In order to determine LC_{50} values, the data were analyzed utilizing the probit procedures with SPSS for Windows® release 24.

3 RESULTS

The Scanning Electron Microscope analysis is an important device for the analysis of the surface morphology of a mesoporous structure. The SEM micrograph of MCM-41@fungus mesoporous is shown in Fig.1. Notably, the particles observe to be in nano range (50-60 nm). The morphology of the mesoporous demonstrates homogeneous, regular, and spherical morphology.

Thermogravimetric analysis (TGA) was employed to select the mass changes of functionalized mesoporous MCM-41.TGA curves for MCM-41 (a) and MCM-41@fungus (b) are depicted in Fig.2. According to the TGA curve b, an initial mass loss was seen at temperatures below 200 °C because of the removal of physically and chemically adsorbed water molecules inside the pores channels and surface hydroxyl groups. Additionally, at temperatures above 200 °C, large mass losses were occurred which were mainly due to the decomposition of

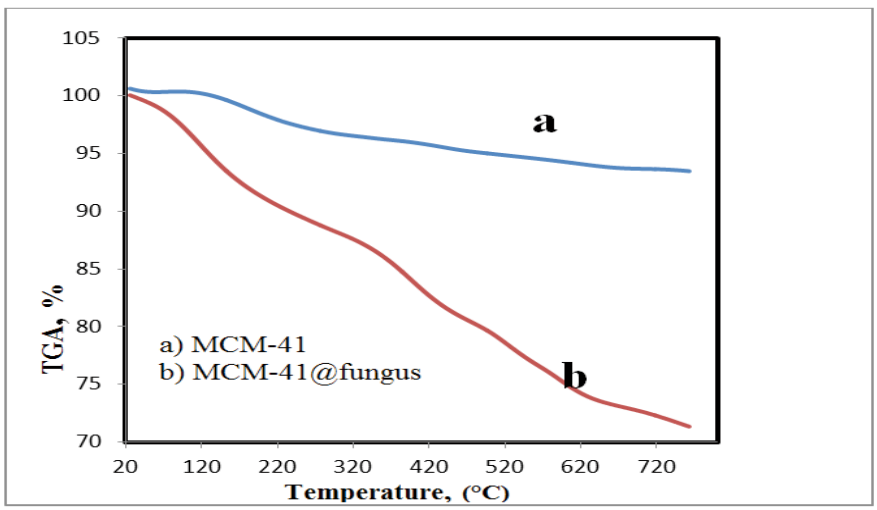

Figure 2: TGA thermograms of MCM-41(a) and MCM-41@fungus (b)

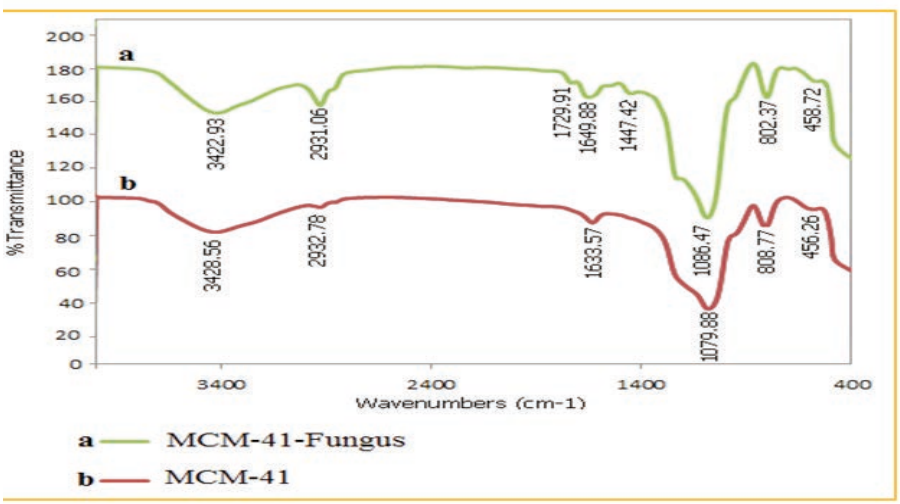

Figure 3: FT-IR spectra of MCM-41@fungus (a) and MCM-41 (b)

Table 1: Toxicity of *Lecanicillium lecanii* and MCM-41@fungus to one-day-old adults of *Brevicoryne brassicae* under laboratory and greenhouse conditions

Experimental conditions	Treatments	Slope ± S. E.	χ^2 (df)	LC_{50} (conidia/ml)	LC_{90} (conidia/ml)
Laboratory	PEF	3.84 ± 0.04	1.58 (3)	1.9×10^6 (6.5×10^5-6.5×10^6)	2.0×10^{10} (1.4×10^9-3.3×10^{12})
	MCM-41@fungus	4.80 ± 0.05	1.53 (3)	2.5×10^4 (9.3×10^3-8.3×10^4)	1.4×10^8 (1.2×10^7- 1.4×10^{10})
Greenhouse	PEF	3.60 ± 0.03	1.26 (3)	2.0×10^7 (7.1×10^6- 6.6×10^7)	1.5×10^{11} (1.2×10^{10}- 1.9×10^{13})
	MCM-41@fungus	4.52 ± 0.05	1.22 (3)	2.0×10^5 (7.8×10^4-6.1×10^5)	8.4×10^8 (8.8×10^7-5.1×10^{10})

PEF: Pure Entomopathogenic Fungus (= non-formulated fungus), 95 % fiducial limit (FL) is shown in parenthesis

covalently bonded organics (200-500 °C) and silanol groups (> 500 °C). This showed that the grafting of fungus had occurred on the inner pore channels of Si-MCM-41.

As shown in Figure 3, curve a, demonstrates stretching vibrations at 1447 cm^{-1} (C-N), 1729 cm^{-1}(C=O) and a broad band at around 3422 cm^{-1} (O-H and N-H) which is related to the fungus wall. Curve b, displays the absorption band at 3428 cm^{-1} is attributed to the stretching vibration of the O-H groups. The bands at 1079, 808 and 456 cm^{-1} are attributed to the symmetric and asymmetric stretching vibrations of the mesoporous framework (Si-O-Si). By comparing the FT-IR spectrums of MCM-41@fungus (a) and MCM-41 (b) in figure 3, the presence of fungus surrounding the MCM-41 can be clearly acclaimed.

LC_{50} values of PEF and MCM-41@fungus were 1.9×10^6 and 2.5×10^4 conidia/ml on adults under laboratory conditions and 2.0×10^7 and 2.0×10^5 conidia/ml in greenhouse conditions, respectively (Table 1). It is obvious that there is a difference between PEF and MCM-41@fungus, as inferred by the confidence limits of LC_{50} values (Table 1).

4 DISCUSSION

MCM-41 has attracted great attention because of its large pore size, high thermal stability and extremely high surface area especially above 1000 $m^2 g^{-1}$ (Nikoorazm et al., 2014). The exceptionally high internal surface area, porous structure and uniformity of MCM-41 make it as a cost-efficient host material for a variety of supported catalysts (Nikoorazm et al., 2014). In this study, fungus was grafted into MCM-41 which led to the synthesis of MCM-41@fungus. During the synthesis, MCM-41 was functionalized with the fungus. Functionalization of MCM-41 was performed by the hydroxyl group of entomopathogenic fungus. Some studies have revealed the efficacy of *L. lecanii* in pest control (Ghaffari et al., 2017; Alavo, 2015; Schreiter et al., 1994; Ramanujam et al., 2017). Our findings confirm *L. lecanii* pathogenicity against adults of *B. brassicae* so it will be an efficient candidate for integrated *B. brassicae* management strategies. According to LC_{50} values, a significant reduction was seen in the amount of entomopathogenic fungus found in the formulated ones. LC_{50} values of PEF and MCM-41@fungus were 1.9×10^6 and 2.5×10^4 conidia/ml on adults of *B. brassicae* under laboratory and 2.0×10^7 and 2.0×10^5 conidia/ml in greenhouse conditions, respectively. Bioassays demonstrated that nano-formulated *L. lecanii* significantly decreased LC_{50} values of fungus and it was more efficient than non-formulated one at adult stage of the pest under laboratory and greenhouse conditions. These results were consistent with the results of Sabbour (2014 b) that studied the efficacy of nano-destruxin of *M. anisopliae* (Metchnikoff) Sorokin (against adult females of *Hetiracris littoralis* Rambur under laboratory conditions. She demonstrated that in nano-formulated samples, LC_{50} values were significantly decreased. The toxicity bioassays of PEF and MCM-41@fungus indicated that adult stage was more sensitive to nano-formulated *L. lecanii* than pure entomopathogenic fungus (i.e. MCM-41@fungus exhibited lower LC_{50} value compared with PEF). Based on our findings, *B. brassicae* adults were more susceptible to infection by MCM-41@fungus than PEF. Based on our knowledge, this is the first research revealing the susceptibility of *B. brassicae* adults to MCM-41@entomopathogenic fungus. The experimental protocol followed in the present study allowed us to provide evidence for MCM-41@entomopathogenic fungus infection.

5 CONCLUSION

Finally, laboratory and greenhouse bioassays have clearly exhibited the pathogenicity of MCM-41@*L. leca-*

nii for adults of *B. brassicae*. These bio-control agents should be considered for the development of a new and environmentally compatible approach for cabbage aphid management in terms of preventing *B. brassicae* infestations. Further research is required to determine the biological activities of MCM-41@*L. lecanii* and its persistence after greenhouse and field applications and other factors that may improve its performance against *B. brassicae* throughout its adult stage.

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