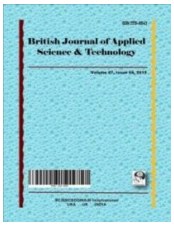

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Mass Production, Yield, Quality, Formulation and Efficacy of Entomopathogenic *Metarhizium anisopliae* Conidia

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Authors' contributions

This work was carried out in collaboration between all authors. Author LI is the principal author of the project, collaborated with all stages. Authors LL and AT contributed in conducting the experiments and laboratory analysis. Author SI contributed in writing and correction of the article. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The aim of this study was to develop an easy but robust method for mass production, to formulate and compare the efficacy of mass produced conidia of a local isolate *Metarhizium anisopliae* against aphids and oil-formulated conidia against whiteflies.
Study Design: The randomized complete block design with replications.
Place and Duration of Study: 2012-2014, Laboratory of Crop Protection Department, Faculty of Agricultural Engineering and Veterinary Medicine, Lebanese University.
Methodology: Isolate of fungal entomopathogen *M. anisopliae* (Metschn.) Sorokin (LIM1) was grown on cooked rice, wheat, vegetable peels and burgul in roasting bags to produce and harvest spore powder. The cultures were dried and total yield of harvested conidia was determined. After harvesting, spores were submitted to quality control to assess concentration, germination, purity, moisture content and pathogenicity against rose aphid, *Macrosiphum rosae* L., mealy plum aphid,

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Hyalopterus pruni and melon aphid, *Aphis gossypii* Glover. Formulated in different oils, conidia were stored at different temperatures for 16 weeks. Oil-formulated conidia were tested against whiteflies under field conditions.

Results: Among the substrates studied, burgul had the highest yield (21 g/100 g substrate). The optimum time for harvesting was 3 weeks. C:N ratio was also the lowest for conidia produced on burgul which caused the highest mortalities and thus appeared the most virulent against all tested insects. Sunflower oil formulated conidia retained viability for at least 16 weeks at room temperature. The preliminary results indicated some but not significant control of whiteflies in the pot experiments.

Conclusion: *M. anisopliae* could be mass produced in large quantities on burgul substrate. The quality of produced inocula as measured by endogenous C:N ratios would be largely affected by substrate used for mass production. The quality would also influence the efficacy against target insects. Mass produced conidia could also be stored as dry or formulated in sunflower oil at room temperature up to 16 weeks. Use of endogenous C:N ratio as quality control indicator for high quality inocula is highly recommended. Use of vegetable peels as potential substrate for mass production combined with a recyclable oil as formulant could be a low cost and environmentally sustainable technology for future mass production of entomogenous fungi.

Keywords: Metarhizium anisopliae; conidia; mass production; C:N ratio; formulation; oils.

1. INTRODUCTION

Most of the vegetable crops in Lebanon are produced in greenhouses. Greenhouse production is more intensive than the open field production allowing rapid spread of problematic pests such as aphids and whiteflies. Their rapid reproductive rates and high resistance to insecticides [1] make them difficult to control. The potential for resistant populations of green aphid *Myzus persicae* (Sulzer) (Hemiptera: Aphididae) and whitefly *Bemisia tabaci* (Genaddius) (Hemiptera: Aleyrodidae) to develop as a result of intensive insecticides use has prompted scientists to conduct studies on integrated pest management (IPM) strategies in which biocontrol may play a significant role. The most promising alternatives to chemical use are the entomopathogenic fungi such as *Beauveria* and *Metarhizium* which kill a number of pest species. Production of adequate quantities of a good quality inoculum is an essential component of any biocontrol strategy. Recent advances in production, formulation and application of biological control agents in the fields and greenhouses have resulted in improvements of mycoinsecticide products such as Mycotal and Ago Biocontrol Verticillium (based on *Verticilium lecanii*), PreFeRal and Pac-Sin (based on *Paecilomyces fumosoroseus*), and BotanyGard, Ago Biocontrol Beauveria, Bea-Sin and Boveril PM (based on *Beauveria bassiana*) [2]. These products have shown the capacity not only to suppress but also to provide a good control of whiteflies and aphids in both greenhouses and field crops [3-8]. However, there are numerous factors that continue to impede the development of commercial products. Strong dependence on environmental conditions, slow action, relatively high cost, negative interactions with commonly used fungicides, poor adulticidal activity, limited shelf life are those limitations that have to be overcome in order to progress the development of strategies for IPM.

Conidia are probably the most appropriate propagules for field use, as they are more infectious units and show greater stability under dry conditions and after application than hyphae or blastospores [9,10]. Herein we report on an investigation into mass production of *M. anisopliae* on solid substrates. The yield, quality, persistence and shelf-life of mass produced conidia are also discussed.

2. MATERIALS AND METHODS

2.1 Mass production

2.1.1 Fungal isolates

Isolate of *M. anisopliae* (Metschn.) Sorokin (LIM1) originally isolated from soils in Lebanon [8] was tested in this study**.** Long term storage of a single spore culture was achieved by freezing conidia in 10% w/w Glycerol at -20°C.

2.1.2 Preparation of inoculums

When required conidia were re-hydrated by suspending in a small volume of sterile water, placed on SDA and incubated at 23±2ºC under

darkness for 14 days. Following incubation conidia were harvested using a spatula and then suspended in 0.03% Tween 80 solution. In order to remove any hyphal fragments the resultant conidial suspension was filtered and used for further experimentation.

2.1.3 Preparation and inoculation of solid substrates

Whole grains of long rice (parboiled), wheat (durum) and fine burgul (a form of whole wheat that has been cleaned, steamed or parboiled, dried, and then ground into grains of several sizes) were tested as solid substrates. In order to mass produce conidia in the shortest period of time, the initial step of the common diphasic liquid–solid fermentation method was eliminated. Instead, the inoculation was done by placing 100 g of each substrate and 80 ml of distilled water (dH_2O) into 36 cm x 15 cm roasting bags (ZEC, China). To reduce substrate contamination, a strip of autoclavable tape (5 cm long) was fixed onto each bag, plugged with cotton wool and sterilized by autoclaving at 121°C, 15 psi for 35 min. After cooling, 2 cm of the tape was peeled off, sterile syringe needle was carefully inserted and 2 ml of the conidial suspension (10^7 conidia ml^{-1}) were injected into each bag.

Upon needle withdrawal the puncture was immediately resealed. Inoculated bags were thoroughly massaged to mix inoculum with the substrate and then placed into plastic containers and incubated at 25°C until the substrates were fully colonized by fungal hyphae. At this point plugs of cotton wool were removed and the content of the bag was kneaded for substrate aeration and maximum sporulation. The experiment was set up as a completely randomized design with a factorial arrangement (4 x 4). The treatments comprised of 4 substrates and 4 different times of harvest, 1, 2, 3 and 4 weeks post inoculation and incubation. Each treatment combination was replicated five times and repeated twice.

2.2 Effect of Mass Production on Conidial Yields

The conidia were harvested at weekly intervals in order to estimate the optimal harvesting period. One g of substrate was drawn from each replicate, placed into 10 ml of 0.03% Tween 80 solution and shaken using a rotary shaker (380 rpm) for 20 min. Spore counts were made and yield estimated using Neubauer haemocytometer. At its maximal production, the bags were removed from the incubator, sides of the bags split open in the middle and kept at fluctuating room temperature of 21±4°C and average relative humidity of 50±5% to allow rapid dehydration of the substrates. After a week of drying the aerial conidia were dislodged from the substrata by gently mixing the grains and then placing them onto 150 µm aperture sieve nested over 106 µm aperture sieve over a collection tray. The set was covered with muslin and manually agitated for approximately 30 min. The resultant powders were collected from the trays and placed into sterile vials, weighed and then dried in silica gel desiccators at 5°C in vacuum for a week. These conidia were also used for quality control assessments, determination of C:N ratios, pathogenicity and shelf-life.

2.3 Effect of Mass Production on Conidial Quality

The quality of the mass produced conidia was assessed for its purity, moisture content, viability and growth.

2.3.1 Purity test and moisture content

Conidial suspension used for yield determination was also assed for its purity using Neubauer haemocytometer and light microscope with phase optics at 400x magnification. Contamination control (CC) and moisture content of produced powder were done according to procedures described in insect pathology manual [11].

2.3.2 Viability and growth tests

Spore germination for each treatment was performed according to Ibrahim et al. [12] before and after desiccation. Briefly, ten µl of conidial suspension in 0.03% Tween 80 (10^5 conidia ml^{-1}) were dispensed onto the center of each of the 10 replicate Petri dishes containing 15 ml of SDA. All plates were sealed with parafilm and incubated in darkness at 25±1°C. After 24 h incubation the incidence of germinated conidia was recorded at 400 magnification using phase-contrast illumination microscope. Total of five hundred conidia (100 conidia per replicate) were examined. Conidia were considered to have germinated when the germ tube of any length was clearly visible. The remaining five replicates were examined every three days over the following 15 days when colony diameters were measured.

2.4 Effect of Mass Production on Conidial C:N Ratios

C:N ratios (carbon and nitrogen composition) from conidia produced on SDA, rice, wheat, vegetable peels and burgul were determined using modified methods of Springer-Klee for organic carbon [13] and Kjeldahl method for total nitrogen [14].

2.4.1 Determination of carbon

Twenty ml $K_2Cr_2O_7$ (0.33 M) and 26 ml conc. H_2SO_4 were added to 0.5 g of desiccated conidia in a glass test tube and left to cool (40-50°C). Tubes were placed in a block digest and heated at 160°C for 10 min. After cooling on icy water bath, the content of each tube was transferred into a 200 ml volumetric flask and the volume was made up to 200 mL with dH_2O. The flasks were shaken to homogenise the solution and then left to settle for 12 h. Titration was done as follows: 20 ml of the solution was mixed with 2 ml of H_3PO_4 (85%) and H_2SO_4 (96%) mixture and 8 drops of indicator in a 100 ml volumetric flask, made up to 100 ml with dH_2O and titrated against F_2SO_4 until colour shifted from violet to pale green. Two control tubes without any sample solution were heated in the block digest and the other two were treated without heating.

2.4.2 Determination of total nitrogen

Each sample of desiccated conidia (0.5 g) together with 3.35 g of catalyst ($K_2SO_4/CuSO_4.5H_2O/Se$ at 100:10:1 ratio) and 10 ml of conc. H_2SO_4 were digested for 30 min at 200°C and then for 1.5 h at 350°C in a fume cabinet. After digestion for each batch of samples at least one blank (no conidia) and one standard ($(NH_4)_2SO_4$) were prepared. Recovery was determined between 95-105%. Distillation was carried out in the distillation unit.

2.5 Effect of Mass Production on Conidial Virulence

2.5.1 Aphids

Mass produced conidia and freshly harvested conidia from SDA cultures were compared for their virulence against three species of aphids. Mixed instar nymphs of rose aphid *Macrosiphum rosae* L. (Hemiptera: Aphididae), melon aphid *Aphis gossypii* Glover (Hemiptera: Aphididae) and mealy plum aphid *Hyalopterus pruni* (Hemiptera: Aphididae) were collected from infested roses grown in the greenhouse, melon plants grown in the open fields and plum trees, respectively. Collected individuals were monitored for symptoms of natural fungal infection or parasitism and only healthy 3rd instars nymphs were used in the pathogenicity assays. For each treatment, a leaf lamina of respective host plant was dipped into 5 ml of the prepared conidial suspension (10^7 conidia ml^{-1}) for 10 sec and the excess removed by placing the leaf on sterile filter paper for 10 min. Each treated leaf then was placed on a moist 9 cm diam. Whatmans filter paper No. 2 in a 9 cm diam. plastic Petri dish. Thirty healthy aphids were placed on the treated leaf lamina and incubated at 25°±2°C with at 16:8 h (Light: Dark) photoperiod. Controls consisted of leaves immersed in 0.03% Tween 80 only. The treatments were replicated 5 times with ten aphids in each replicate. The number of dead aphids was recorded daily over a period of 7 days. In order to confirm that death was due to fungal infection, the cadavers were transferred to Petri dishes lined with moist tissue paper to encourage fungal growth and sporulation.

2.6 Effects of oil Formulations on *M. anisopliae* Conidial Viability and Shelf-life

Conidia of *M. anisopliae* isolate LIM1 obtained from burgul substrate were used in this experiment because they retained high viability after desiccation and were highly virulent against three different species of aphids.

Oil formulations were prepared by suspending 3 mg of desiccated conidia (4.5-5% moisture content) in 10 ml of each: fresh refined sunflower oil (Spinney's own brand, Spinney's supermarket, Beirut, Lebanon), extra virgin olive (home produced, Kfar Hammam, South Lebanon) and reutilized/recycled oil (mixture of vegetable oils previously used for deep frying potato, meat or fish, Zaatar W Zeit restaurant, City Centre, Hazmieh, Beirut, Lebanon) to give a final concentration of 10^9 conidia ml^{-1}. Filtered through sterilised muslin cloth and rested for 3 hr the stock oil suspensions were placed into 20 ml Universal glass bottles and thoroughly mixed for 1 min using vortex mixer (Lab Dancer S42, VWR (IKA), UK). To maintain moisture content between 4.5-5% five dry non-indicating silica gel beads were added to each treatment. All bottles containing oil suspensions and bottles containing 3 mg of pure dry conidia as a standard for

general comparisons were wrapped in foil to prevent any exposure to light. Resultant formulations were stored at 4ºC, room (actual fluctuating) temperatures and 28ºC.

Viability tests were conducted by diluting stock oil formulations with respective oils and by adding 0.03% Tween 80 solution to dry conidia to desired 10^5 conidia ml^{-1}. Resultant dilutions were vortexed for 3 min to obtain homogeneous suspensions. Conidial germination for each treatment was assessed as described above [12] at 24 hr, 1, 4, 6, 9, 12 and 16 weeks.

2.7 Effect of Oil-formulated Conidia on Whiteflies under Field Conditions

Twenty seedlings of cucumber plants *Cucumis sativus* L. (Cucurbitaceae) variety "Hiba" and 20 seedlings of egg plants *Solanum melongena* L. (Solonaceae) variety "Dream" were planted in 35 cm diameter pots. Potted plants were grown under filed conditions until formation of at least 3-5 true leaves and natural infestation by whiteflies *Bemisia tabaci* Gennadius (Homoptera: Aleyrodidae). Five potted plants were randomly assigned to each treatment and density of whiteflies was estimated one day before treatment by picking one middle leaf per plant. Eggs and nymphs of whiteflies were counted in the laboratory using a dissecting microscope. Leaf area was determined and densities of whiteflies were calculated as per cm^2. Treatments were consisting of control 1 (Tween 0.03% only), control 2 (Tween 0.03% + 60% sunflower oil), conidial suspension (Tween 0.03% + dry conidia that has been stored for 16 weeks at 4°C) and emulsified sunflower oil conidial formulation (Tween 0.03% + 60% sunflower oil formulated conidia that has been stored for 16 weeks at 4°C). Each plant was sprayed with 15 ml of respective formulation using hand sprayer. Formulations containing fungal conidia were calibrated to give final concentration of 1 x 10^8 conidia ml^{-1}. Two weeks after treatment, one leaf from each replicate plant was sampled and density of eggs and nymphs was determined. To see if death has occurred and was due to fungal infection, the leaves carrying dead and alive individuals were transferred to Petri dishes lined with moist tissue paper to encourage fungal growth and sporulation. The number of surviving eggs and nymphs was analyzed using analysis of variance (TWO way ANOVA).

2.8 Statistical Analysis

All data were subjected to analysis of variance (TWO way ANOVA) using a statistical software package (SPSS Inc. 2013 v.22). Differences among treatments were compared using Tukey's mean separation test ($P<0.05$).

3. RESULTS

3.1 Effect of Mass Production on Conidial Yields

When isolate of *Metarhizium anisopliae* LIM1 was grown under standard conditions on different solid substrates and harvested at 1, 2, 4 and 6 weeks intervals, significant differences in conidial yield were found between the substrates. Highest total conidial yields (as determined by spore counts using Neubauer haemocytometer) were obtained after 4 weeks for rice, wheat, burgul and vegetable peel substrates (2.9 x 10^9/g, 5.0 x 10^8/g, 4.9 x 10^9/ g and 2.9 x 10^9/g, respectively, Table 1). The spore concentration per gram of substrate was highly variable ($P<0.001$). This concentration was highest for burgul, followed by rice, vegetable peel and then wheat. These results were comparable with the spore powder weights where 16 times more powder was obtained from burgul than from wheat, 4.2 times more than from rice (Table 1). It was observed that after harvesting considerable amount of LIM1 spores still remained on the substrates. Following sieving, these substrates were washed out with 0.03% Tween 80 solution to extract the spores for counting and to calculate the total concentration for each treatment. The extraction of the remaining spores from the cultures showed that a highest number of spores (5.1 x 10^8/g) remained on the rice (Table 1).

3.2 Effect of Mass Production on Conidial Quality

3.2.1 Purity test

Microscopic examination of spore powder extracted from each substrate revealed negligible 0.001% contamination with *Aspergillum* or *Penicillium* spp. only in wheat powder. Burgul culture was pure and free from any microbial or other contaminants. Rice spore powder contained insignificant amount of rice starch.

3.2.2 Viability and growth tests

The viability test of desiccated and non-desiccated conidia was conducted at 24 h only since germination counts at 48 hours were difficult to perform as they were overgrown with mycelia. Overall, the germination for all treatments before desiccation was over 85% and germination after desiccation was over 70%. The highest germination of 99.8% and 95.3% was recorded for conidia produced on burgul before and after desiccation, respectively. The lowest rate of germination was observed for conidia obtained from wheat (86.7% and 70.2%, respectively) (Table 2). The speed of germination was directly reflected in speed of fungal growth, where fastest germinating conidia were also fastest in radial growth (Table 2).

3.3 Effect of Mass Production on Conidial C:N Ratios

Mass produced conidia should always be tested for quality control. One of many attributes of such quality is endogenous C:N ratios. Dry conidia used in our study and grown on different substrates were tested for carbon and nitrogen content. The results obtained were expressed in percentages as a mean of three replicates and then C:N ratio was determined for each treatment. The results in Table 3 illustrate that conidia harvested from burgul contained the lowest amount of carbon (31.2%) and conidia from SDA showed the highest 47.7% quantity of carbon. Nitrogen content, on the other hand, did not significantly differ if grown on different substrates. C:N ratios of conidia harvested from burgul and vegetable peel were significantly lower ($F_{4,14}$=33.186; P=0.001) than those obtained from SDA, rice or wheat (Table 3).

3.4 Effect of Mass Production on Conidial Virulence

Virulence of mass produced inoculum was tested against M. *rosae* and mealy plum aphid *H. pruni* and melon aphid *A. gossypii* (Fig. 2). Dry conidia of LIM1 isolate grown on burgul and rice were more effective against rose aphids killing 100% within 2 days post treatment. Conidia from the same substrates killed 83% and 63% of mealy plum aphids, respectively. The lowest mortality (16.7%) 2 days post treatment was observed for mealy plum aphids treated with inocula from wheat (Fig. 1).

Table 1. Mean yield of aerial conidia produced by *Metarhizium anisopliae* on different solid substrates over 4 weeks period and concentration of conidia after harvesting

Substrate / Weeks	Rice 10^9 ml^{-1}±SE	Wheat 10^9ml^{-1}±SE	Burgul 10^9 ml^{-1}±SE	Vegetable peel 10^9 ml^{-1}±SE*
1	0.77±0.02c	No conidiation	1.5±0.16c	No conidiation
2	1.3±0.11b	0.49±1.43a	2.4±0.03b	2.2±1.11a
3	2.9±0.07a	0.50±0.53a	4.9±0.09a	2.4±0.09a
4	2.4±0.03a	0.48±0.10a	4.8±0.06a	2.8±1.23a
Dry spore powder weight,(g/100g)	5.0±0.09B	1.3±1.11C	20.7±0.03A	-[A]
After harvesting	0.51±0.03A	0.44±0.07B	0.38±0.01C	-

*[A] – not determined; *-standard error (SE); a,b,c - Means of 5 replicates followed by the same small letter in a column are not significantly different according to Tukey's test (P≤0.05); A,B,C - Means of 5 replicates followed by the same capital letter in a row are not significantly different according to Tukey's test (P≤0.05)*

Table 2. Germination (%) after 24 h post inoculation and radial growth (mm) after 2 weeks post inoculation of LIM1 conidia harvested from different substrates

Substrate	Germination (%±SE*) before desiccation	Germination (%±SE) after desiccation	Growth (mm±SE) of desiccated conidia
SDA	89.4±0.11b	81.3±0.09c	80.3±0.33b
Rice	98.2±0.07a	89.5±0.11ab	82.4±0.29b
Wheat	86.7±0.14b	70.2±1.32d	69.7±0.76c
Burgul	99.8±0.02a	95.3±0.03a	86.9±0.91a
Vegetable peel	98.9±0.03a	90.1±0.09a	87.7±0.90a

**-standard error (SE); a,b,c,d - Means of 5 replicates followed by the same small letter in a column are not significantly different according to Tukey's test (P≤0.05)*

However, 100% control of rose and mealy plum aphids were achieved within 7 days post inoculation for all treatments irrespective to culture substrate. Furthermore, more than 80% of dead aphids treated with conidia harvested from burgul, rice and vegetable peel have developed external conidiation on rose aphids' cadavers (Table 4). Rose aphids (Table 4) and mealy plum aphids (Fig. 1) treated with conidia produced on wheat, on the other hand, have shown only 65% and around 16.7% of external mycosis, respectively, suggesting of being less fit and aggressive than conidia from cultures mentioned (Table 4 and Fig. 1).

3.5 Effects of Oil Formulations on *M. anisopliae* Conidial Viability and Shelf-life

Conidial viability test on SDA after 24h of incubation did not present significant differences between formulations resulting in germination values above 90% (data not presented) and indicating that none of oil formulations had any adverse effect on conidial germination. However, the viability of conidia stored in four different oil formulations at three different temperatures during 16 weeks was significantly affected by formulations ($F_{3,359}$ = 113.504, P<0.001), temperature ($F_{2,359}$ = 1897.1, P<0.001) and time of storage ($F_{5,359}$ = 520.494, P<0.001) (Table 5). There were also significant interactions between the main factors: formulations x temperature ($F_{6,359}$ = 24.421, P<0.001), formulations x time of storage ($F_{15,359}$ = 25.520, P<0.001), temperature x time of storage ($F_{10,359}$ = 102.458, P<0.001) and formulations x temperature x time of storage ($F_{30,359}$ = 19.961, P<0.001). Depending on the oil used in the formulations conidial viability gradually declined over time at different rates. Viability of conidia was better at 4ºC than at room and constant 28ºC for all tested formulations. Dry conidia and conidia formulated and stored in different oils at 4ºC for 16 weeks maintained viability above 80%. Olive oil and recycled vegetable oil had negative effect on conidial survival at 28ºC after 12 weeks of storage and after 16 weeks of storage at room temperature (Table 5).

Table 3. Endogenous C:N ratio of LIM1 conidia produced on different substrates

Substrate	C(%)	N (%)	C:N±SE* ratio
SDA	47.74	6.2	7.7±0.01b
Rice	42.52	6.0	7.1±0.04b
Wheat	45.76	6.1	7.5±0.06b
Burgul	31.18	5.5	5.7±0.03a
Vegetable peel	40.93	6.9	5.9±0.01a

-standard error (SE); a,b - Means of 3 replicates followed by the same small letter in a column are not significantly different according to Tukey's test (P≤0.05)

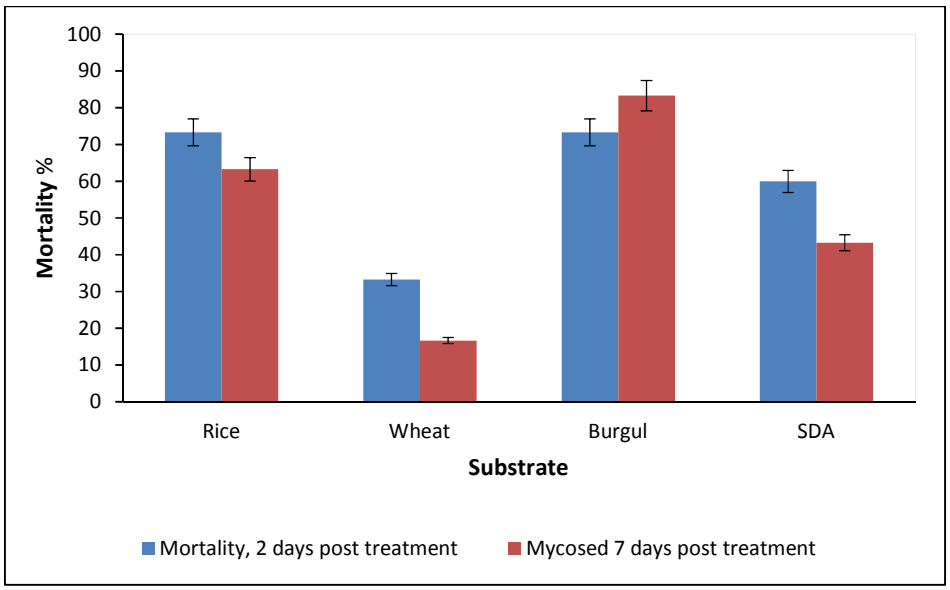

Fig. 1. Effect of dry conidia of *M. anisopliae* LIM1 produced on different substrates against mealy plum aphid *Hyalopterus pruni*. Bars represent average values±SE

Table 4. Virulence of *M. anisopliae* LIM1 conidia produced on different substrates against *Macrosiphum rosae* L.

Substrate	Total mortality (%±SE*) (2 days post treatment, %)	Mycosed (%±SE) (7 days post treatment, %)
SDA	81.6±6.67[b]	73.3 ±2.50[a]
Rice	100.0±0.00[a]	81.7±8.83[a]
Wheat	71.6±8.81[b]	65.0±16.67[a]
Burgul	100.0±0.00[a]	81.7±6.67[a]
Vegetable peel	87.6±11.11[b]	80.1±4.56[a]

-standard error (SE);a,b - Means of 3 replicates followed by the same small letter in a column are not significantly different according to Tukey's test (P≤0.05)

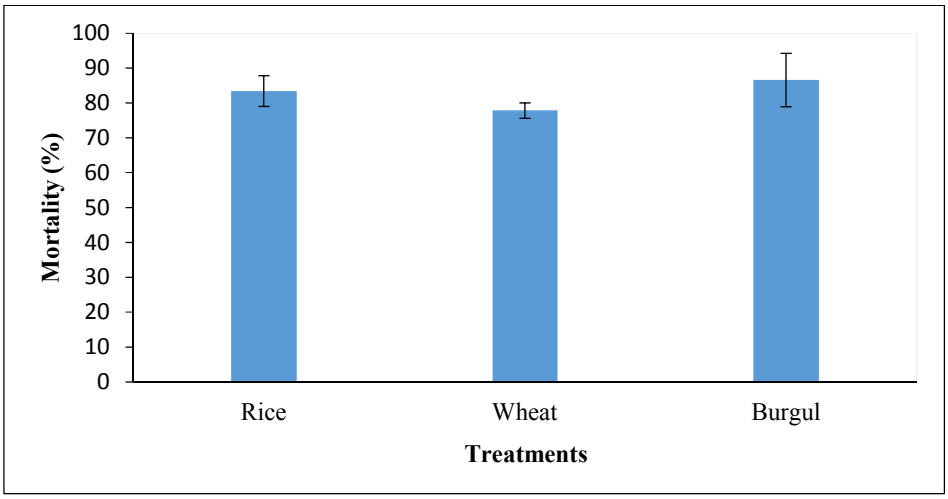

Fig. 2. Effect of dry conidia of *M. anisopliae* LIM1 produced on different substrates against melon aphid *Aphis gossypii* 7 days post-treatment. Bars represent average values±SE

3.6 Effect of Oil-formulated Conidia on Whiteflies under Field Conditions

The carriers used to formulate conidia did not have any effect on whiteflies either on cucumber ($F_{1,39}$=0.0377, P=0.847) nor on egg plants ($F_{1,39}$=1.804,P=0.189) (Figs. 3A and B). The density of whitefly eggs and nymphs determined before treatment was not significantly different between treatments ($F_{3,39}$=1.45, P=0.247 (Fig. 3A) and $F_{3,39}$=1.4, P=0.261 (Fig. 3B), respectively). Two weeks post-treatment densities of eggs and nymphs of whiteflies treated with Tween conidial suspension and oil formulation were slightly but not significantly lower than in both controls on both tested plants $F_{3,39}$=1.503, P=0.232 (Fig. 3A) and $F_{3,39}$=0.884, P=0.840 (Fig. 3B), respectively).

4. DISCUSSION

The data of this study show that burgul substrate was considerably better substrate for mass production of aerial conidia of *M. anisopliae* (LIM1) in terms of yield. Nutritional values of solid substrate would greatly influence the yield of conidia [15,16]. Compared to unenriched white rice, bulgul has more fiber and protein, a lower glycemic index, and higher levels of most vitamins and minerals [17]. In addition, utilisation of solid substrates by microorganisms is affected by many other factors such as particle size, shape, surface-to-volume ratio, crystallinity, and porosity of the substrate, all factors that can influence the accessible surface area to both organism and enzymes [18]. Also, production of conidia of *M. anisopliae* on coarse grain is significantly greater than that on whole grain [19]. Yypsilos [20] reported that burgul wheat supported a higher yield of better quality conidia compared to millet grains because burgul provided a more accessible substrate. This difference was marked in the case of millet where the hard shell of the grains could have considerably prevented the fungus from easy access when small volumes of water were used. This case could be applied to our study where rice and whole wheat grains were used.

In relation to what has been discussed, present study also suggests that the nutritional content of burgul wheat is more favourable for conidial production of higher quality compared to other substrates and that the physical properties of burgul wheat grains allowed the fungus to access more nutrients. In addition, it appears that interaction between culture substrate and moisture availability would also affect the conidial production of *M. anisopliae* [15]. When compared corn, millet and wheat grain with different water volumes it was found that highest conidial production was achieved on wheat at 1:1.5 (substrate : water) ratio [15]. In our study the ratio 1:0.8 was tested.

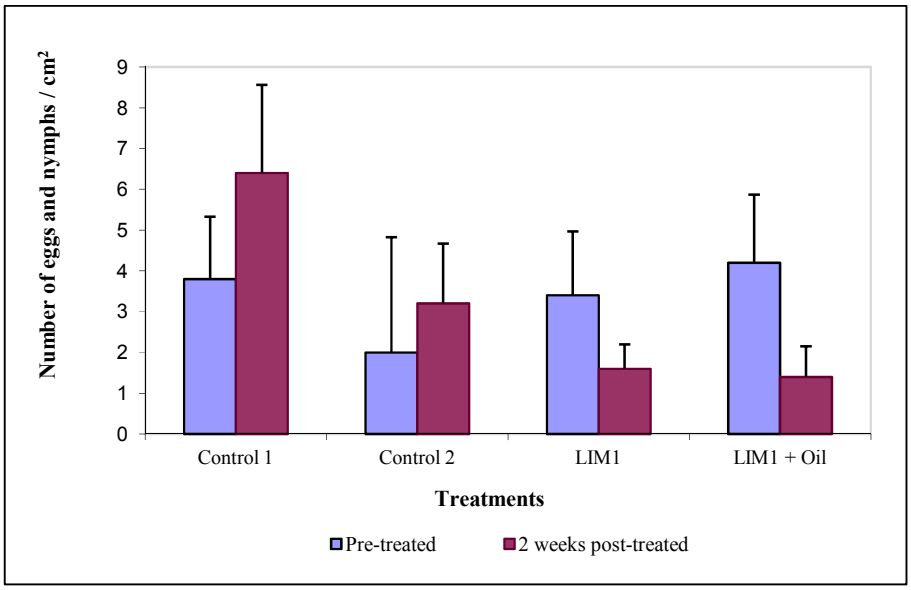

Fig. 3A. Efficacy of oil –formulated conidia of *M. anisopliae* against eggs and nymphs of whiteflies on cucumber plants. Bars represent average values±SE

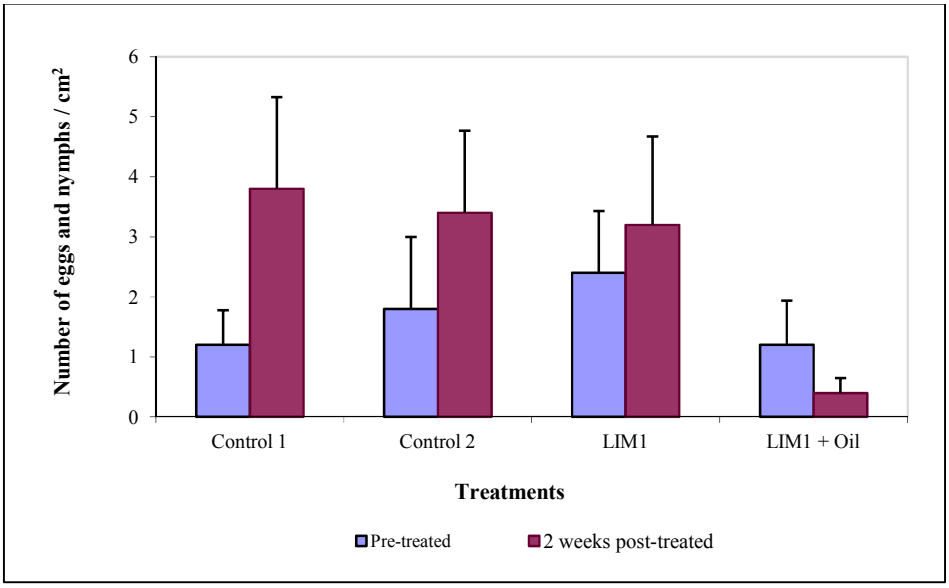

Fig. 3B. Efficacy of oil –formulated conidia of *M. anisopliae* against eggs and nymphs of whiteflies on egg plants. Bars represent average values±SE

^{Control1} -0.03%Tween solution only; ^{Control2} -Sunflower Oil emulsion only; ^{LIM1} -Fungal conidia + 0.03% Tween solution; $^{LIM1 + Oil}$ -Fungal conidia + Sunflower Oil emulsion

Table 5. Effects of oil formulations on *M. anisopliae* conidial viability and shelf-life after 16 weeks of storage at different temperatures

Oil formulation	Conidial viability (%±SE*)								
	1 week			4 weeks			6 weeks		
	4ºC	Room	28ºC	4ºC	Room	28ºC	4ºC	Room	28ºC
Dry	91.2±6.38a	41.8±11.95b	63.8±22.79b	91.8±4.44a	56.4±8.08b	59.0±12.90a	87.4±4.45a	55.6±11.87c	22.6±3.97b
Sunflower	97.0±3.16a	89.6±8.20a	93.6±5.40a	94.2±1.30a	85.8±4.76a	62.6±19.58a	94.2±0.45a	87.8±2.39ab	56.6±8.35a
Olive	99.0±2.24a	97.2±2.77a	96.9±3.28a	94.4±1.94a	92.8±1.30a	45.2±10.45a	92.0±3.54a	76.2±4.32b	32.6±12.36b
Recycled	98.2±3.03a	96.6±4.56a	93.8±4.92a	95.6±2.30a	85.8±1.79a	58.0±9.95a	92.0±2.00a	91.0±4.64a	32.6±6.50b
	9 weeks			12 weeks			16 weeks		
	4ºC	Room	28ºC	4ºC	Room	28ºC	4ºC	Room	28ºC
Dry	81.2±3.19a	58.8±7.95b	19.8±18.41b	86.2±2.59a	40.4±7.60c	7.8±2.28b	80.2±2.95a	38.2±8.47a	0.0±0.00a
Sunflower	88.6±2.07a	75.6±6.54a	55.4±3.58a	86.4±9.56a	70.4±5.13a	17.4±4.50a	87.6±8.56a	53.4±17.06a	0.0±0.00a
Olive	84.4±6.66a	76.6±2.07a	26.4±2.51b	86.0±3.32a	0.0±0.00d	0.0±0.00c	81.4±4.45a	0.0±0.00b	0.0±0.00a
Recycled	87.2±0.45a	80.2±3.56a	21.2±1.92b	82.4±1.52a	62.6±5.03b	0.0±0.00c	81.4±4.62a	0.0±0.00b	0.0±0.00a

**- standard error (SE);a,b,c,d - Means of 5 replicates followed by the same small letter in a column are not significantly different according to Tukey's test (P≤0.05)*

Although the total spore production of LIM1 was higher on burgul than on the other substrates, it is still below 1.0 x 10^{10} spores/g which is the target level for production of reliable mycoinsecticidal products [21]. It was also suggested that when entompathogenic fungal spores are considered for industrial production it would be necessary to adapt or change production methods to increase spore production and harvest with greater efficiency [22,23]. In the case of LIM1 products based on burgul, it will be necessary to improve the extraction method to remove the spores that are retained on the burgul particles.

The speed of germination could be affected by conidial quality since the endogenous reserves of such inoculum are initially the sole source of nutrients available to conidia during the process of germination. There were significant compositional differences reported in conidia of *B. bassiana, M. anisopliae* and *P. fumosoroseus* [24,25] and in blastospores of *B. bassiana* [26] produced in different nutritional environments. Conidia of three *M. anisopliae* isolates [27] accumulated significantly higher levels of polyols and total proteins when grown on insect cuticles or nitrogen-limited medium (MM) than conidia cultured on YEA or SDAM media and contained higher amounts of total endogenous nitrogen. In the study reported here it was evident that spores containing lower amounts of C but higher quantities of N had also a greater germination rate. Moreover, studies with pathogenic fungi have suggested that carbon and nitrogen concentrations of nutritional conditions are responsible for the pathogenicity of resultant inocula [24,28,29]. Shah et al. [30] have recommended endogenous C:N ratio (<5.2:1) of conidia in combination of other parameter as a virulence indicator in quality control.

In this study inoculum produced on different substrates has shown different level of virulence against different species of aphids suggesting that any isolate considered for mass production should be tested against as many pest insects as possible. Also, the virulence of *M. anisopliae* inoculum was reported to be influenced by the culture medium [12,30]. For example, inoculum of *B. bassiana* from insect cadavers was shown to be less virulent than that produced on rice or other synthetic substrate [31]. Isolate LIM1 in our study was noticeably more aggressive towards all target insects if grown on burgul or rice. These conidia were also identified with lower endogenous C:N ratios.

Spores of *M. anisopliae* (LIM1) produced on burgul and formulated in different vegetable oils when subjected to short term storage have shown gradual drop in viability in temperature and oil type dependent manner. Storage at high temperatures resulted in a faster rate of decline than at cooler temperatures. Conidia of *B. bassiana* was also shown to survive better if maintained at 25°C than 30°C retaining viability above 24% after three weeks of storage [32]. Moore et al. [33] suggested that if more than three weeks of storage were required for minimal loss of viability then cooled storage would be necessary. High germination of our formulated spores after 24 h of incubation indicated that none of vegetable oils used had any initial adverse effect on conidial germination. Survival of spores over period of 16 weeks at room and 28°C, however, was greatly affected by the oil used. Rapid decline in viability was observed for olive oil followed by recycled vegetable oil. Olive oil contains more monounsaturated oleic acid and less polyunsaturated linoleic and linolenic acids than other vegetable oils and thus renders olive oil more resistant to oxidation. However, it is generally accepted that a warm region olive oil will be richer in polyunsaturated fatty acids than a cool region oil. Vegetable oils rich in polyunsaturated fatty acid are more prone to oxidation compared to the oils which are rich in monounsaturated fatty acids [34]. Since olive oil used in this experiment was extra virgin oil from South Lebanon, it is possible that this oil was richer in polyunsaturated than in monounsaturated fatty acids which made it more unstable and easily broken down by heat. When these essential fatty acids are destroyed and certain fat soluble vitamins (antioxidants) disappear the oil ages (oxidizes) very quickly. Rapid disintegration of olive oil could have been the reason for poor spore survival. Recycled vegetable oil, on the other hand, had been previously subjected to extremely high heat as it was repeatedly used for deep frying fish, meats and potatoes. When heated repeatedly, changes in physical appearances and chemical content of the oils will occur [35]. Heating causes the oil to undergo a series of chemical reactions like oxidation, hydrolysis and polymerization [36]. Changed chemical composition of recycled oil combined with effects of high storage temperatures could have been a contributing factor to accelerated spore deterioration. In contrast, fresh refined sunflower oil used in our experiment, presumably have undergone solvent extraction, de-gumming, neutralization and bleaching processes that made it more stable

and heat resistant and thus considered more suitable for long term storage at room temperatures. Although good survival rate of dry LIM1 spore powder was achieved at room temperature over period of 16 weeks, there is scope for further improvements. Data from other studies identified spores of *B. bassiana* stored under ideal conditions of low moisture content and low temperature had survived for over two years [37]. Also, some commercial products based on *B. bassiana* isolates that currently used in agriculture [38] have shelf lives of 12 months or more at room temperature.

Dry and sunflower oil formulated conidia that have been stored for 16 weeks at 4°C were tested against eggs and nymphs of *B. tabaci* on cucumber and egg plants. Both, Tween and oil formulated conidial suspensions were to some extent but not significantly effective against white flies in comparison to controls on both tested plants. Ineffective results of our test under open field could be attributed to either very low relative humidity or insufficient number of spray applications. With respect to whiteflies in particular, effectiveness of many mycoinsecticides under field conditions is not as good as in protected agriculture [2]. This is attributed to unfavorable environmental conditions, economic constrains and high susceptibility of crops to mass migration of whiteflies from surrounding vegetation or harvested fields [2]. However, when incubated at optimal conditions, half of those eggs and nymphs that have died showed fungal growth and sporulation suggesting of pathogenesis taking place. Efficacy of this entomopathogen under field conditions, however, could be improved by increasing dosage, rate of applications or even changing formulants. For example, *M. anisopliae* formulated in Shellsol T paraffin oil was more effective against *Phaedon coclеariae,* mustard beetle than a mixture of Shellsol T and sunflower [39].

5. CONCLUSION AND RECOMMENDATIONS

Our studies have demonstrated that LIM1 isolate could be mass produced in large quantities on burgul substrate. The quality of produced inocula as measured by endogenous C:N ratios would be largely affected by substrate used for mass production. The quality would also influence the efficacy against target insects. Mass produced conidia of LIM1 could also be stored as dry or formulated in sunflower oil at room temperature up to 16 weeks. Four–month-old conidia could still be successful in controlling whiteflies. The authors strongly recommend to use the endogenous C:N ratio as quality control indicator for high quality inocula. Use of vegetable peels as potential substrate for mass production combined with a recyclable oil as formulant could be a low cost and environmentally sustainable technology for mass production of entomogenous fungi.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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