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Research Article
Physicochemical Properties Affects on Different Oil Formulations on Fungus *Metarhizium anisopliae* for Control of *Oryctes elegans*

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Abstract

Background and Objective: Date palm horned beetle *Oryctes elegans* Prell. caused serious damage to date palms. *Metarhizium anisipliae* is suitable for biological control of this pest in Iran. This study was carried out with the bioassay of *M. anisipliae* in conditions with different vegetable oils formulation, their synergistic and antagonistic effects and the evaluation of the effect of oil formulation on maintaining the germination capacity of fungi at different temperatures. **Materials and Methods:** The oils used included Canola, Soybeans, Sesame, Corn and odoriferous oils that their physico-chemical characteristics were studied including non- soap [Iodine] index, soap index and viscosity. About 250 mL of each oil were sterilized for 24 h at -20°C. The LC_{50} equivalents to 5.69±108 spores/mL of the pathogen were on each oil formulation for inoculating the larvae of *O. elegans*. **Results:** The results showed that the highest reduction of the lethal time was observed in Sesame oil formulation and the lowest in odorless oil formulation. All oils formulations had synergistic effects and increased the pathogenicity of *M. anisipliae* on the larvae of *O. elegans* the highest and the lowest synergistic effect were recorded for Sesame oil and odorless oil formulations respectively. Only iodine index had strong and negative effects on Synergist indices. Other physicochemical properties (soap index and viscosity) had medium and positive effects on it. The formulation of *M. anisipliae* with different oils increased its resistance to thermal shocks and thus its germination increased strength compared to control at higher temperatures. So that the reduction rate of *M. anisipliae* fungus spore germination was lower than control in high temperature. The highest median life expectancy and the hazard rate of *M. anisipliae* spore germination recorded in Soybean and Sesame oil formulations and the lowest average median life expectancy and the highest hazard rate in odorless oil formulation. The soap index had negatively affected on the gradient reducing spore germination (C_w) but w the non-ionic index (iodine) and viscosity had positively affected on it. **Conclusion:** According to this study, Sesame and Canola vegetable oils were recommended for formulation of *M. anisopliae* isolate DEMID 01.

Key words: Formulation, *Metarhizium anisopliae*, *Oryctes elegans*, sesame oil

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Competing Interest: The author has declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The importance of date palm in the economy of many of the producing countries is very high[1]. Date palm horned beetle *Oryctes elegans* Prell. caused serious damage to date palms. The adults attack to the bunch's tail and the stems of the leaf. The larvae attacked to the stems of leaf, trunk and root[2]. The adults transmit *Fusarium proliferatum* Matsushima to date palm[3,4]. Direct pest damage to the bunch's tail occurs after the fruit setting in April[5,6]. The pest damage to young trees (aged 10-20 years) and with planting distance less than 5 m are more[7].

Some studies have been done on the use of *Metarhizium anisipliae* for biological control of this pest in Iran. The unfavorable environmental factors are the most important problems in the development of microbial control, which are largely modulated through appropriate formulation[8]. The suitable physical conditions, especially humidity, temperature and light are importance factors in increasing the epizootic of entomopathogenic fungi in nature[9].

The added carbohydrate in some formulations stimulates the germination entomopathogenic fungi spores and increases the fungal spores persistence in environment[10]. After spraying, there is not enough time and sufficient moisture around entomopathogenic spores for germination because the droplets water evaporate and very quickly[11]. Application of oily formulations increases the efficiency of *M. anisopliae* in comparison with aqueous formulations relative humidity less than 35% in arid areas[12]. In addition, oily formulations maintain spores against ultraviolet radiation[13].

Formulation of *M. anisipliae* spores by using vegetable oils were necessary following the application of microbial control program of *O. elegans*. This study was carried out with the bioassay of *M. anisipliae* in conditions with different vegetable oils formulation, their synergistic and antagonistic effects and the evaluation of the effect of oil formulation on maintaining the germination capacity of fungi at different temperatures.

MATERIALS AND METHODS

The research was conducted in Entomology Laboratory of Date palm and Tropical Fruits research center over the years 2016-2017 for one year and six months.

Horned beetle breeding: The adults of *O. elegans* were reproduced into plastic cages with height and diameter equal to 10 and 7 cm, respectively in the laboratory. Each pair of male and female beetles was transferred into a cage containing 400 g of apical meristem tissue as feeding source and laying site. The cages were kept in incubator at temperature 25±5°C and humidity 75±5%. The eggs were separated from the meristem tissue daily and transferred to similar cages for incubation period.

Culture entomopathogenic fungi: The isolate of *M. anisopliae* was prepared through the Plant Protection Institute of Iran. The isolate was named DEMID 01, which was collected from Saravan area in Sistan and Baluchistan province in the eastern of Iran. The fungus isolate was cultivated in the SDAY media. The concentration of spore was measured with an improved Neubar[14].

Study of the oils properties: The oils used included Canola, Soybeans, Sesame, Corn and odoriferous oils that their physico-chemical characteristics were determined by the following methods.

Non-soap (iodine) index: Five grams of tested oil, 60 mL of ethanol and 100 mL of ether of petroleum were boiled in a balloon attached to the refrigerator for half an hour and added 80 mL of distilled water to it. Then, the contents of the balloon were transferred to a decanter. The balloons were distilled with 80 mL of distilled water and added to the contents of the decanter. Adding 100 mL of ethyl ether to decanter and mixing the two phases completely. The lower phase was transferred to another lattice and mixed well with 50 mL of ethyl ether and then again separated by two phases. This phase was dislodged and the supernatant was transferred to the first factor and after mixing completely with 100 mL of water. The bottom phases were discarded and again washed with distilled water until the substrate titration with phenol-phthalate had not been altered and the dehydration completely completed. Then discarded the underlying phase and distilled off the non-soap compounds and left the remaining ether with a teaspoon of sodium sulfate anhydrous in a small eagle and then filtered with the filter paper inside the funnel. Non-soap combinations in a previously weighed test tube the ether was evaporated and weighed again. The weight (%) of non-soap compounds was obtained in mg/100 g[9].

Soap index: Two grams of oil and 25 mL of half-normal alcoholic potassium were boiled in a balloon for 1 h after being bonded to the refrigerant. A few drops of phenol-

phthalate were added to it and then titration was performed by Burette using half-normal chloride acid to make the solution colorless. Control titration (semi-normal alcoholic potassium) was performed with chloride. As a result of the incorporation of the resulting numbers in equation 1, the soap index (SV) was obtained in mg/100 g[15] as shown in Eq. 1.

$$SV = \frac{(B-A)\times 56.1\times N}{W} \quad (1)$$

Where:

B = Control volume (mL)
A = Chloride acid volume (mL) used for titration
N = Acidity normal value equal to 0.5
W = Initial sample weight (g)

Viscosity: First, the viscometer (the SVM™ Stabinger Viscometer™ series and the Lovis 2000 M/ME rolling-ball viscometer) was calibrated with normal water and then the temperature of the oils was measured at the time of the test (25°C). The oil was placed in an appropriate container and the scraper was viscose in oil. The resulting numbers include the number of revolutions per minute (RPM) and Sol's error of the machine and the final number of oil concentrations. Viscosity measured with centipoise unit (cp)[15].

Formulation of fungal spores in oil: About 250 mL of each oil were sterilized for 24 h at -20°C. The amount of volatile oil was reached to the desired volume to preparation concentrations.

Effect of oil formulations on *M. anisopliae* pathogenicity: The LC_{50} equivalent to 5.69 ± 10^8 spores mL^{-1} of the pathogen was on each oil formulation for inoculating the larvae of *O. elegans*. Bioassay tests were conducted by them. The third instar of larvae was treated by oil formulated suspensions Experiments were done by 5 replications. The larvae were immersed in spore suspension for 20 sec and they have been kept at temperature 25±1°C and humidity 85±5% for two days. The separate spore suspension was prepared for each replication. Then infected larvae were incubated at humidity 40±5% and photoperiod (12D:12L) for the following days. The larvae mortality had been recorded daily for 14 days. Then, the cumulative mortality table was prepared[16].

Data analysis: The distribution of natural mortality data were normalized based on the Abbott method by transforming them to $A \sin \sqrt{x}$. The average time of 50% mortality (LT_{50}) and 90% mortality (LT_{90}) were estimated by logistic regression for each treatment. The SR indices were calculated to increasing or decreasing effects of oil formulation by the Eq. 2:

$$SR = \frac{LT_{50}\ (\text{Entomopathogen in control})}{LT_{50}\ (\text{Entomopathogen in formulation})} \quad (2)$$

If 1 SR<1, then the composition has antagonistic effects and if SR>1, then the composition has antagonistic effect.

Effect of formulation on *M. anisopliae* spore germination in different temperatures: The spore suspensions were in oils with concentration equal to 10^5 spores mL^{-1}. Then, 0.5 mL of each formulated suspensions was coated over a thin layer of SDA media in petridish. The petridishes were closed by parafilm. All treatments have been incubated at 25, 30, 35, 40 and 45°C and completely dark condition for 24 h. Then, 1 mL of 0.5% formic aldehyde was shed into each petridish to stopping spores germination. The germination (%) were calculated by counting 100 spores from each replication by 40X Microscope magnifications. Each treatment was conducted with 4 replications[16].

Data analysis: The means values of spore germination in oil formulations had been compared by Student-Newman-Keuls (SNK) method is a stepwise multiple comparisons procedure used to identify sample means at 0.05% significant level after variance analysis was done[17]. The simulation model for the variation (%) of spore germination in different temperatures is based on Eq. 3:

$$G(\%) = m\text{-}C_t t \quad (3)$$

In Eq. 3 G (%) is the mean of germination percentage, Ct is the gradient spore germination in various formulations due to changes in temperature, t temperature changes in studied condition and m is constant[18].

Study of *M. anisopliae* spore survival in oil formulations: Spore survival in oil formulations and control were evaluated in a completely randomized statistical design with four replications. For this purpose, 250 mL of each oil were prepared. The oils were sterilized as described above. Fungal spores with concentration equal to 10^5 spores mL^{-1} were formulated in oils. Each replicate was packed in cylindrical polyester containers (5×10 cm) and covered with cellophane. Packs have been stored in laboratory conditions with temperature 25±5°C and humidity 40±5% for three weeks. The control included 100 g of fungal spore powder, which was stored in similar containers and conditions. Then, the germination of fungus spores were evaluated in the treatments once a week during the 3 week period. For this purpose, at first initially, the formulation containing packs

have been shaken for 2 min and then 5 mL of each of them were randomly sampled. The sampled spores were examined for germination.

Data analysis: The means values of spore germination in oil formulations had been compared by Student-Newman-Keuls (SNK) method is a stepwise multiple comparisons procedure used to identify sample means at 0.05% significant level after variance analysis was done. Then, the risk of survival decreasing of the fungus spores were estimated by calculating hazard rate and median life expectancy of the fungus spores the oil formulation and control using the Eq. 4 and 5[19]:

$$HR = \frac{2q_i}{b_i(1+p_i)} \quad (4)$$

$$M = (t_j + t_i) + \frac{b_j(s_j - \frac{S_j}{2})}{s_j - s_j + 1} \quad (5)$$

In the Eq. 4 and 5, q_i the degree of germination reduction at the time i is the ratio of reduction of germination interval time i to the previous time of the sampling, b_i is the distance between the two sampling times, P_i is the cumulative probability of germination reduction to the interval time i , the starting point of the experiment until the time j, S_j is the cumulative survival of germination until time j and t_i and t_j are interval time between two samples i and j, respectively. Statistica software 13.0.159.7 was used for this purpose[20].

The simulation model for the variation percentage of spore germination in different physico-chemical characteristics of the oil formulations is based on Eq. 6:

$$\text{Log } [Q] = K\text{-}C_w \text{Log } [I] \quad (6)$$

where, Q, C_w and k are variations of the standard germination percentage, gradient germination reduction in different formulations due to changes in physico-chemical characteristics and constant of equation respectively[18]:

RESULTS

Bioassay *M. anisipliae* in different oils formulations: The oil formulations had a significant effects lethal time of the larvae of *O. elegans*. So that in Canola, Soybeans, Sesame, Corn and odoriferous oils formulation reduce the lethal times in comparison with control Table 1.

Against larvae of *O. elegans*: The highest reduction of the lethal time was observed in Sesame oil formulation and the lowest in odorless oil formulation.

Synergistic effects of oils formulations on *M. anisipliae* pathogenicity: The SR indices were estimated in order to study the synergistic or antagonistic effects of oils formulations on the pathogenicity of the *M. anisipliae* in the larvae of *O. elegans*, the results of which were shown in Fig. 1. It should be noted that if the coefficient for oil formulation is greater than 1, it has a synergistic effect and if smaller than 1, has an antagonistic effect.

All oils formulations had synergistic effects and increased the pathogenicity of *M. anisipliae* on the larvae of *O. elegans*. The highest and the lowest synergistic effect were recorded

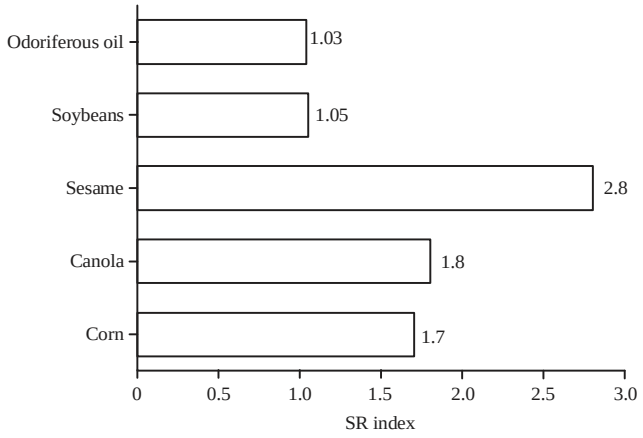

Fig. 1: SR indices of *M. anisopliae* in different oils formulations and control

Table 1: LT_{50} and LT_{90} values *M. anisopliae* formulated in different oils and control against larvae of O. *elegans*

Treatments	LT_{50} (days) (95% fiducial limits)	LT_{90} (days) (95% fiducial limits)	Slope(±SE)	$X^2_{\text{-test}}$
Control	3.35 (3.11-3.47)	11.29 (10.38-11.92)	1.89±0.02	5.78
Corn	2.01 (1.97-2.17)	4.39 (3.93-4.61)	2.94±0.04	2.98
Canola	1.87 (1.73-1.98)	3.99 (3.49-4.23)	3.05±0.04	1.09
Sesame	1.18 (0.98-1.26)	2.26 (2.11-2.43)	3.54±0.03	1.27
Soybeans	3.17 (2.91-3.38)	10.34 (10.11-10.74)	1.95±0.02	6.64
Odoriferous oils	3.24 (3.09-3.8.45)	11.25 (10.96-11.73)	1.85±0.02	6.61

for Sesame oil and odorless oil formulations respectively. In order to investigate the effects of physicochemical properties of tested oils on the synergistic effect, the correlation coefficient these characteristics with SR indices were calculated (Fig. 2). The results showed that only iodine index had strong and negative effects on SR indices. Other physicochemical properties (soap index and viscosity) had medium and positive effects on it.

Effect of oil formulation on *M. anisopliae* spore germination at different temperatures: There was a significant difference between the mean *M. anisipliae* spore germination formulated with different oils (MS = 3356.8, df =5) in different temperatures (MS = 5229.3, df = 4) and interactions of temperature and oils (MS = 785.3, df = 20) at one percent significant level. The comparison of the spore germination mean in treatments was showed in Fig. 3.

As seen in Fig. 3, there is no significant difference between germination of *M. anisipliae* at 25 and 30°C in vegetable oils and control based on the mean comparison test so all treatments were grouped as a. But at higher temperatures the difference is significant and vegetable oils formulation and control were in different groups. The formulation of *M. anisipliae* with different oils increased its resistance to thermal shocks and thus its germination increased strength compared to control at higher temperatures. So that the reduction rate of *M. anisipliae* fungus spore germination was lower than control in high temperature. The rate of spore germination reduction varied for oils formulations in different temperatures. The gradient of the variation spore germination of *M. anisiplia*e were estimated based on the mode were inscribed in Fig. 4.

The slopes of the equation line in Fig. 4 were equivalent to the spore germination gradients of *M. anisipliae* fungus relative to the temperature changes under the conditions of the oils formulations treatments and control. Based on Fig. 4, the highest grain germination gradient was observed in the control (-4.17) and the lowest in sesame (-1.27). The gradients of reductions of Soybean and corn oil formulations were lower than Canola, but the base abilities of fungi spore germinations were lower than canola oil formulation.

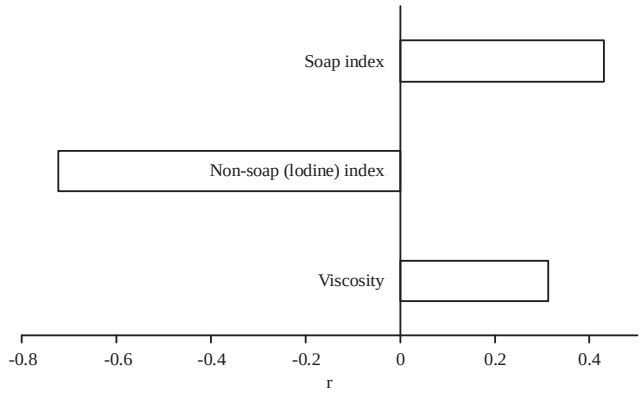

Fig. 2: Correlation coefficient of physicochemical properties of the oils used in formulation of *M. anisipliae* for Microbial control of *O. elegans*

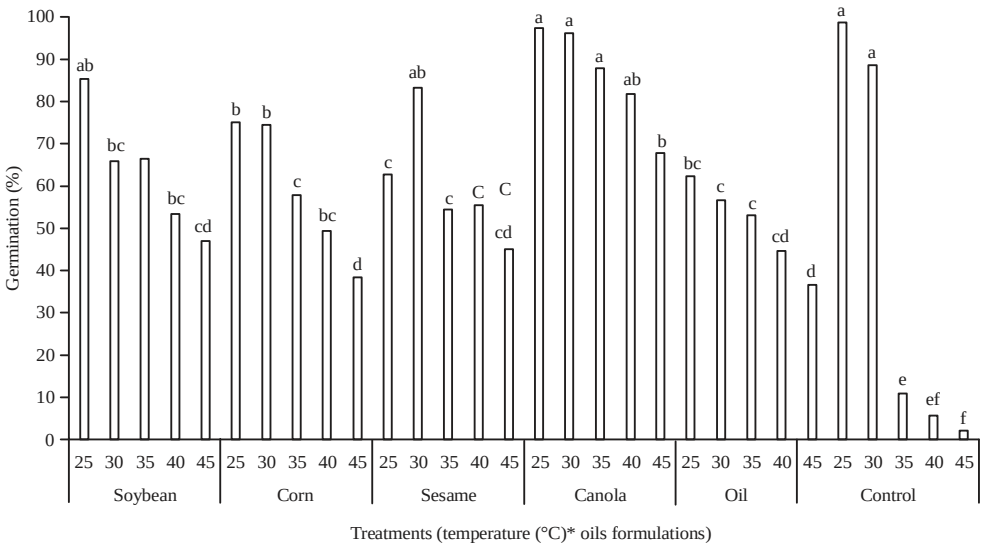

Fig. 3: Comparison of the spore germination of *M. anisipliae* in different treatment (temperature×oils formulations) based on SNK method

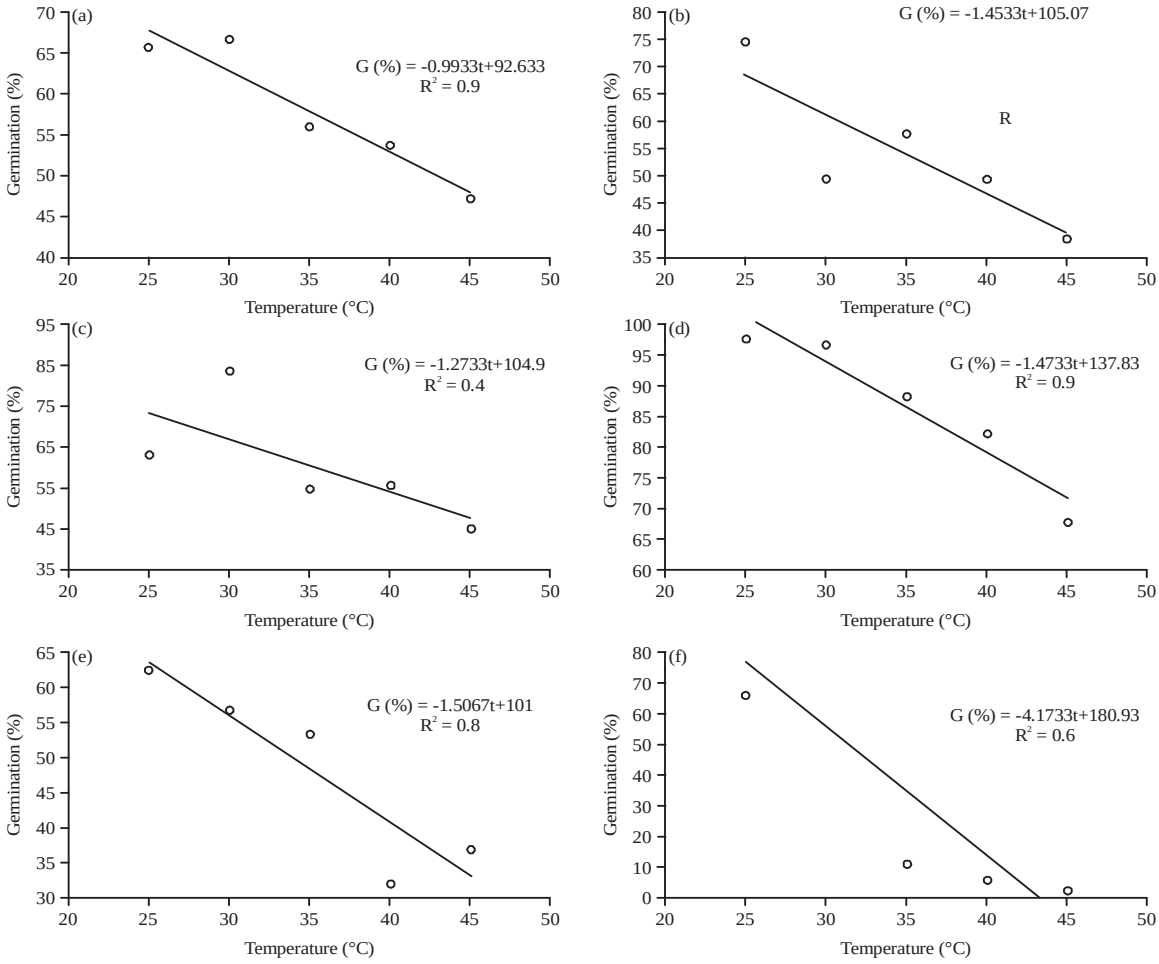

Fig. 4(a-f): Models of *M. anisipliae* spore germination variation related to temperature variations in different oil formulations and control, (a) Soybean, (b) Corn, (c) Sesame, (d) Canola, (e) Odorless oil and (f) Control

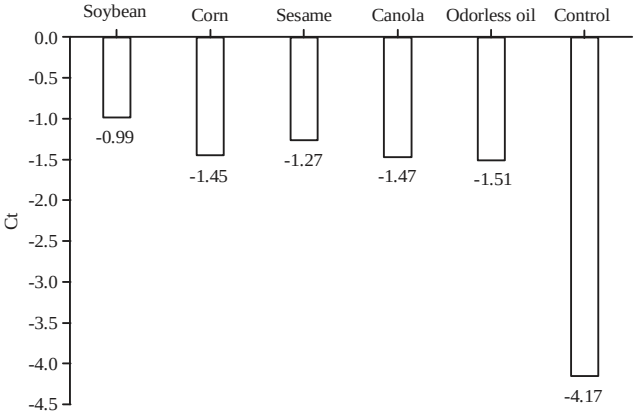

Fig. 5: Gradients of *M. anisipliae* spore germination in the conditions that formulated by oils and control

The reduction rates of spore germinations in all oils formulations were much lower than control. The lowest germination have been recorded in Sesame formulations. Therefore, the formulation of this vegetable oil provided the highest tolerance to temperature for the pathogenic fungus.

Survival of *M. anisopliae* spores in oil formulations: The results of variance analysis of *M. anisipliae* spores survival ability in oils formulations and control showed had significant at a probability level of 1% based on the kind of oils formulations treatments (MD = 681.7, df = 5), duration of storage (MS = 4002.5, df = 2) and the interaction between oils formulations and duration of storage (MS = 18.6, df = 10). The means of the spore germinations in interaction treatments were compared by SNK method (Fig. 5). According to results, the spore germinations abilities of the fungi were maintained in Canola and Sesame oils formulation more than other formulations and control for two weeks.

As seen in Fig. 6, the highest germination ability was observed in three vegetable oils of Maize, Canola and Sesame

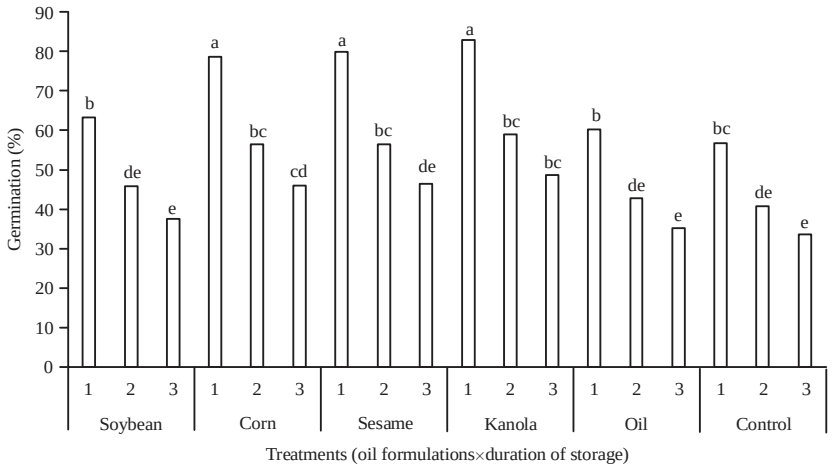

Fig. 6: Comparison of the effect of oils formulations and storage time on the mean of the spores germination ability of *M. anisipliae*

Table 2: Estimation of germination hazard rate and median life expectancy (M.) of *M. anisipliae* in oil formulations and control

Treatments	Hazard rate	Median life expectancy
Soybean	2.24	94.99
Corn	3.28	89.26
Sesame	2.81	91.76
Canola	3.22	89.96
Odorless oil	4.41	87.35
Control	9.57	11.18

(group a) during the first month which showed a significant difference with other oil formulations and control. The germination ability of these treatments were higher than other treatments and control and they were placed in different groups based on the mean comparison test in the second and third months of experiment. Hazard rate and median life expectancy of the fungus spores for the oil formulation and control were estimated based on the Eq. 4 and 5. The results were showed in Table 2.

The highest Median life expectancy and the lowest Hazard rate of *M. anisipliae* spore germination recorded in Soybean and Sesame oil formulations and the lowest average Median life expectancy and the highest Hazard rate in odorless oil formulation. All oils have been effective in increasing the Median life expectancy and reducing the Hazard rate of *M. anisipliae* germination capacity. The relationship between the gradient germination reduction in different formulations with the changes in the physicochemical properties of the oil formulations were simulated by the Eq. 6, the results of which were shown in Fig. 7.

Bases on Fig. 7, soap index had negatively affected (-0.758) on the gradient reducing spore germination (C_w) but the non-ionic index (iodine) (0.893) and viscosity (0.133) had positively affected on it. In other words, increasing the viscosity and non-soap index had negative effects on the survival of *M. anisipliae* in oil formulations.

DISCUSSION

Some factors were described as physiological causes of entomopathogenic fungi tolerance to high temperatures due to the formulation of fungus spores with vegetable oils[9,21]. The most important factor in increasing the *B. bassiana* resistance to thermal shock is the trachalose accumulated in the cell structures that contribute to the stability of the natural structure of the essential proteins. Trehalose is the most active in acidic environments in fungi. Vegetable oils, saturated and unsaturated fatty acids provided the necessary environmental conditions for trehalose enzyme activity and increase resistance to high temperatures in fungi spores[22].

Similar researches had been conducted on other entomopathogenic fungi in this field[23,24]. Germination of *B. bassiana* spores isolate FHD13 studied in condition that formulated by vegetable oils. Results showed that there was no significant difference between vegetable oils on spore germination at temperature 4°C after 28 days[25]. The effects of additives material such as Agrocer®, Addit®, PA_1 and Tween80 were studied on the protection of *M. anisopliae* spores. The results showed that oils formulations had protective effects on the survival of fungi spores[20]. The effective application of a formulated fungus strain against Plutella xylostella larvae constitutes the first step towards its use in pest management of this insect. The formulated *Zoophthora radicans* in inverted emulsion could be used as an alternative tool to

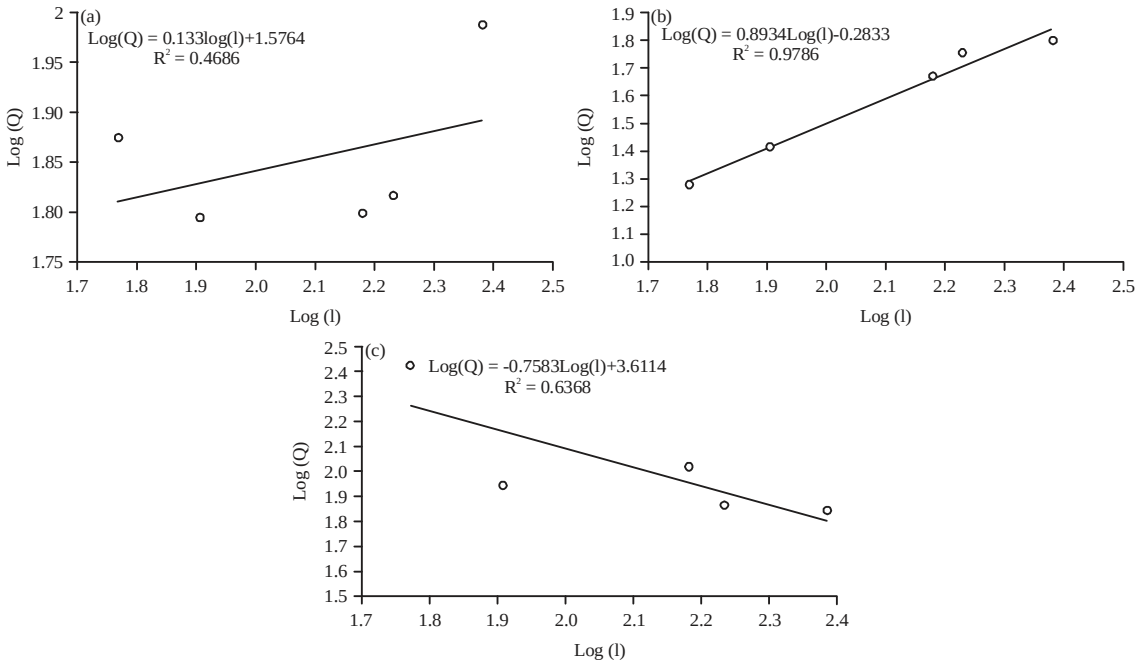

Fig. 7(a-c): Relationships between germination reduction of *M. anisipliae* spores in with the changes in the physicochemical characteristics of the oil formulations, (a) Viscosity, (b) Non-soap (Iodine) index and (c) Soap index

insecticides in pest management of *P. xylostella* larvae because of the development of resistance to insecticides in the treated larvae[26]. There are specific fatty acids in rotting oils that prevent germination of the spores of the *Metarhizium flavoviride*[27]. The reasons for the low fungal survival in undiluted oils are unclear and maybe the oils are suitable for use in completely dry formulations, because the incompatible factors may not be controlled by drying the oil[28].

According to this study, Sesame and Canola vegetable oils were recommended for formulation of *M. anisopliae* isolate DEMID 01. Among the tested formulations, the lowest LT_{50} was on Sesame and Canola oil formulations. These formulations had also the most synergistic effects on the pathogenicity of the *M. anisopliae*. Consequently, less time is needed for effective control of O. *elegans*. Formulation of *M. anisopliae* with different vegetable oils increased its resistance to thermal shock and pathogenicity in higher temperatures. So that the rate of host mortality was more than control in the higher temperatures. The best germination and pathogenicity of *M. anisopliae* were recorded in Canola and Sesame oil so these vegetable oils were recommended for formulation of it for microbial control of *O. elegans*. Mineral oils act like vegetable oils for formulation of entomopathogenic fungi spores, but they are dense in the long term at high temperatures and have an unpleasant odor. In all studies, such as the results of this study, odorous oil was not better than other oils[15]. These results indicated that, the beetle larvae did not inoculate by effective dose of spores in dry conditions and the development of applied application technologies such as the development of vegetable oil formulations with greater stability helps to microbial control of this pest. The amount of spore moisture is another factor that affects the ability to spores storage. The spores moisture content up to 5% is necessary to survive. Using oil formulations were helped spore survival by maintaining moisture[29].

CONCLUSION

The present research constitutes the first investigations into the formulation of *M. anisopliae* against the larvae of *O. elegans*. However, further research studies are needed in this respect especially under date palm garden conditions before using this fungus in microbial control of *O. elegans* or of other pests that may be infected with these entomopathogenic fungi.

SIGNIFICANCE STATEMENTS

The formulation of *M. anisopliae* with vegetable oils increases its resistance to temperature shocks and thus increases its pathogenicity in higher temperatures. The use of vegetable oils increases the tolerance of *M. anisopliae* fungus

to heat. so that, it can be used biological control of *O. elegans* by vegetable oil formulations of *M. anisopliae* in subtropical conditions of date palm growing areas. This situation warranted shifts the use of vegetable oil formulation of *M. anisopliae* which showed good results of biological control of *O. elegans* in Date palm plantation conditions.

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