

Diversity of Entomopathogenic Fungi in Argane Forest Soil and Their Potential to Manage Mediterranean Fruit Fly (*Ceratitis capitata*)

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Abstract: The present study forms a part of biological control against the medfly (*Ceratitis capitata*) and seeks to isolate and determine the diversity of the medfly-associated entomopathogenic fungi in soil samples collected from Argane (*Argania spinosa*) forest, in different localities of Souss-Massa region. Search and isolation of the indigenous populations of *Ceratitis capitata* entomopathogenic fungi was carried out by using larvae of this pest as baits. After trapping, isolation and identification of fungal isolates, a pathogenicity test was used to select fungal strains that have significant virulence potential against the Mediterranean fruit fly. The degree of virulence was estimated by the ability of the fungus to induce the disease and/or the death in the insect and also by calculating the lethal time 50 (LT₅₀). The obtained results were clearly demonstrated the sensitivity of medfly to tested fungal strains and particularly to strains of *Fusarium* sp., *Aspergillus niger* and *Scopulariopsis* sp. that have shown high mortality rates (more than 84%) and to *Trichoderma harzianum*, *Scedosporium* sp., *Epicoccum* sp. and *Ulocladium* sp. with more than 70% mortality for the two tested concentrations. Furthermore these strains showed short LT₅₀ (less than 83 hours). All these results confirm the presence of entomopathogenic fungi of *Ceratitis capitata* in Argane soil, and prove the potential of entomopathogenic fungi for biocontrol of the Mediterranean fruit fly under laboratory conditions.

Key words: Biological control, *Ceratitis capitata*, entomopathogenic fungi, *Argania spinosa*.

1. Introduction

Ceratitis Capitata (Wiedmann) (Diptera: Tephritidae) also called the Mediterranean fruit fly, (Medfly) is considered as one of the most dangerous pest of fruit crops in the world and especially in the tropical and Mediterranean regions [1, 2]. In Morocco, the medfly survives in the *Argania spinosa* forests and invades continuously the agricultural areas [3, 4]. Affecting in larval stage over 300 varieties of forest and fruits tree [5], this polyphagous pest is responsible for most of the economical damages and both of quantitative and qualitative losses in the Mediterranean area, particularly in Morocco [6]. In addition, due to quarantine regulation against this pest in several

importing countries, producer countries lose their international markets.

Currently, control of *C. capitata* is often based on chemicals by using attractive baits mixed with insecticides that have toxicological and ecological bad effects [7-9] which required the development of alternative management strategies. Therefore, the medfly current control is relied to adults [10]. Third instar medfly larvae are usually pupariating in soil; however the control measures against pre-pupating larvae and pupae in soil are poorly applied [11]. In order to reduce chemicals, EF (entomopathogenic fungi) could be applied as microbial insecticides in integrated pest management programs or even as alternative [12, 13]. The possibility of medfly management with entomopathogenic fungi was

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supported by many researches which have shown the susceptibility of Medfly larvae, puparia and adults to infection by strains of *Beauveria bassiana* and *Metarhizium anisopliae* in Kenya [7, 11], Spain [8, 10], Greece [14] and Morocco [15] and strains of *Paecilomyces lilacinus* in Morocco [16]. The approach of medfly control with entomopathogenic fungi include disseminating the fungi among medfly populations by attracting adults to bait stations where they are infected with the pathogen and transmit the disease to other individuals [17] as well as targeting soil pupariating larvae and puparia by soil inoculation [7, 11].

The soil is the natural reservoir of entomopathogenic fungi since it's protected from biotic and abiotic influences [18]. Therefore, entomopathogenic fungi are isolated from soil using several selective media [19, 20] or by using baiting method [18, 21]. With the exception of the studies by Imoulan in 2011 [16] and 2014 [15], the entomopathogenic strains tested against the medfly have never been isolated from a soil that naturally contains the *C. Capitata* larvae and pupae from infected individuals. Using of introduced entomopathogenic strains would probably reduce the effectiveness of the biocontrol agents and may have ecological risks compared to indigenous strains which could be adapted to the Mediterranean fruit fly as well as to the particular environmental conditions (humidity, temperature) [22]. Furthermore, the potential of entomopathogenic fungi often vary among fungal species and strains. Therefore, highly virulent and specific fungal strains against *C. capitata* can be isolated from *Argania spinosa* forest which is the natural refuge of the medfly.

Consequently, our work focuses on the search for efficient Moroccan entomopathogenic agents. It aims to determine the occurrences and diversities of medfly-associated entomopathogenic fungi in Argane forest soil, and investigate their *in vitro* biocontrol potential towards *C. capitata*.

2. Method and Materials

2.1 Soil Sampling

A total of nine soil samples were collected from Argane forest in the SM (Souss-Massa) region (Fig. 1). To represent the variability of soil and climate in this region, soil was sampled from three different localities in the order of three sub-samples per locality. The selected Sampling sites are Agadir (Amsekroud), Taroudant and Ait-Baha (Biougra) (Table 1). For each sub-sample, about 2 kg of soil is taken from 3 random points and mixed to make a homogenous sample. Sampling was carried out in the sub-trees area at depth of 0 to 20 cm after removal of surface litter. The soil of sub-trees area is the natural living environment of the L3 larvae and pupae of the medfly (*C. capitata*) [4, 16, 23]. Consequently, the choice of this soil can increase

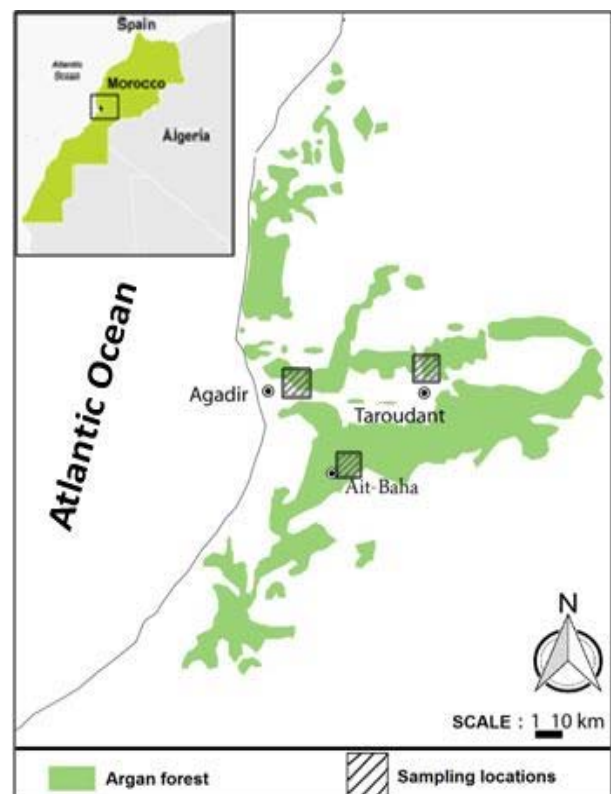


Fig. 1 Map showing locations of the soil sampling sites in Souss-Massa Argan (*Argania spinosa*) forest (base map: Msanda, 2005 [26]).

Table 1 Sampling sites climate information [24] and soil types [25].

Sampling Site	Altitude (m)	AAT (°C)	AAR (mm)	Type of Soil
Agadir	30 ± 20	18.6	246	Brown soil
Taroudant	240 ± 40	20.1	226	Sieroze-ms and collapse-ble
Ait-Baha	130 ± 20	19.3	214	Sandy

AAT: average annual temperature (°C); AAR: average annual rainfall (mm per year).

the chance to trap specific entomopathogenic fungi of the medfly.

Soil samples were placed in plastic bags to prevent water loss, and transferred immediately to laboratory then stored at 4 °C in the dark for less than 1 month until use.

2.2 Trapping of Entomopathogenic Fungi

To trap the indigenous populations of entomopathogenic fungi, the technique of using larvae of *C. capitata* as baits was adopted. This method was originally used to isolate entomopathogenic nematodes from soil samples, and then developed to isolate entomopathogenic fungi [18, 27].

In order to prepare baits, fifteen living L3 *C. capitata* larvae were buried in Petri dishes containing about 40 g of humidified soil. Then the dishes were closed with Parafilm and incubated at 25 °C to enable growth of fungi on larvae and/or on pupae. To yield reliable data, for each soil sample three repetitions and a total of 45 larvae were used. The *C. capitata* larvae used in this study were isolated from infested *Argania spinosa* fruits collected from Argane forest in SM.

2.3 Isolation of Entomopathogenic Fungi

In order to isolate EF, two methods have been adopted according to the condition of the dead insect [28-30]. Firstly, dead insects (larvae, pupae and adults) of *C. Capitata* were observed using a dissecting microscope. Insects without any external growth of fungi on their surface were surface disinfected, using 0.1% of sodium hypochlorite for 3 minutes, rinsed three times by sterile distilled water, and then transferred to a moist chamber. After 7 to 10 days

incubation at 25 °C, fungi that had developed were isolated, using a sterile needle under a dissecting microscope (× 60 magnification), either by conidial transfer or by removing a piece of mycelium of the fungus, onto PDA plates, supplemented with antibiotics. However, insects with external mycelial growth of fungi were placed directly in Petri dishes containing PDA (potato dextrose agar), supplemented with chloramphenicol (0.25 g/L).

The colonies obtained were purified and identified on the basis of macroscopic and microscopic criteria, using the specialised key of Pitt (2009) [31].

2.4 Tests

Once isolated and purified, the fungal strains were tested *in vitro* to determine their entomopathogenic abilities against the Mediterranean fruit fly (*C. capitata*). After two weeks of incubation in 25 °C, a spore suspension intended for the infection of larvae of the Mediterranean fruit fly were prepared. Conidia were harvested directly by scraping from the surface culture and suspended in sterile distilled water containing 0.02% Tween to produce a homogenous suspension. The concentration of the suspension was determined by using a haemocytometer, and diluted with sterile distilled water plus 0.01% Tween 80 to obtain the conidial concentrations for each trial. For each strain, two concentrations of spores were tested, 10⁶ spores/mL and 10⁵ spores/mL. After preparation of the inoculums, living L2 and L3 *C. capitata* larvae were placed in Petri dishes in order of five larvae per Petri dish, and then they were inoculated by spraying about 2 mL of the test spore suspension. Three repetitions were performed for each concentration.

Sterile distilled water containing 0.02% of Tween 80 was used as control. The treated insects were maintained at a temperature of 25 °C. The mortality rates were recorded after 24 hours of inoculation and after each 48 hours for 12 days. A re-isolation of the entomopathogen from the insects was done in order to verify that the fungi tested are responsible of observed mortality (Koch's postulates). Due to Mortality natural mortality in control dishes (MRc), the percentage of larval mortality in each Petri dish (MR) was calculated according to Abbott (1925) [32]:

$$MR (\%) = \frac{(MRt - MRc)}{(100 - MRc)} \times 100$$

MR: percentage of larval mortality, MRt: percentage of Mortality in the treatment, MRc: percentage of Mortality in the control.

2.5 Data Analysis

The degree of virulence of the different isolates was estimated based on the ability of the fungi to induce death in the insect and also by calculating the lethal time 50 (LT₅₀). To evaluate the influence of different strains and concentrations, two way analysis of variance (ANOVA) were applied. Mortality data were analyzed using two-way analysis of variance (ANOVA), and the means were compared with the fisher's LSD test. All Statistical analyses were performed using Statistica V6 software [33].

3. Results and Discussion

3.1 Entomopathogenic Fungi Isolated

As already described, *C. capitata* larvae were used as baits to trap entomopathogenic soil fungi from samples of *Argania spinosa* soil, the fungi trapped were isolated on PDA medium directly or after passing through the moist chamber. In our knowledge, this paper reports for the first time the possibility of using *C. Capitata* larvae as bait to trap EF. Thus, during the baiting test 34 larvae from 405 larvae used were infected by fungi. Entomopathogenic fungi were detected in all the 9 sub-samples analyzed (100%). Comparable results

were found in the Moroccan endemic forests of *Argania spinosa* (91.62%) [16], but higher than in other countries: over 71.7% in Spain [28, 34], 55.5% in China [35], 43% in Southern Italy [36], 33.6% in Palestine [37], 20.59% in Turkey [22] and 17.5% in the UK [21]. These comparisons must be taken with precautions because in all this studies the numbers and the type of larvae and number of repetitions used to trap entomopathogenic fungi from soil samples were different. Dead insects were showed varying symptoms as observed in Fig. 2, this multitude of symptoms depend on the genus of the parasitic fungus and/or the stage of the host development and it can be explained by the attack of different pathogens or the symptoms of the same fungus at different stages of its development in the host [30].

A total of 29 strains were isolated and purified from the dead insects by plating or live baiting from soil samples. Identification of the isolated entomopathogenic fungi revealed that *Fusarium* was the most frequent genera with 44.8%, followed by *Aspergillus* with 13.79% and *Ulocladium* with 10.34% of the total of isolated fungi. *Beauveria bassiana*, *Trichoderma harzianum* and *Scopulariopsis* sp. were isolated from 6.89% of larvae. On the other hand, *Epicoccum* sp., *Scedosporium* sp. and *Penicillium* sp. were very rare (Fig. 3). The present results have demonstrated that entomopathogenic fungi are common

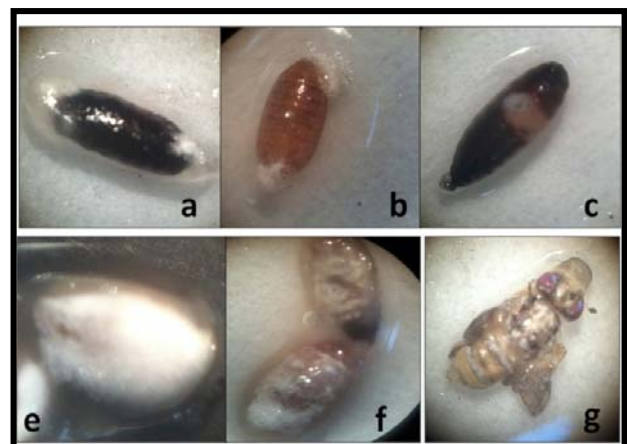


Fig. 2 Different symptoms showed by the infected *C. Capitata* individuals in different stages of development: larvae (a, c, e), pupae (b, f), adults (g).

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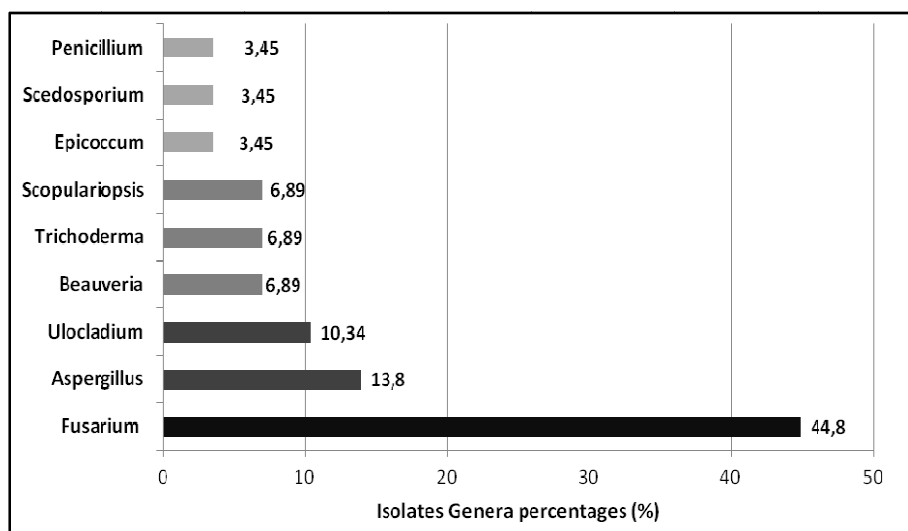


Fig. 3 Diversity of entomopathogenic fungi isolated from *C. capitata* larvae.

inhabitants of the Souss-Massa *Argania spinosa* forest soil. Our findings support the results obtained in the endemic Moroccan *A. spinosa* forest [16]; however, the diversity of fungi recorded (9 genus) in this study was higher than obtained by Imoulan et al. [16]. The most commonly isolated species belonged to genera of *Fusarium*, *Aspergillus* and *Ulocladium*. These genera are recorded by their high occurrence rates in soil in china and Palestine [35, 37]. Species in these genera, especially the genus *Fusarium*, display a high diversity of life strategies including associations with insects. These species were recorded as entomopathogen in several researches [30, 37-39]; whereas, they were classed as opportunistic pathogens by other authors [35]. A pathogenicity test (Koch's postulates) was necessary to prove their pathogenicity.

3.2 Tests

3.2.1 Mortality Rates

To confirm their pathogenicity using Koch's postulates, sixteen of the isolated strains were tested against the *C. capitata* L3 larvae and pupae. Two concentrations of spore suspensions were tested for each strain (10^5 and 10^6 spores/mL). The strains studied demonstrated varied pathogenicity by causing different mortality rates ranged from 15 to 100 % for the two concentrations used (Fig. 4). The mortality levels were

significantly different between strains and between trains and control ($p = 0.000$), as well as between concentrations ($p = 0.000039$). The concentration of 10^6 spores/mL recorded the highest mortality rates for all the strains.

For the first concentration tested (10^5 spores/mL), the highest mortality rate was recorded by the strain S6 of *Fusarium* sp. (92.3%) followed by *Aspergillus niger* S5, *Epicoccum* sp. S3 and *Scopulariopsis* sp. S11 with more than 84%, and *Aspergillus fumigatus* S9, *Fusarium* sp. S16 with 74.9% each. The lowest mortality levels for this concentration were obtained by *Beauveria bassiana* (S1) and *Fusarium* sp. S15 with 15% of mortality and no significant difference with the control. Concerning the treatments with the concentrations of 10^6 s/mL, the mortality percentages have been relatively increased for the majority of strains. As for the first treatment, the most virulent strain for this concentration was the strain S6 of *Fusarium* sp. with 100% of mortality followed by the strains S5 and S13 of *A. niger* and *Scopulariopsis* sp (S11), *Fusarium* sp. (S16) with mortalities of up to 92%. In a second range, the strains of *Beauveria bassiana*, *Aspergillus fumigates* and the unidentified species *Epicoccum* sp. (S3), *Scedosporium* sp. (S12) and *Fusarium* sp. (S4 and S14) showed mortality rates ranged from 77% to 84,6%. The lowest mortality level

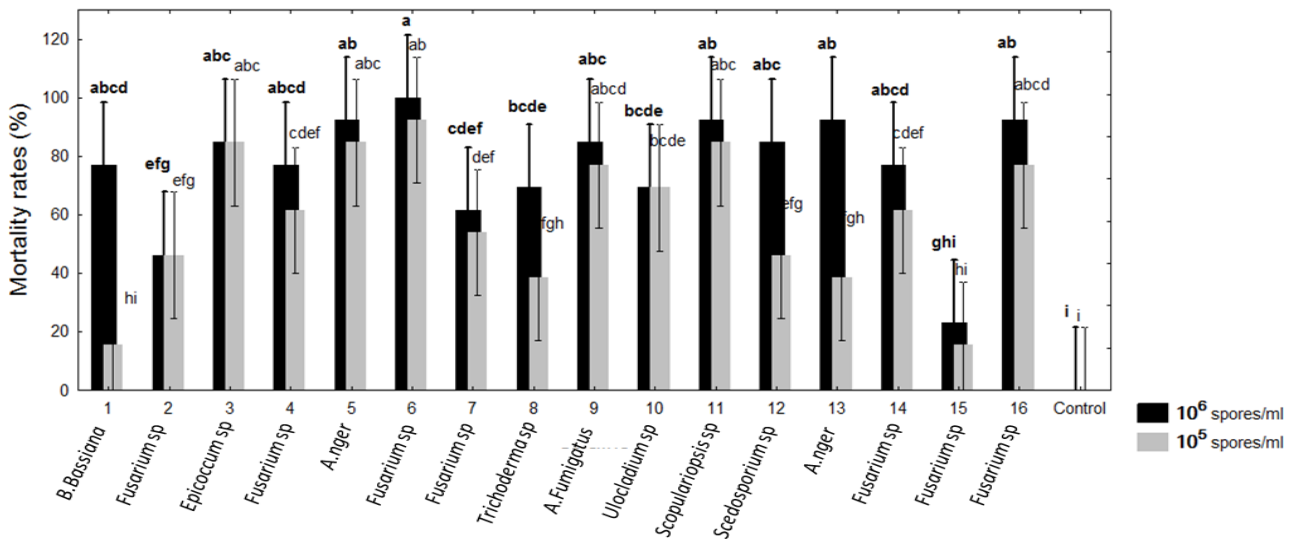


Fig. 4 Mortality rates recorded by tested strains for the two concentrations used, the vertical bars represent the CI (confidence intervals) at 0.95.

for this concentration was recorded by the strain S15 of *Fusarium* sp. with no significant difference from the control.

This work may be considered as the first record of the high pathogenicity of species of *Fusarium*, *Epicoccum*, *Scopulariopsis* and *Aspergillus niger* against *C. capitata*. *Fusarium* species were considered as saprophytic fungi [35] and also reported to be pathogenic to *Aphis gossypii* Glover [30], *Scirtothrips dorsalis* Hood [40, 41], *Polyphagotarsonemus latus* Banks [40], *Aleurodicus dispersus* Russel [42] and to other pests [37]. Species of *Aspergillus* have been found associated with a large number of insects such as larvae of bees (*A. niger*), *Troglophilus neglectus* (*A. niger*, *A. ochraceus*), and *Culex pipiens* (*A. ochraceus*) [38, 39, 43]. *Beauveria Bassiana* is common in *Argania spinosa* soil and can easily be grown in culture [15, 16]. It has also been found to be pathogenic to the olive fruit flies *B. Oleae*, adults of *C. capitata* and other pests [8, 14, 15].

Our results confirm the virulence of *Beauveria Bassiana* against larvae and pupae *C. capitata* with more than 75% of mortality for the concentration of 10⁶ spores/mL. Two way ANOVA results showed the absence of a significant effect for the combination between the two tested factors (Strain*Concentration)

on the mortality levels ($p = 0.1187$). This result could be explained by the fact that the virulent strains keep their high levels of mortality for the two tested concentrations excepting for *Beauveria Bassiana*.

3.2.2 The Lethal Time 50 (LT₅₀)

In order to get reliable data, the time required to kill 50% of treated insects or the lethal time 50 (LT₅₀) should be taken into account. During pathogenicity tests, mortalities rates were recorded everyday for 12 days. The results showed that LT₅₀ varies depending on strains and concentrations of the used suspensions as represented in Table 2. The shortest LT₅₀ at the concentration of 10⁶ spores/mL were recorded by the strain *Epicoccum* sp. (1.48 days) followed by *Scopulariopsis* sp (S11) *Scedosporium* sp. (S12) with less than 77 hours. In contrary strains S2, S7 of *Fusarium* sp. and S8 of *Trichoderma harzianum* required more than 200 hours to reach 50% mortality. While *Fusarium* sp. S15 did not get mortalities up to 50% at this concentration. As for the concentration of 10⁵ spores/mL, 50% of mortalities were reached after 94 hours by *Fusarium* sp. (S7), followed by *Epicoccum* sp. S3 with less than 114 hours (4 days 18h); whereas treatment with *Fusarium* sp. S6 and *Scopulariopsis* sp. S11 induced the same mortalities after 130 hours (5j 10 h). While *Fusarium* sp. S15 and *Beauveria bassiana*

Table 2 The lethal time 50 (LT₅₀) recorded by tested strains.

Strains	Concentration (s/ml)	LT50 (Days)	LT50 (d/h/m)	Strains	Concentration (s/ml)	LT50 (Days)	LT50 (d/h/m)
S1	10 ⁶	6.76	6 d 18 h 14 min	S9	10 ⁶	4.50	4 d 12 h 00 min
	10 ⁵	-----	-----		10 ⁵	4.86	4 d 20 h 38 min
S2	10 ⁶	8.80	8 d 19 h 12 min	S10	10 ⁶	4.50	4 d 12 h 00 min
	10 ⁵	10.34	10 d 8 h 9 min		10 ⁵	5.73	5 d 17 h 31 min
S3	10 ⁶	1.48	1 d 11 h 31 min	S11	10 ⁶	3.22	3 d 5 h 17 min
	10 ⁵	4.77	4 d 18 h 29 min		10 ⁵	5.35	5 d 8 h 24 min
S4	10 ⁶	4.09	4 d 2 h 9 min	S12	10 ⁶	3.22	3 d 5 h 17 min
	10 ⁵	8.23	8 d 5 h 31 min		10 ⁵	10.74	10 d 17 h 45 min
S5	10 ⁶	5.99	5 d 23 h 46 min	S13	10 ⁶	4.68	4 d 16 h 19 min
	10 ⁵	7.16	7 d 3 h 50 min		10 ⁵	13.16	13 d 3 h 50 min
S6	10 ⁶	6.21	6 d 5 h 2 min	S14	10 ⁶	4.52	4 d 12 h 28 min
	10 ⁵	5.82	5 d 19 h 41 min		10 ⁵	7.83	7 d 19 h 55 min
S7	10 ⁶	9.69	9 d 16 h 33 min	S15	10 ⁶	-----	-----
	10 ⁵	3.93	3 d 22 h 19 min		10 ⁵	-----	-----
S8	10 ⁶	9.17	9 d 4 h 5 min	S16	10 ⁶	3.18	3 d 4 h 19 min
	10 ⁵	6.48	6 d 11 h 31 min		10 ⁵	5.99	5 d 23 h 45 min

(S1) did not get mortalities up to 50% at this concentration. The obtained results showed that strains with high mortality levels have also short LT₅₀. The correlation coefficient between the mortality rates and the LT₅₀ obtained was negative (R = -0.786).

This result justifies the short LT₅₀s obtained by the strains which showed a high efficiency. Indeed, the virulent strains reached 50% of mortality more faster [15].

4. Conclusions

During this study we have isolated several entomopathogenic fungi from soil samples taken from the Souss-Massa *Argania spinosa* forest using the baiting method. As expected, our results confirm the presence of different entomopathogenic fungal species in the Souss-Massa *Argania spinosa* forest soil and the most frequent genera were *Fusarium* (44.8%) and *Aspergillus*. These indigenous strains showed high levels of pathogenicity against the *C. Capitata* larvae and pupae and are an option to control *C. capitata*. Despite the fact that our experiments *in vitro* do not simulate the real conditions of the natural environment and soil, the obtained results are useful in indicating the potential of the local strains of entomopathogenic fungi

against *C. capitata* puparia and larvae. Nevertheless, the application of these strains in the medfly control necessities more studies in order to understand the factors controlling their effectiveness and their survival in the natural environment and the key factors affecting their migration and retention in the soil. An *ex situ* test is also needed before considering a large-scale application. Furthermore, the possibility of mixing these agents with pesticides in integrated pest management programs could be tested.

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