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Biological control of *Ectomyelois ceratoniae* Zeller (Lepidoptera: Pyralidae) using the egg parasitoid *Trichogramma cacoeciae* Marchal (Hymenoptera: Trichogrammatidae) in a Tunisian citrus orchard

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Abstract
The carob moth, *Ectomyelois ceratoniae* (Lepidoptera: pyralidae) is a polyphageous pest that ingest several important economic host plants in Tunisia such as dates and pomegranates. During the latest years, damages on citrus fruits were reported in Tunisian citrus orchards. As this pyralid has an endophytic behavior which makes its control with insecticides inefficient, biological control could be a crucial tool in the fight against it. For that reason, our study was conducted to investigate the efficacy of an oophagous parasitoid, *Trichogramma cacoeciae,* for biological control of this pest thanks to three releases conducted in a Tunisian citrus field. Results showed a significant difference between laboratory and field conditions for both emergence and parasitism rates of *Trichogramma cacoeciae*. Moreover, a significant difference in the infestation rates at harvest between the control and the two treated blocks after *T. cacoeciae* releases in the citrus field was obtained, thus the efficacy of this parasitoid was about 44.5% at harvest. Several factors that affect *T. cacoeciae* efficacy in the citrus field were discussed. However, despite the infestation level decrease in the treated blocks, this rate was not sufficient to limit economic losses suggesting that, this biological agent could be used as a part of an integrated pest management program of this pest.

Keywords: biological control, citrus, *Ectomyelois ceratoniae***,** oophagous parasitoid, Tunisia

1. Introduction
Biological control is a crucial tool in the fight against crop pests. It is defined as the reduction of plant enemies' populations by natural agents and typically involves an active human role (Holmes *et al.* 2016) [1]. The aim of this method is to reduce populations of pest organisms below levels that would have a negative economic impact (Bale *et al.* 2008) [2]. Biocontrol measures included the use of natural enemies such as predators, microbes, beneficial nematodes and parasitoids (Mahr *et al.* 2008) [3]. The larvae or pupae of the latter group feed exclusively on other arthropods, mainly insects, resulting in the death of the host (Eggleton *et al.* 1990) [4]. Parasitoids represent an extremely diverse group, though mainly belonging to Hymenoptera. Trichogrammatids (Chalcidoidea: Trichogramma) are the most widely used egg parasitoids to control several lepidopterous pests such as *Cydia pomonella* (Lepidoptera: Tortricidae), *Helicoverpa armigera* (Lepidoptera: Noctuidae) *Chilo suppressalis* (Lepidoptera: Crambidae) (Li, 1994; Mansfield *et al.* 2002; Oztemiz *et al.* 2009; Yuan *et al.* 2012) [5-8].
These wasps lay their eggs in pests' eggs and develop at their expense, eliminating them even before they began to cause damage. This is an important asset, which justifies the place granted to them in the biological control (Hawlitzky, 1992) [9].
Ectomyelois ceratoniae (Lepidoptera: Pyralidae), known as the carob moth, has a cosmopolitan distribution. In Tunisia, it is found in the north as well as in the south of the country. It is a polyphageous insect [10], which ingests several important economic host plants in Tunisia such as dates (*Phoenix dactylifera*), pomegranates (*Punica granatum*) and recently citrus (*Citrus sinensis*) (Dhouibi, 1989; Hached *et al.* 2017) [11, 12]. The endophytic behavior of this pyralid, makes his control with insecticides inefficient (Dhouibi *et al.* 2000) [13]. Taking into account this barrier, other strategies were tested to control this pest such as autocidal control in a Tunisian pomegranate orchard (Mediouni, 2005) [14]; releases of parasitoids like *Phanerotoma ocularis* (*Hymenoptera: Braconidae*) (Khoualdia *et al.* 1996a) [15] and *Habrobracon hebetor* (Hymenoptera: Braconidae) (Dehliz *et al.* 2016) [16] were carried out in date palm fields.

Trichogramma cacoeciae (Marchal) (Hymenoptera: Trichogrammatidae) is a thelytokous species with a broad geographic distribution (Pinto, 1999) [17]. It is mainly found in arboreal habitats and orchards. In Tunisia, a native strain selected by (Khoualdia *et al*. 1995) [18], has proven efficacy to control *E. ceratoniae* on palm dates (Khoualdia *et al.* 1996b) [19] and in pomegranate and citrus orchards (Lebdi and Ben Ayed, 2005; Dhouibi *et al*. 2016) [20, 21]. In this order, and with the scarce information about its efficiency in citrus orchards, this work was conducted to evaluate the power of *T. cacoeciae* to control *E. ceratoniae* in a Tunisian citrus field.

2. Materials and Methods
2.1. Experimental site
The study was conducted in 2015 in an old citrus orchard located in Mornag, the governorate of Ben Arous (North-Eastern region of Tunisia). The field covered an area of 0.9 ha planted with 174 trees with a line spacing of 7 x 7 m.

2.2. Rearing of *Trichogramma cacoeciae*
Ultraviolet-irradiated eggs of a substitute host, *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) were used for maintaining and rearing *T. cacoeciae* in the laboratory [20]. These eggs were sprinkled on pieces of carton paper (40 x 15 mm) which were previously painted in one side with an (Arabic Gum) (10%) to glue them. These cardboards were provided by a private company (ControlMed, Tunisia).
Then, carton papers were kept in culture glass tubes plugged with cotton containing *Trichogramma* adults, which will lay on it. Glass tubes were placed in an acclimatized room (16L: 8D) under constant conditions of temperature (25 ± 1 °C) and relative humidity (60± 10%). The obtained parasitized eggs were kept in glass tubes until hatching.
Upon their emergence, a drop of honey was placed in the tube to feed the wasps. These adults were provided with newly laid *E. kuehniella* eggs.

2.3. *Trichogramma cacoeciae* releases in the field
For proper timing of treatments, the flight activity of *Ectomyelois ceratoniae* was monitored by Delta pheromone traps (ISCA® Technologies) baited monthly with the sex pheromone citrus moth. Two traps were installed in the control block at a height of 1.5m of citrus trees and were checked weekly for captures of males. Three inundative releases of *T. cacoeciae* were conducted, early in the morning, on August 24th, September 7th and November 3rd to synchronize treatments with the egg-laying period of the citrus moth (Figure 1).

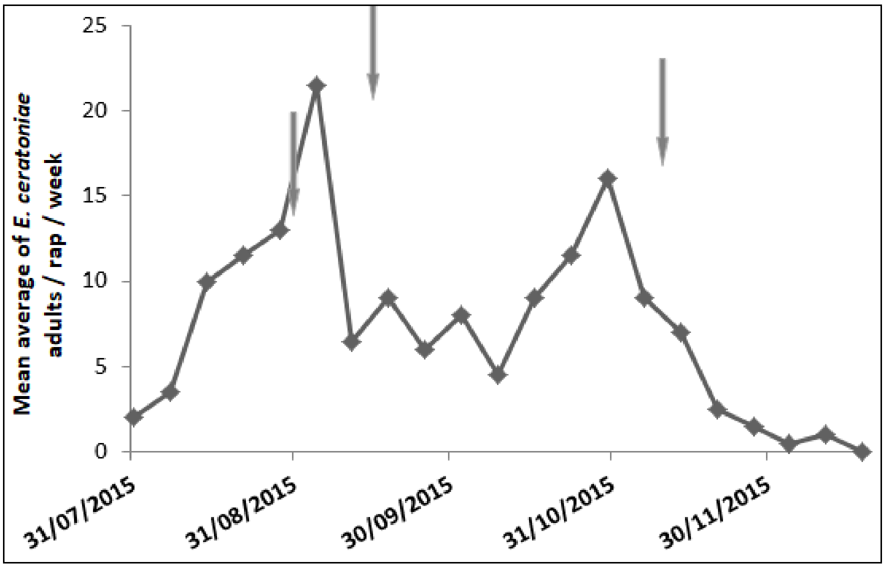


Fig 1: Flight activity of *E. ceratoniae* adults and dates of *Trichogramma* releases (indicated by the arrows) in the citrus orchard in Mornag region in 2015

The releasing units were cardboard cards each with about 1 000 *E. kuehniella* eggs parasitized by *T. cacoeciae*. Each carton paper was placed in a mosquito netting (with mesh sizes of 2 mm^2 which allows the escape of emerging parasitoids) and hanged to each tree belonging to the treated block by a thread soaked in glue to prevent any predation by ants. These carton papers were placed on the interior terminal branches of the citrus tree, at a height of 1.5m, to protect the parasitized eggs from varying temperature conditions at the outer area of the canopy.
Three cards were placed in each tree (in the northern east, southern east and southern west direction) to apply 3000 wasps per tree per release. A total of thirty trees were used for the assay. Ten trees constituted an elementary block: one for control and two for the treatment.
A total of thirty cardboard cards were used per *Trichogramma* release per treated block.At the end of the whole treatment, each treated tree has received 9000 *E. kuehniella* eggs parasitized by *T. cacoeciae*. A total of 180 000 wasps were released in the field (twenty treated trees). That means that one hectare (≈ 200 trees) of the targeted orchard received ca. 1 800 000 females of the parasitoid during the season.
Two cardboard cards which contained 1000 unparasitized *E. kuehniella* eggs were placed weekly in the field since June to check the presence of native parasitoids in the citrus orchard.

2.4. Emergence rate of *Trichogramma cacoeciae*
In order to determine the emergence rate of *T. cacoeciae,* the carton papers were recuperated ten days after each release and inspected under a binocular microscope. Ten cardboards were evaluated under laboratory conditions to compare the emergence rate between controlled and open field conditions. The emergence rate was calculated as follows:

$$\text{Emergence rate} = \frac{\text{Number of hatched eggs of } E.\ kuehniella}{\text{Total number of released eggs}} \times 100$$

2.5. Estimation of *Trichogramma* parasitism rate in the field

The parasitism rate was estimated by installing cardboard cards containing 1000 *E. kuehniella* unparasitized eggs put in mosquito netting and attached to the treated trees. Each treated citrus tree received one cardboard card placed at the trunk at a height of 1.5m. These cards were installed the day of each release and one week after. This rate was also calculated under laboratory conditions using ten cardboard papers.
The parasitism rate was calculated as follows:

Parasitism rate = (Total number of parasitized eggs of *E. kuehniella*/total number of eggs recorded) x 100

2.6. Effectiveness of *T. cacoeciae* to control *E. ceratoniae* in the citrus field

The efficacy of the parasitoid to control the pyralid, was evaluated by the examination of all the dropped fruits from each tree belonging to the experimental plot (Control + treated). The fallen fruits, from every single tree, (attacked by the moth and safe) were collected separately and examined the day of the first release then weekly until harvest (mid-December 2015). Damaged fruits were counted as a percent of the total number of citrus fruits in each tree.
The success of *Trichogramma* releases was evaluated by the decrease of pest density using the formula according to (Henderson and Tilton, 1955) [22].

% efficacy = (1- (Ta/Ca). (Cb/Tb)) x 100

Where: Tb is the infestation in the treated plot before application
Ta is the infestation in the treated plot after application
Cb is the infestation in the check plot before application
Ca is the infestation in the check plot after application
The infestation level of *E. ceratoniae* in the three blocks (control + block 1 + block 2) was equal in the start of the experiments. In fact these blocks have the same method for crop management thus Cb was equal to Tb so (Cb /Tb) = 1.

2.7. Climatic data

In Mornag, the mean temperature ranged from 28.8 °C (August) to 17.6 °C (November) and the mean relative humidity ranged from 65% (August) to 75% (November) (Table 1).

Table 1: Monthly average of temperature (°C) and relative humidity (%) in Mornag 2015

	July	August	September	October	November	December
T°	29.1	28.8	26.6	22.8	17.6	13.6
RH°	55	65	62	69	75	81

(The Tunisian Meteorological Institute)

2.8. Statistical analyses

The obtained data corresponding to *T. cacoeciae* emerged adults from *E. kuehniella*, the parasitism rate of *T. cacoeciae* on *E. kuehniella* eggs and the infestation rate of *E. ceratoniae* on citrus fruits were analyzed using one-way ANOVA. Means of treatments were separated using Duncan's multiple range test at *P* = 0.05. All statistical analyses were performed using the software SPSS 16, SPSS Inc. (2007).

3. Results and Discussion

3.1. Emergence rate of *Trichogramma cacoeciae*

The determination of the emergence rate gives an idea about the real emerged number of wasps in the field. On average, the number of emerged adults during the three releases was 595, 8/1000 parasitized eggs, which means 59, 58% of the released eggs emerged in wasps. The results are given in Table 2. A significant difference in the emergence rate between the control (under laboratory conditions), the first and the second release compared to the third one (*F = 18.441, df = 189, P = 0.000)* was recorded. The difference between laboratory and field conditions could be explained by several factors such as the quality and the storage conditions of *E. khueniella* eggs used to rear these wasps (Blibech *et al.* 2010; Ozder, 2004) [23, 24].

Table 2: Mean number (± SE) of *T. cacoeciae* adults emerged from parasitized eggs of *E. kuehniella* and the respective emergence rate (%) under laboratory conditions and after ten days of each release

	Number of emerged adults/Number of parasitized eggs recorded	Emergence rate
Laboratory conditions	904, 5 ± 26,8 c	90.45%
Release 1	627, 2 ± 127, 1 b	62.72%
Release 2	641, 2 ± 152, 7 b	64.12%
Release 3	518, 9 ± 206 a	51.89%

Means followed by a different letter in the same column differ significantly at the 5% level of significance (Duncan's multiple range test).

Climatic factors such as rain and temperature could play a key role to explain this difference. Indeed, the rainfall recorded in the first week of November has caused leaching of the egg-cards (can reach ≈ 97% on two cardboard papers). Fallen rains dislodge many eggs causing their falls and then their destruction on the ground, which led to the decrease of the emergence rate between the two first releases and the latest one. Also, the optimal temperature to rear *T. cacoeciae* on the alternative host (*E. kuehniella*) is 25 °C while our experiments were carried out on August (28.8 °C) and November (17.6 °C). Nevertheless, previous studies conducted by Pizzol *et al.* (2010) [25] and Zouba (2016) [26] demonstrated that emergence rates were stable at the four temperatures tested (15, 20, 25 and 30 °C) on *T. cacoeciae* reared on *E. kuehniella.* Besides, Krechemer and Foerster (2015) [27] have shown that the emergence of three *Trichogramma* species *T. pretiosum*, *T. atopovirilia* and *T. acacioi* reared on *Trichoplusia ni* (Lepidoptera: Noctuidae) eggs were not affected by the same temperatures tested. Consequently, this factor could not be considered. Finally, we noticed that cardboard papers were attacked by several predators despite all the precautions taken (mosquito netting and the glue) which corroborate with Pereira *et al.* (2004) [28] who have demonstrated that parasitized eggs of *T. cacoeciae* can be attacked by different ants and predators mainly (Hymenoptera: Formicidae) where the percentage of predation reached 97.8% seven days after releases.

3.2. Estimation of the parasitism rate

Parasitized host eggs turned black during the third instar of parasitoids larvae (Vinson and Iwantsch, 1980) [29]. The parasitism rate is defined as the number of blackened eggs compared to the number of offered ones. The results as described in Table 3.a showed a significant difference in the parasitism rates between laboratory and field conditions (*F = 420.186, df = 15, P = 0.000)*. In fact, it ranged between 88.2% and 23% respectively. This weakness of field performance due to the parasitoid difficulty to locate the host egg can be influenced by environmental factors such as wind as shown by (Yu *et al.*1984) [30] who confirmed that wind affects adult dispersal. In addition, low temperatures and rain can reduce searching and parasitism Suh (1998) [31]. Moreover, Pizzol *et al.* (2010) [25] confirmed that humidity and photoperiod could influence the parasitism effectiveness by *Trichogramma* parasitoids.

Table 3: Mean number (± SE) of parasitism rate of *T. cacoeciae* on *E. kuehniella* eggs

a. Comparison between laboratory and field conditions

	Laboratory	Field
Parasitism rate of *T. cacoeciae* on *E. kuehniella*	88.2%±0.060 a	23%±0.064 b

Means followed by a different letter in the same line differ significantly at the 5% level of significance (Duncan's multiple range test).

b. Comparison between the three releases under field conditions

Period of check	Parasitism rate
Release 1	22 ± 1% a
Release 2	30.5 ± 2.5% b
Release 3	17±1% a

Means followed by a different letter in the same column differ significantly at the 5% level of significance (Duncan's multiple range test).

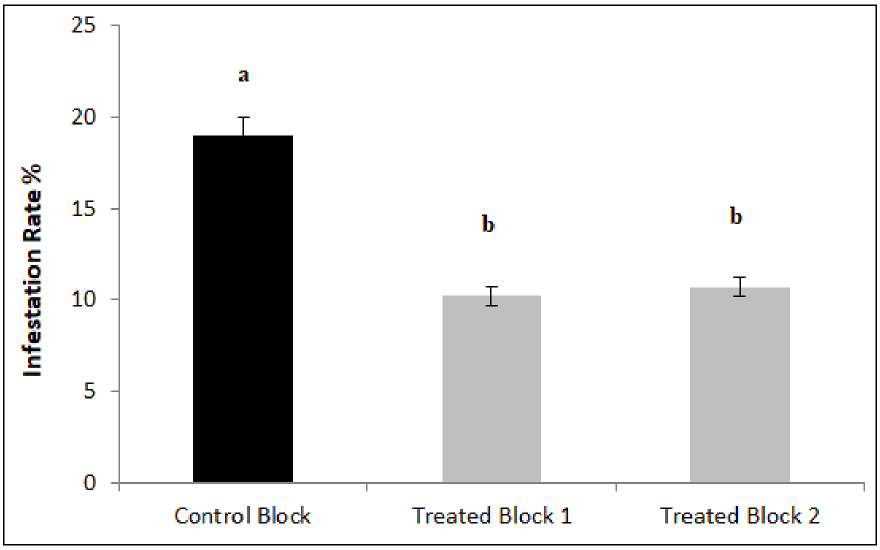
Besides *Trichogramma* species use various semiochemicals including host kairomones in their search for host eggs Noldus (1989) [32] which can be affected under field conditions. Furthermore, we noticed that there was a significant difference in parasitism rates between the three releases (*F = 16.936, df = 5, P = 0.023*). Indeed the Table 3.b showed that this parameter was more important in the second release (30.5%) compared to two others (22 and 17%). However, compared to previous studies, in arboreal orchards, the parasitism rates recorded in these releases were relatively low. In fact, in a trial conducted by (Khouladia *et al.* 2008) [33] in a Tunisian palm field, the parasitism of *T. cacoeciae on E. ceratoniae* eggs reached 78.7%. Applied on the cherry bark tortrix (*Enarmonia farmosana*) the parasitism levels of *T. cacoeciae* ranged between 72 and 74% in cherry orchards (Breedveld and Tanigoshi, 2007) [34].

Codling moth, (*Cydia pomonella*) eggs parasitized by *T. cacoeciae* reached respectively 45% and 31% in Crimean mountain orchards and in India (Dyadechko *et al.* 1978; Pawar *et al.* 1980) [35, 36]. The parasitization level of Grapevine Moth (*Lobesia botrana*) eggs with *T. cacoeciae* in vineyard plots reached 45 and 50% respectively for the first and the second generation of the moth in the reared plots (Hommay *et al.* 2002) [37]. These findings suggested that field performance could be affected by the size and the structure of the crop besides the host egg size (Bigler *et al.* 1997; Roriz *et al.* 2006) [38, 39]. Other *Trichogramma* species were evaluated on *E. ceratoniae* eggs. In Algerian palm fields, the parasitism rates of *Trichogramma embryophagum* and *Trichogramma cordubensis* were about 45% and varying from 47 to 64% respectively (Doumandji-Mitiche and Idder, 1986; Idder *et al*. 2009) [40, 41]. In Iranian pomegranate orchards, Mirkarimi (2000) [42] confirmed that thanks to releases of *Trichogramma embryophagum*, the percentage of parasitized eggs increased from 17.5% under natural conditions to 53.1% in the mass release plot.

3.3. Evaluation of *T. cacoeciae* efficacy against *E. ceratoniae* in the field

The flight activities of *E. ceratoniae* as well as the dates of releases are shown in (Figure 1). Releases started with citrus moth the second generation, we noticed that during 2015 the adults flight activity started later than the previous crop years (data not shown). In fact, the second and the third generations of the pest, which were the most important (in terms of a number of adults caught in traps), extended from the 31st July to 11th September. Two other generations have followed and extended to the end of December. Thus, releases were effectuated three days after the peak of the most important generations (the second, the third and finally the fifth) which corresponded to the egg-laying period.

We remarked that before *Trichogramma* releases, there were no significant differences in fruit damages between untreated and treated blocks. Besides, the cardboard cards which contained unparasitized *E. kuehniella* eggs indicated the absence of native parasitoids in the field. Results as described in (Figure 2) showed a significant difference in the infestation rates between the control and the two treated blocks (*F = 16.250, df = 29, P = 0.000)* after *T. cacoeciae* releases in the citrus field. Indeed the infestation, at harvest, was higher in the untreated block (19.03%) compared with the two treated blocks in which it was similar (10.56% on average). The efficacy of this parasitoid obtained by Henderson and Tilton (1955) [22] was about 44.5% at harvest.



Means followed by a different letter for bars differ significantly at the 5% level of significance (Duncan's multiple range test).

Fig 2: Infestation rates variability between the control and two treated blocks after *T. cacoeciae* releases in a citrus orchard in Mornag region in 2015

Several factors may influence the efficacy of *Trichogramma* releases in the citrus orchard. Firstly, the choice of the strain as advised by Hommay *et al.* (2002) [37] who confirmed that autochthonous *Trichogramma* strains are generally better adapted to local conditions and are the favored option for the biological control of pests. This factor justifies our application of the Tunisian strain *T. cacoeciae* which has proven its efficacy in the control of *E. ceratoniae* both in palm and pomegranate field. Secondly, the dose of released *Trichogrmama:* in this trial, the number of *E. kuehniella* parasitized eggs released was 9000 per tree but only ca. 5362 have emerged on wasps (The emergence rate obtained was 59.58%) that means that in one targeted ha which had received 1 800 000 *E. kuehniella* parasitized eggs, only 1072440 *T. cacoeciae* were released. Different doses of *T. cacoeciae* were tested in Tunisian fields against *E. ceratoniae*. In palm date fields, Khoualdia *et al.* (2008) [33] by releasing 20 000 wasps per palm date hectare, obtained 78.7% of parasitized eggs. In pomegranate fields, Lebdi and Ben Ayed (2005) [20] by releasing 15000 *T. cacoeciae* per pomegranate tree, decreased the infestation rate from 13.2% to 2.4% respectively in control and treated plots at harvest. Besides, Dhouibi *et al.* (2016) [21] by releasing 25000 of these wasps per pomegranate hectare, the infestation level decreased from 82% to 22%. In citrus orchards, Dhouibi *et al.* (2016) [21] demonstrated that the release of *Trichogramma cacoeciae* (300 eggs/tree) lowered the infestation level to ≈ 8%.

Several studies have evaluated the efficacy of this parasitoid; on *Cydia pomonella* in apple orchards, Stein (1960) [43] confirmed that pest damage decreasing was about 35-65% during normal season, but under high host density population and very hot weather the codling moth reduction was only 10-45%. Besides, Almatni *et al*. (2002) [44] obtained an efficacy of 34.37% (by releasing 300 parasitoid /apple tree).While Sakr *et al.* 2000 [45] obtained the highest efficacy with 80.6%. Against the grape vine moth, *Lobesia botrana* in French vineyards, *T. cacoeciae* provided moderate success (Barnay *et al.* 2001) [46]. Thirdly, the frequency of parasitoid releases depends on the availability besides the length of time in which parasitoids must remain actively foraging (Smith, 1994) [47]. Finally, the artificial conditions of mass rearing can select for genetic changes that reduce the effectiveness of the *Trichogramma* in the field (Bigler, 1994) [48].

These findings suggest that the applied dose was high enough to control effectively *E. ceratoniae* in the citrus field. However, the efficacy of the selected parasitoid even against the same pest depends on the crop type. In addition, Li (1994) [5] pointed out that the total number of released parasitoids should be large enough to control the population of target insect pests at the permissible economic level, which varies depending on the type and growth of crops, climate, species and density of pest.

Several parasitoids were tested to control *E. ceratoniae*. In pomegranate orchards, the parasitism rate of *H. hebetor* reached 55% and 95% respectively (Dhouibi and Jemmazi 1993; Dhouibi *et al.* 2000) [49, 13]. By releasing *T. embryophagum* the percentage of damaged pomegranate fruits at harvest, was reduced from 72.2% in the control plot to 24.2% in the treated plot (Mirkarimi, 2000) [42]. In the oases of Tozeur, Khoualdia *et al*. (1996.a) [15] pointed out that the parasitism rate of *Phanerotoma ocuralis* reached approximately 80%.

4. Conclusion

In conclusion, the decrease of the infestation level in the treated blocks was not sufficient to limit economic losses suggesting that, this biological agent could be used as a part of an integrated pest management program of this pest. Further studies are needed to test doses parasitoids in citrus orchards.

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