Efficacy of three entomopathogenic agents for control the tomato borer, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae)

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Abstract: In This study, efficiency of four concentrations (10^7; 10^8; 10^9; 10^{10}) of *Bacillus thuringiensis* var. *kurstaki* (Btk), *Beauveria bassiana* and *Metarhizium anisopliae* were prepared and tested on *T. absoluta* larvae (Neonate "newly hatched", 2nd & 3rd instar) to study the effect of these agents on larval mortality. In addition, eggs of *T. absoluta* were exposure to *B. bassiana* and *M. anisopliae* to evaluate their effect on hatchability under laboratory conditions. Results showed that; the estimated LC_{50} of Btk was 3.25 x 10^6, 5.47 x 10^6 & 3.28 x 10^6 spores/ml for neonate, 2nd instar & 3rd instar of *T. absoluta* larvae, respectively. While the LC_{50} values of *B. bassiana* and *M. anisopliae* were (0.28 x 10^6 & 0.11 x 10^6), (0.45 x 10^6 & 0.46 x 10^6) and (0.32 x 10^6 & 0.27 x 10^6 conidia/ml) for neonate, 2nd instar & 3rd instar *T. absoluta* larvae, respectively. According to LC_{50} values, *B. bassiana* and *M. anisopliae* were most effective on larval phase of *T. absoluta* than Btk. The effect of pathogen application was dependent on the instar phase at which the larvae were fed on pathogen-treated leaves also, the greatest percentage of mortality occurred on the neonate larvae for three agents. Concerning the most effective concentration of each agent, the higher concentration (10^{10}) the higher mortality. The greatest percentage of mortality occurred in the newly hatched "neonate" followed by the third instar larvae when fed with leaves treated with Btk. Also, the greatest percentage of pathogen effect occurred in the newly hatched "neonate" followed by the second and the third instar larva which gave similar larval mortality values when larvae fed with leaves treated with *B. bassiana* and *M. anisopliae*. After exposure of the eggs to the three agents, the pathogen effect was evident by the fourth day of evaluation after exposure in the four concentrations. The black appearance of eggs of *T. absoluta* took place after the exposure to the four concentrations of the two pathogenic fungi starting from the day five after exposure, and thereby no hatched larvae were detected in the four concentrations of the two pathogenic fungi comparing the control where the hatchability reached 87.7% and 80.0% during the evaluation time for *B. bassiana* and *M. anisopliae,* respectively. Thus our laboratory experiment suggested that *B. bassiana* and *M. anisopliae* has a potential effect on both egg and neonate "newly hatched larvae" of *T. absoluta* followed by Btk where was had moderate effect on neonate and third larval stage.

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1. Introduction:

The tomato borer, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), first described in Peru in 1917, is now found throughout South America, where it is considered to be one of the most devastating pests for tomato crops (**Barrientos *et al.*, 1998; Estay, 2000**; **EPPO, 2006**). In Spain, this pest was first detected at the end of 2006 in the north of Castellon (Eastern Spain) (**Urbaneja *et al.* , 2008b**). During 2007, *T. absoluta* was detected in several locations throughout the Spanish Mediterranean Basin, the most important tomato growing region in the country. Since then, its presence has also been confirmed in Algeria, Canary Islands, France, Italy, Morocco, and Tunisia in 2008, and in Albania, Bulgaria, Cyprus, Germany, Malta, Portugal, Switzerland, The Netherlands, and the United Kingdom in 2009 (**Desneux *et al.* , 2010**; **EPPO, 2010**). The tomato leafminer *T. absoluta* (Meyrik) is one of the most devastating pests of tomato in South America (**Barrientos *et al.* ,1998)**. This pest was initially reported in estern Spain in late 2006 (**Urbaneja *et al.* , 2007)** and has subsequently spread throughout the Mediterranean Basin and Europe **(Potting, 2009)**. Since the time of its initial detection, the pest has caused serious damages to tomato in invaded areas **(Germain *et al.* , 2009)** and it is currently considered a key agricultural threat to European and North African tomato production. If no control measures are taken, then the pest can cause up to 80-100% yield losses by attacking leaves, flowers, stems and especially fruits **(Lopez, 1991)**.

The current management of *T. absoluta* in the Mediterranean Basin is mainly based on treatments with chemical insecticides. Nevertheless, few active ingredients are effective against *T. absoluta* and selective to beneficial and pollinators at the same time.

Therefore, integration with other control methods (cultural, biological and biotechnological methods) becomes imperative, as the continued use of chemical insecticides could harm non-target organisms **(Landgren *et al.* , 2009)** and the environment. Also, prolonged use could lead to resistance. Few studies have evaluated the efficacy of *B. thuringiensis* on *T. absoluta*, although 3000 species, belonging to 16 orders of insects, have been reported as susceptible to *B. thuringiensis* **(Huang *et al.*, 2004)**. Commercial formulates based on this bacterium have been used for decades to control insect pests as an alternative to chemicals. Most of the studies that focused on the effect of *B. thuringiensis* on *T. absoluta* have been performed in the region of origin of *T. absoluta* **(Niedmann and Meza-Basso 2006). Giustolin *et al.* (2001)** found that Btk can cause mortality in all *T. absoluta* instars and that the use of Btk has synergistic or additive effects when applied to tomato resistant genotypes. A pathogenicity study of 64 *M. anisopliae* var. *anisopliae* and 70 *B. bassiana* isolates against tomato moth *T. absoluta* eggs, was carried out under laboratory conditions. The first evaluation was accomplished by spraying suspensions of 10^7 conidias ml^{-1} of each isolate directly on eggs, through a Potter tower. Mortality and conidia production on the eggs were significantly higher with the isolates *M. anisopliae* Qu-M558 and *B. bassiana* Qu-B911, Qu-B912 and Qu-B928 **(Marta Rodríguez *et al*., 2006).** The objective of this study was to estimate the susceptibility of *T. absoluta* larvae to Btk, *B. bassiana* and *M. anisopliae*, and the effect of *B. bassiana* and *M. anisopliae* on egg hatchability.

2. Material and Methods

Tomato plants

Tomato seeds (Castle Rock) was sown in nursery in 209 cell foam trays and kept for 45 days until transplanted to the laboratory under conditions (24±3 °C, 65±5% R.H.) at Plant Protection Research Institute, Dokki, Giza. Seedlings of 45 days old were transplanted in 30 cm diameter plastic pots containing a sterilized soil- peat moss mixture, one seedling per pot. Pots were held in rearing cages (60 cm^2 high, 50 cm^2 wide, 50 cm^2 long).

Tuta absoluta colony

A laboratory colony of *T. absoluta* was established with pupae from field strain. This colony was maintained in the laboratory. Pupae were dislodged from leaves and were housed in a wooden and nylon cage. Adults were fed on 10% honey solution (Taphla leaves were used as a carrier for honey droplets as a food source for adults) and provided with tomato terminal buds and leaves for oviposition overnight so that *T. absoluta* pupation could take place either on leaves or on soil. When pupation was completed, the cocoons were carefully collected to be used for starting the experimental. *T. absoluta* adults were reared on tomato plants (45 days old). Tomato plants were placed in Pots and held in rearing cages (60 cm^2 high, 50 cm^2 wide, 50 cm^2 long) provided weekly by seedlings for feeding and egg laying. When required for our assays, newly emerged adults were collected using an aspirator **(Fargalla and Shalaby, 2013).**

Materials used:

1- *Bacillus thuringiensis* var. *kurstaki* (10^7; 10^8; 10^9; 10^{10} spores/ml)

2- *Beauveria bassiana* (10^7; 10^8; 10^9; 10^{10} conidia/ml)

3- *Metarhizium anisopliae* (10^7; 10^8; 10^9; 10^{10} conidia/ml)

1-Entomopathogenic bacteria:

In this study, one strain of *Bacillus thuringiensis* was used as follow:

Btk was kindly provided from Insect Pathogen Production Unit, plant protection Research institute, ARC, Ministry of Agriculture, Egypt.

Culture conditions of *Bt*:

Culture conditions of *Bt* were carried out according to **Attathom *et al.* (1995)** as follows:

T3 medium was prepared which composed of tryptone 3.0g (Sigma), tryptose 2.0g (Sigma), yeast extract 1.5g (Sigma), $MnCl_2$ 0.005g (Sigma) and NaH_2PO_4. H_2O 8.9g (Adwic), adjusted pH to 6.8 and the final volume was made up to 1 liter with distilled water. The medium was sterilized at 121°C for 20 min, and inoculation with standard inoculum. Inoculated flask was incubated on a shaker (142 rpm) at 28 °C for 72 hrs. The number of spores/ml of the suspension, which resulted from the previously technique of production, was determined by plate count method, and then the suspension was stored at 4˚C until use.

2-Entomopathogenic fungi

Tow Entomopathogenic fungi were used in this study, *B. bassiana* and *M. anisopliae*. The first fungus was isolated from the white fly in the Sharkia Governorate and the second fungus was isolated from the red palm weevil in Ismailia Governorate. **(Ibrahim, 2006).**

- Culture conditions of *B. bassiana* and *M. anisopliae*

Stock cultures of the isolates were stored at -80 ˚C. Conidia obtained from fungal cultures of *B. bassiana* and *M. anisopliae* were grown at 25±1 ºC, in dark, on Sabouraud dextrose agar (SDA), consisted of peptone 10 g/l, glucose 20 g/l, & agar-agar 20 g/l., (constant volume of 15 ml) in standard Petri-dishes (90 mm diameter). Conidia were harvested from 15 days old plates by scraping into sterile tween-80 (polyoxyethylene sorbitan monooleate; 1ml/l). The suspension was vortexed for 2 min and agitated for 1.5

hrs on a flask shaker (Griffen and George Ltd.) at room temperature before filtering through four layers of sterile muslin. The conidial concentration of the resulting stock suspension was estimated using an improved Neubauer bright line hemocytometer (Reichart) under a Leitz Dialu x 20 EB microscope (400 x magnifications). A series of dilutions were made to give concentrations range of 10^7, 10^8, 10^9 and 10^{10} conidia/ml. Suspensions were held overnight on ice at 4 ºC and then routinely checked for conidial germination prior to use in bioassays as described by **Yeo *et al*., 2003.**

Treatment procedures:

Four concentrations of each agent three replicate for each were tested on *T. absoluta* larvae (Neonate "newly hatched", 2nd & 3rd instar) to study the effect of these materials on larval mortality. *B. thuringiensis* spores/ml, *B. bassiana* and *M. anisopliae* were prepared with concentrations of (10^7; 10^8; 10^9; 10^{10}). Tomato seedlings pots of approximately 45 days old were placed in rearing cages (60 cm^2 high, 50cm^2 wide, 50 cm^2 long) and were exposed to ten *T. absoluta* couples in rearing cages for 24 hrs. Then *T. absoluta* adults were removed and the plants were checked daily until egg hatching. Potted plants were removed after exposure period and transferred in other cages until eggs hatching.

Nine randomly selected leaves each concentration were cut and dipped into the suspensions (three leaves per replicate), transferred onto white clean paper for water evaporation then treated leaves were put in Petri dishes with filter papers and supplied with moisture as needed, then treated leaves infested with neonate larvae obtained from the laboratory colony (15 larvae/replicate). The treated disks were only used once at the beginning of the bioassay. Subsequently, the larvae were fed untreated leaves when needed. Similar method of experiments was performed to estimate the effect of the three entomopathogenic materials on larvae from the second instar and third instar. In addition, eggs of *T. absoluta* were exposure to *B. bassiana* and *M. anisopliae* to evaluate their effect on hatchability. In these cases, the experiments were conducted in the same way. In order to obtain larvae of the 2nd & 3rd instar used in these experiments, larvae were reared to the desired instar on tomato plants. Leaves were collected from the tomato plants, arranged in Petri dishes and infested with larvae obtained from the laboratory colony. Larvae were allowed to feed on untreated leaves until they reached the second and third instar. Discs were transferred to Petri dishes and larvae in the appropriate instar were placed in the dishes. The bioassay lasted for 7 days and the median lethal concentration (LC_{50}) values were obtained by the software computer probane. The larval mortality was evaluated daily for 10 days and or until the end of the experiment. The mortality was corrected using Abbott's formula (1925).

$$\text{Corrected Mortality \%} = 100 \times \left(1 - \frac{\text{n in T after treatment}}{\text{n in Co after treatment}}\right)$$

n = insect population
T = treated, Co = control

3. Results and Discussion

Efficiency of four concentrations of *Bacillus thuringiensis*, *Beauveria bassiana* and *Metarhizium anisopliae* were prepared with concentrations of (10^7; 10^8; 10^9; 10^{10}) were tested on *T. absoluta* larvae (Neonate "newly hatched", 2nd & 3rd instar) to study the effect of these materials on larval mortality. In addition, eggs of *T. absoluta* were exposure to *B. bassiana* and *M. anisopliae* to evaluate their effect on hatchability under laboratory conditions.

The estimated LC_{50} of Btk were 3.25 x 10^6 spores/ml, 5.47 x 10^6 & 3.28 x 10^6 for neonate, 2nd instar & 3rd instar *T. absoluta* larvae, respectively. While the LC_{50} values of *Beauveria bassiana* & *Metarhizium anisopliae* were (0.28 x 10^6 & 0.11 x 10^6), (0.45 x 10^6 & 0.46 x 10^6) and (0.32 x 10^6 & 0.27 x 10^6 conidia/ml) for neonate, 2nd instar & 3rd instar *T. absoluta* larvae, respectively. According to LC_{50} values, *B. bassiana* and *M. anisopliae* were most effective on larval phase of *T. absoluta* than Btk (**Table 1**).

Daily mortality (%) of larval phase of *T. absoluta* fed in the newly hatched, second instar and third instar larva with leaves treated with Btk illustrated in **Figs. 1, 2 & 3**. The results revealed that when neonate larvae fed on Btk **(Fig. 1)**; the pathogen effect was evident by the third day of evaluation after exposure in the four concentrations (10^7; 10^8; 10^9; 10^{10} spores/ml) with recorded mortality (13.3, 13.3, 33.3, 33.3%) respectively. Thereafter, the values of the corrected mortalities of neonate larvae increased gradually from the 4th day after exposure until the last day (10th) with mortality 88.8% for the first concentration 10^7 spores/ml. For the second concentration (10^8 spores/ml) the mortality values were increased and reached its maximum in the 8th day of exposure (100% reduction). The mortality values reached its maximum in the 7th day and in the 5th day

for the third and fourth concentration (10^9 & 10^{10} spores/ml) and recorded 100% reduction for the two concentrations. Thus, it was evident that the higher effective concentration of Btk on neonate larvae of *T. absoluta* was 10^{10} spores/ml followed by 10^9 spores/ml while the other two concentrations (10^7 & 10^9 spores/ml) showed moderate effect.

For the second instar larva of *T. absoluta* **(Fig. 2)**, the pathogen effect was evident by the fifth day for (10^7 spores/ml) & (10^8 spores/ml) and by the 4th day for (10^9 spores/ml) and by the 3rd day for (10^{10} spores/ml) of evaluation after exposure with corresponding mortalities (53.3, 40.0, 6.6 and 6.6 %) for the four concentrations, respectively. Then, the values of the corrected mortalities of the second instar larvae increased gradually until the 9th day after exposure to record 88.8% mortality for the first concentration (10^7 spores/ml). The mortality values reached its maximum in the 9th day, 8th day and in the 7th day for the second, third and fourth concentration ($10^{8,}$ 10^9 & 10^{10} spores/ml) and recorded 100% reduction for the three concentrations. Therefore, it was obvious that the higher effective concentration of Btk on second instar larvae of *T. absoluta* was 10^{10} spores/ml while the other two concentrations (10^7 & 10^9 spores/ml) showed moderate effect.

For the third instar larva of *T. absoluta* **(Fig. 3)**, the pathogen effect was evident by the 3rd day of evaluation for the four concentrations (10^7, 10^8, 10^9 & 10^{10} spores/ml), with corresponding mortalities (13.3, 13.3, 33.3 and 53.3 %) for the four concentrations, respectively. Then, the values of the corrected mortalities of the second instar larvae increased gradually until the 8th day after exposure to record 70.o% mortality for the first concentration (10^7 spores/ml). The mortality values reached its maximum in the 8th day, 7th day and in the 5th day for the second, third and fourth concentration ($10^{8,}$ 10^9 & 10^{10} spores/ml) and recorded 100% reduction for the three concentrations. Thus, it was evident that the higher effective concentration of Btk on the third instar larvae of *T. absoluta* was 10^{10} spores/ml followed by 10^9 spores/ml while the other two concentrations (10^7 & 10^9 spores/ml) showed moderate effect.

Daily mortality (%) of larval phase of *Tuta absoluta* fed in the newly hatched "neonate", second instar and third instar larva with leaves treated with *Beauveria bassiana* illustrated in **Figs. 4, 5 & 6**. The results stated that when neonate larvae fed on *Beauveria bassiana* **(Fig. 4)**; the pathogen effect was evident by the 3rd day of evaluation after exposure in the first concentration (10^7 conidia/ml) with recorded mortality (20.0%). While the pathogen effect was evident by the 2nd day of evaluation after exposure in the 2nd, 3rd & 4th concentrations (10^8, 10^9 & 10^{10}) with recorded mortalities (6.7%) for the three concentrations. Thereafter, the values of the corrected mortalities of neonate larvae increased gradually until the 7th day with maximum mortality 100.0% for 10^7 & 10^8 conidia/ml. While for the 3rd and 4th concentration (10^9 & 10^{10} conidia/ml) the mortality values were rapidly increased and reached its maximum in the 5th and 4th day of exposure (100% reduction). Thus, it was evident that the higher effective concentration of *B. bassiana* on neonate larvae of *T. absoluta* was 10^{10} conidia/ml where this concentration gave 100% reduction rapidly in the 4th day of the evaluation, followed by 10^9, 10^8 and 10^7 conidia/ml, respectively.

For the second instar larva, the results confirmed that when larvae fed on *B. bassiana* **(Fig. 5)**; the pathogen effect was evident by the 3rd day of evaluation after exposure in the concentration (10^7 & 10^8 conidia/ml) with recorded mortality (6.7 & 13.3%), respectively.

While the pathogen effect was evident by the 2nd day of evaluation after exposure in the 3rd & 4th concentrations (10^9 & 10^{10} conidia/ml) with recorded mortalities (6.7%) for the two concentrations. After that, the values of the corrected mortalities of the second instar larvae increased gradually until the 7th day and 8th day with maximum mortality 100.0% for 10^7 & 10^8 conidia/ml, respectively. While for the 3rd and 4th concentration (10^9 & 10^{10} conidia/ml) the mortality values were rapidly increased and reached its maximum in the 5th day of exposure (100% reduction). Thus, it was clear that the higher effective concentration of *B. bassiana* on the second instar larvae of *T. absoluta* resulted from the two concentrations 10^{10} and 10^9 conidia/ml evenly, followed by 10^8 and 10^7 conidia/ml, respectively.

When the third instar larvae fed on *B. bassiana* **(Fig. 6)**; the pathogen effect was evident by the 3rd day of evaluation after exposure in the first concentration (10^7 conidia/ml) with recorded mortality (20.0%). While the pathogen effect was evident by the 2nd day of evaluation after exposure in the 2nd, 3rd & 4th concentrations (10^8, 10^9 & 10^{10}) with recorded mortalities (6.7%) for the three concentrations. Afterward, the values of the corrected mortalities of the third instar larva increased gradually until the 7th day and 6th day where reached its maximum mortality 100.0% for 10^7 & 10^8 conidia/ml, respectively. Whereas, for the 3rd and 4th concentration (10^9 & 10^{10} conidia/ml) the mortality values were rapidly increased and reached its maximum in the 5th day of exposure (100% reduction). Thus, it was evident that the higher effective concentration of *B. bassiana* on the second instar larvae of *T. absoluta* was 10^9 & 10^{10} conidia/ml where these concentrations gave 100% reduction rapidly in the 5th day of the evaluation, followed by 10^8 and 10^7 conidia/ml, respectively.

Daily mortality (%) of larval phase of *T. absoluta* fed in the newly hatched "neonate", second

instar and third instar larva with leaves treated with *M. anisopliae* illustrated in **Figs. 7, 8 & 9**. When neonate larvae fed on *M. anisopliae* **(Fig. 7)**; the pathogen effect was evident by the 3rd day of evaluation after exposure in the first concentration (10^7 conidia/ml) with recorded mortality (20.0%). While the pathogen effect was evident by the 2nd day of evaluation after exposure in the 2nd, 3rd & 4th concentrations (10^8, 10^9 & 10^{10}) with recorded mortalities (6.7%) for the three concentrations. Subsequently, the values of the corrected mortalities of neonate larvae increased gradually until the 7th day with maximum mortality 100.0% for 10^7 & 10^8 conidia/ml. While for the 3rd and 4th concentration (10^9 & 10^{10} conidia/ml) the mortality values were rapidly increased and reached its maximum in the 5th day of exposure (100% reduction) for the two concentrations. Hence, it was evident that the higher effective concentration of *M. anisopliae* on neonate larvae of *T. absoluta* was 10^9 & 10^{10} conidia/ml evenly, where these two concentrations gave 100% reduction rapidly in the 5th day of the evaluation, followed by 10^8 and 10^7 conidia/ml, respectively.

The results confirmed that, when the second instar larvae fed on *M. anisopliae* **(Fig. 8)**; the pathogen effect was evident by the 3rd day of evaluation after exposure in the concentration (10^7 & 10^8 conidia/ml) with recorded mortality (6.7 & 13.3%), respectively. While the pathogen effect was evident by the 2nd day of evaluation after exposure in the 3rd & 4th concentrations (10^9 & 10^{10} conidia/ml) with recorded mortalities (6.7%) for the two concentrations. After that, the values of the corrected mortalities of the second instar larvae increased gradually until the 7th day with maximum mortality 100.0% for 10^7 & 10^8 conidia/ml. While for the 3rd and 4th concentration (10^9 & 10^{10} conidia/ml) the mortality values were rapidly increased and reached its maximum in the 6th and 5th day of exposure (100% reduction) for the two concentrations, respectively. Thus, it was clear that the higher effective concentration of *M. anisopliae* on the second instar larvae of *T. absoluta* resulted from the concentration 10^{10} conidia/ml followed by the concentration 10^9 conidia/ml, 10^8 and 10^7 conidia/ml. While the third instar larvae when fed on *M. anisopliae* **(Fig. 9)**; the pathogen effect was evident by the 3rd day of evaluation after exposure in the first concentration (10^7 conidia/ml) with recorded mortality (20.0%). While the pathogen effect was evident by the 2nd day of evaluation after exposure in the 2nd, 3rd & 4th concentrations (10^8, 10^9 & 10^{10}) with recorded mortalities (6.7%) for the three concentrations. Afterward, the values of the corrected mortalities of the third instar larva increased gradually until the 7th day and 6th day where reached its maximum mortality 100.0% for the four concentrations, (10^7, 10^8, 10^9 & 10^{10} conidia/ml).

Eggs of *T. absoluta* were exposure to *B. bassiana* and *M. anisopliae* to evaluate their effect on hatchability with concentrations of (10^7; 10^8; 10^9; 10^{10} conidia/ml) under laboratory conditions (**Tables 2 & 3**).

Hatchability of *T. absoluta* eggs treated with *B. bassiana* and *M. anisopliae* demonstrated in (**Tables 2 & 3)** revealed that; the pathogen effect was evident by the fourth day of evaluation after exposure in the four concentrations (10^7; 10^8; 10^9; 10^{10} conidia/ml). The black appearance of eggs of *T. absoluta* took place after the exposure to the four concentrations of the two pathogenic fungi starting from the day five after exposure, and thereby no hatched larvae were appeared in the four concentrations of the two pathogenic fungi comparing the control where the hatchability reached 87.7% and 80.0% during the evaluation time for *B. bassiana* and *M. anisopliae,* respectively. In this regard **(Torres *et al.* , 2001and Fargalla and Shalaby, 2013)** stated that; eggs of *T. absoluta* are deposited singly or (rarely) in batches. Immediately after egg deposition they are yellowish becoming coppery-red and with two red eye-spots about 1 day before hatching. The incubation period is between 4 and 7 days at 278 C. Also, **Inanli *et al.*, 2012** revealed that, According to statistical analysis of observed data, effect of *B. bassiana* was 41.67% and 66.67% while effect of *M. anisopliae* was 91.67% and 100.00% on egg stage of *T. absoluta* at the end of 7th and 9th days after application. In spite of that, on first larval stage of *T. absoluta* at the end of 9th day after application, effect of *B. bassiana* and *M. anisopliae* were 12.50% and 91.67% respectively. Consequently, this laboratory experiment suggested that *M. anisopliae* has a potential effect on both egg and first larval stage of *T. absoluta* but *B. bassiana* is effective just on egg stage.

Generally, results of total larval mortality of larval phase of *Tuta absoluta* fed with leaves in the newly hatched "neonate", second instar and third instar larva during the evaluation period treated with Btk, *B. bassiana* and *M. anisopliae* illustrated in (**Figs. 10, 11 & 12**).

The total larval mortality of larval phase of *T. absoluta* fed with leaves treated with Btk (**Fig. 10**), showed that; the greatest percentage of mortality occurred for in the newly hatched "neonate" where recorded 44.9, 56.1, 67.3 & 71.3% for the four concentrations (10^7; 10^8; 10^9; 10^{10} spores/ml), respectively followed by the third instar larva and the second instar larva (33.20, 37.41, 47.62, 44.82%) for the four concentrations , respectively which gave the lowest larval mortality. Thus, this effect was

dependent on the instar at which the larvae were fed Btk-treated leaves.

This results agree with findings of **Giustolin *et al.* (2001)** where stated that; the higher mortality of neonate larvae, than later instars can be explained by feeding behaviour differences. Neonate larvae scratch the leaf for 20±45 min before penetrating the mesophyll and are therefore exposed to a higher dose of bacterial spores and toxins. For second instar larvae as well, the low mortality on Btk-treated `Santa Clara' leaves was probably related to the lack of leaf scratching as observed with neonates. For the third instar larvae, high mortality was probably due to greater leaf consumption since this instar consumed the entire treated leaf disc, consequently ingesting a higher dose of the pathogen and its toxin.

The accumulative larval mortality of larval phase of *T. absoluta* fed with leaves treated with *B. bassiana* (**Fig. 11**), showed that; the greatest percentage of pathogen effect occurred for in the newly hatched "neonate" where recorded 64.4, 67.1, 73.3 & 74.7% for the four concentrations (10^7; 10^8; 10^9; 10^{10} conidia/ml), respectively followed by the second and the third instar larva which gave similar larval mortality values. Similarly, when larval phase of *T. absoluta* fed with leaves treated with *M. anisopliae* (**Fig. 12**), the pathogen effect was clearly high on the newly hatched "neonate" where recorded 61.7, 65.8 , 72.0 & 72.7% for the four concentrations (10^7; 10^8; 10^9; 10^{10} conidia/ml), respectively followed by the second and the third instar larva which gave similar larval mortality values. In this regard (**Inanli *et al.*, 2012**) stated that, the experiment used the immersion method was conducted with four replicates in laboratory. After application, leaves on which larvae and eggs were laid on blotting paper in order to dry and then they were placed in petri dishes. Dead and survival individuals were taken census at 3rd, 5th, 7th and 9th days. According to statistical analysis of observed data, effect of *B. bassiana* was 41.67% and 66.67% while effect of *M. anisopliae* was 91.67% and 100.00% on egg stage of *T. absoluta* at the end of 7th and 9th days after application. In spite of that, on first larval stage of *T. absoluta* at the end of 9th day after application, effect of *B. bassiana* and *M. anisopliae* were 12.50% and 91.67% respectively.

Generally, it could be concluded that; the effect of pathogen application was dependent on the instar at which the larvae were fed on pathogen-treated leaves. Where in our experiment, the greatest percentage of mortality occurred on the neonate larvae for three treatments (Btk, *B. bassiana* & *M. anisopliae*). In addition, the pathogen *B. bassiana* gave the higher effect on larval phase of *T. absoluta* during the evaluation time and similar to the effect of *M. anisopliae* followed by the effect of Btk. Concerning the most effective concentration of each agent, it was found that the higher concentration (10^7) the higher mortality. Thus our laboratory experiment suggested that *B. bassiana* and *M. anisopliae* has a potential effect on both egg and neonate "newly hatched larvae" of *T. absoluta* and Btk is effective on neonate and third larval stage.

Table (1): Efficacy of three entomopathogenic agents against *Tuta absoluta*

Entomopathogenic	LC_{50}		
	Neonate	2nd instar larvae	3rd instar larvae
Bacillus thuringiensis var. *kurstaki*	3.25×10^6	5.47×10^6	3.28×10^6
Beauveria bassiana	0.28×10^6	0.45×10^6	0.32×10^6
Metarhizium anisopliae	0.11×10^6	0.46×10^6	0.27×10^6

Table (2): Hatchability of *Tuta absoluta* eggs treated with *Beauveria bassiana*.

Concentration	1st day	2nd day	3rd day	4th day	5th day	6th day	7th day	8th day	9th day	10th day
					Hatchability%					
10^7	15	15	15	15	0.0	0.0	0.0	0.0	0.0	0.0
10^8	15	15	15	15	0.0	0.0	0.0	0.0	0.0	0.0
10^9	15	15	15	15	0.0	0.0	0.0	0.0	0.0	0.0
10^{10}	15	15	15	15	0.0	0.0	0.0	0.0	0.0	0.0
Control	15	15	15	15	86.7	86.7	86.7	86.7	86.7	86.7

Table (3): Hatchability of *Tuta absoluta* eggs treated with *Metarhizium anisopliae*.

Concentration	1st day	2nd day	3rd day	4th day	5th day	6th day	7th day	8th day	9th day	10th day
					Hatchability%					
10^7	15	15	15	15	0.0	0.0	0.0	0.0	0.0	0.0
10^8	15	15	15	15	0.0	0.0	0.0	0.0	0.0	0.0
10^9	15	15	15	15	0.0	0.0	0.0	0.0	0.0	0.0
10^{10}	15	15	15	15	0.0	0.0	0.0	0.0	0.0	0.0
Control	15	15	15	15	80.0	80.0	80.0	80.0	80.0	80.0

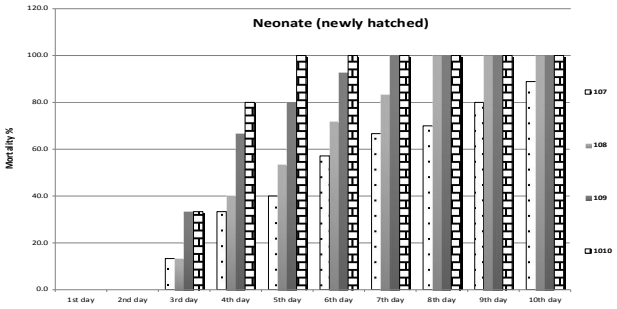

Figure 1. Daily mortality (%) of larval phase of *Tuta absoluta* fed in the newly hatched with leaves treated with *Bacillus thuringiensis* var. *kurstaki*.

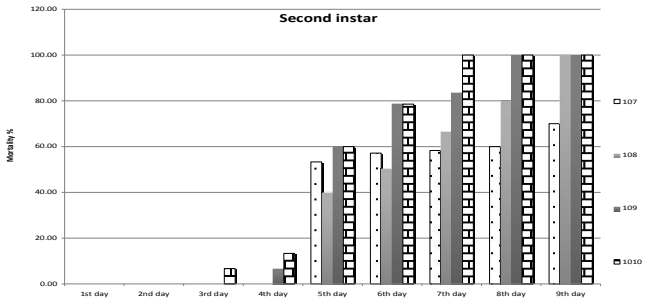

Figure 2. Daily mortality (%) of larval phase of *Tuta absoluta* fed in second instar with leaves treated with *Bacillus thuringiensis* var. *kurstaki*.

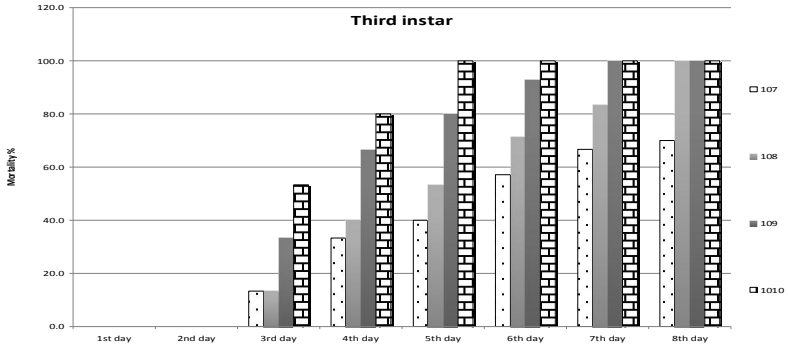

Figure 3. Daily mortality (%) of larval phase of *Tuta absoluta* fed in the third instar with leaves treated with *Bacillus thuringiensis* var. *kurstaki*.

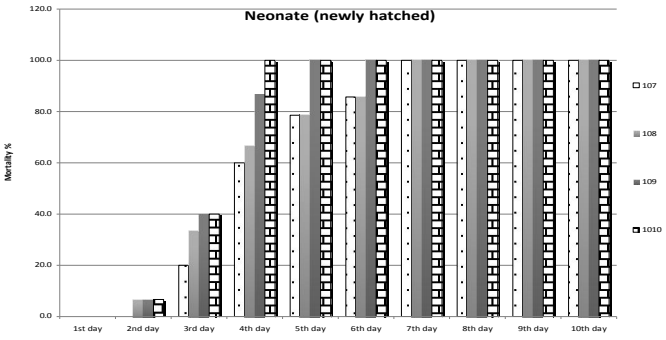

Figure 4. Daily mortality (%) of larval phase of *Tuta absoluta* in the newly hatched fed with leaves treated with *Beauveria bassiana*.

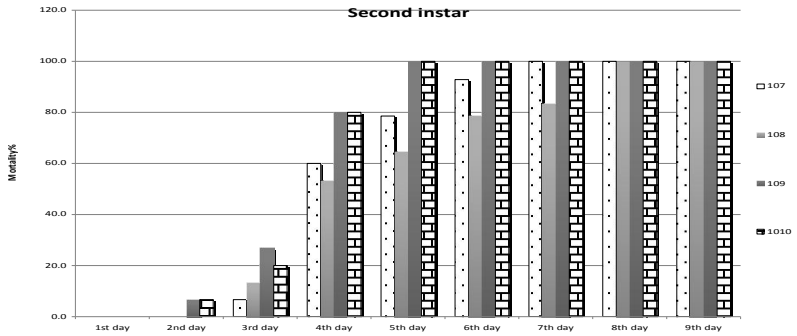

Figure 5. Daily mortality (%) of larval phase of *Tuta absoluta* in the second instar fed with leaves treated with *Beauveria bassiana.*

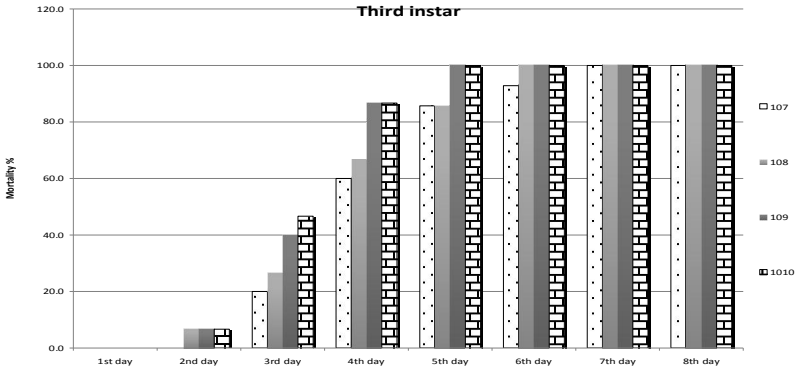

Figure 6. Daily mortality (%) of larval phase of *Tuta absoluta* in the third instar fed with leaves treated with *Beauveria bassiana.*

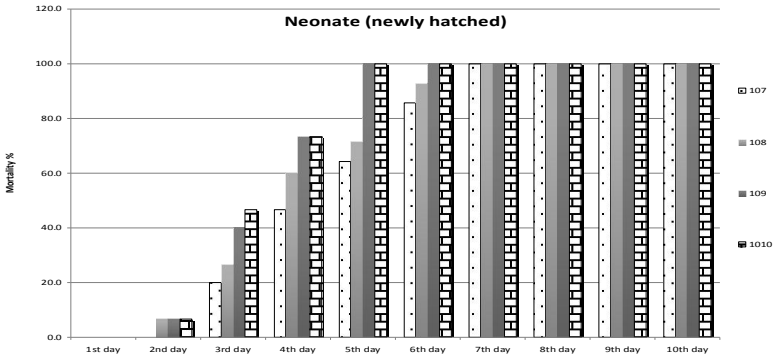

Figure7. Daily mortality (%) of larval phase of *Tuta absoluta* in the newly hatched fed with leaves treated with *Metarhizium anisopliae*

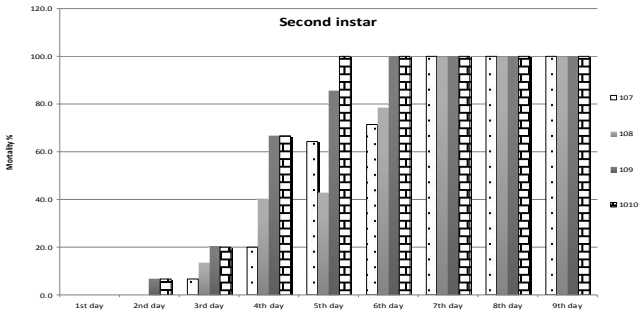

Figure 8. Daily mortality (%) of larval phase of *Tuta absoluta* in the second instar fed with leaves treated with *Metarhizium anisopliae*

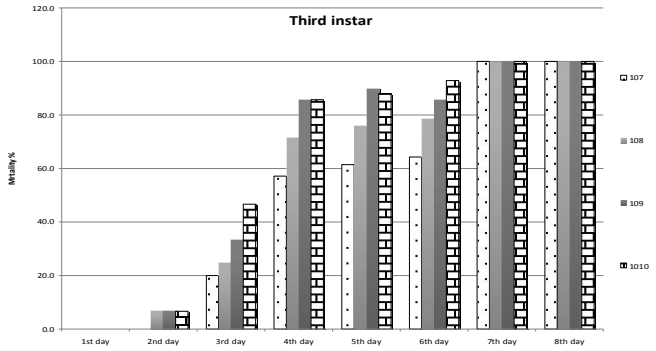

Figure 9. Daily mortality (%) of larval phase of *Tuta absoluta* in the third instar fed with leaves treated with *Metarhizium anisopliae*

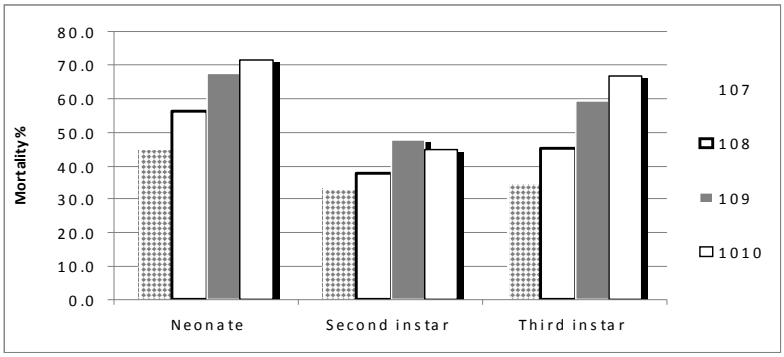

Fig. 10. Total larval mortality of *Tuta absoluta* fed with leaves treated with *Bacillus thuringiensis* var. *kurstaki.*

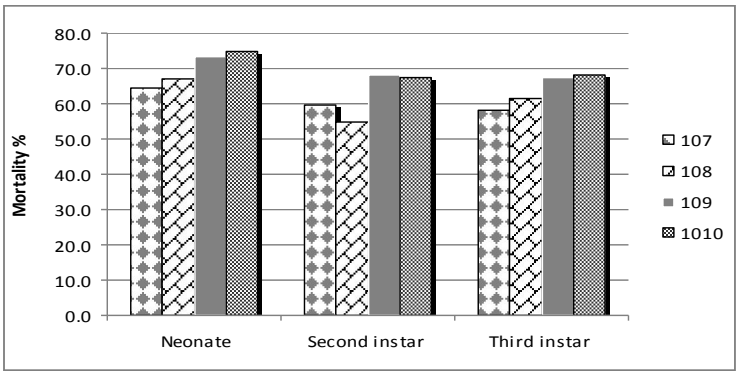

Fig. 11. Total larval mortality of *Tuta absoluta* fed with leaves treated with *Beauveria bassiana.*

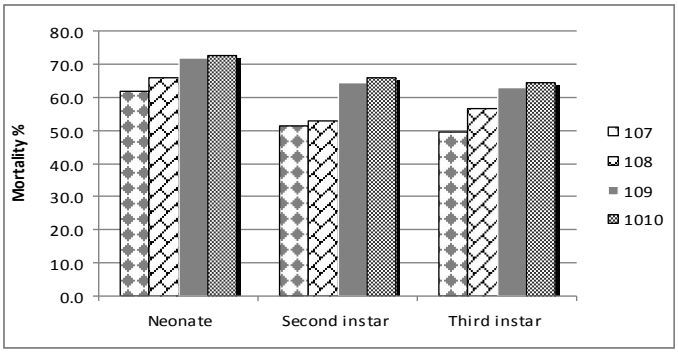

Fig. 12. Total larval mortality of *Tuta absoluta* fed with leaves treated with *Metarhizium anisopliae.*

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