Molecular study of the Mediterranean fly insect *Ceratitis capitata* and the effect of the direction factor on its density on citrus trees

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Abstract

An ecological and molecular study was conducted for the Mediterranean fruit fly *Ceratitis capitata* in one of the citrus orchards of Ishaqi district during the season 2020-2021. PCR technique was used to determine the gene (COXI) in mitochondria after extracting DNA from the insect. The nucleotides sequences were determined for the product of the polymerase chain reaction gene (COXI) of the insect *C. capitata*. The sequences obtained from the Korean company Bioneer were analyzed., then alignment and matching was performed with the strains registered in the World Genetic Bank, the Iraqi isolate showed 100% identical with the American strain *Ceratitis capitata* isolate 030220-11 TNE11 under the accession number JN705021.1 and with a percentage of 99.17 identical with the American strain *Ceratitis caetrata* isolate 030221-04 TNE12 under the accession number JN705054.1 and with a percentage of 99.17 identical with the Belgian strain *Ceratitis caetrata* voucher RMCA:1245 under the accession number GQ154186.1 and 99.00% identical to American strain *Ceratitis caetrata* isolate 071102-3 TNE13 under accession number JN705049.1 and 99.00 identical to American strain *Ceratitis caetrata* isolate 030221-03 TNE12 under accession number JN705053.1. The results of the genetic tree showed that the percentage of kinship of the insect with other recorded strains, the Iraqi isolate *Ceratitis capitata* rand1 appeared 100% identical with the American isolate *Ceratitis capitata* isolate 030220-11 TNE11 under the accession number JN705021.1, and the value of the genetic distance between them reached 0.002, then the Iraqi isolate was recorded *Ceratitis capitata* isolate rand1 in the World GenBank under accession number MW889894.1. The results of distributing pheromone traps (Jackson) on Citrus aurantium trees, oranges and tangerine showed that the total number of the highest number of hunted males was in the middle of the orchard for Citrus aurantium trees, which reached to 904 males, while for the orange trees, the total density of the highest number of hunted males was in the western side, which reached to 710 hunted males while the total number of the highest population density in the north side was for the tangerine trees (162 hunted males), then the insect ended up appearing in the first half of March of 2021 for all directions in all the trees of the orchard.

Keywords: Mediterranean fly, insect, Ceratitis capitata and citrus trees

1. Introduction

The Mediterranean fruit fly *Ceratitis capitata* is one of the most dangerous major insect pests that infect fruit trees with a great economic impact [1], this insect infects the fruits of citrus, stone-core trees and vegetable crops, causes great economic losses in tropical and subtropical regions of the world [2]. *Ceratitis capitata* belongs to the family Tephritidae, which includes more than 4000 species, of which approximately 250 species are considered economically important [3]. The damage is caused by the larvae of this insect, where the female lays her eggs by the egg-laying machine and appears in the form of scars on the outer peel of the fruits, which leads to the distortion of the citrus fruits and other fruits that infect them, as well as the rotting of the fruits as a result of infection due to attacking the pathogens, and usually these infected fruits fall before their maturity In addition to the holes resulting from the larvae on the fruits peel reduces its marketing value [4].

The Mediterranean fruit fly, *Ceratitis capitata*, ranks first among the economic fruit fly species because it infects more than 350 different plant hosts from fruits and vegetables in all over the world belonging to 70 plant families, and the damage it causes is estimated at no less than 2 billion dollars annually in all over the world [5]. This insect entered the citrus orchards in Iraq for the first time, specifically in Diyala Governorate in 1947, and tampered with some orchards, causing severe economic damage and heavy losses in production, at that time, severe preventive measures were taken against it. It is believed that harsh environmental factors limited the spread of this insect in Iraq and led to its complete disappearance [4], then appeared again during the October 2006 due to the lack of agricultural quarantine and the import of many different citrus fruits, peaches, apricots and vegetables from neighboring countries [4]. This insect has spread and succeeded in invading different regions of the world and became well-established first in the Mediterranean Basin, Central America, South America, Australia and Huawei, and also progressed in all islands in the western Indian Ocean. The mitochondrial DNA was relied upon to obtain a modern geographical base for its spread throughout the world [6]. The PCR technique was used in this study to diagnose this insect by determining the

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nucleotide sequences of the product of the polymerase chain reaction of the COX 1 gene in the adults mtDNA, where the use of this gene in classification has become widely used in recent years, and the identification of this insect and its conformity with global insects helps to apply a unified global strategy in an integrated management to control it. Pheromone traps have been widely used in integrated management programs for this insect and this is done by using a specialized pheromone, as the knowledge of population monitoring plays an important role in chemical control using the pesticides [7].

2. Materials and Methods

Adult insect's collection

Citrus fruits infected with the Mediterranean fruit fly were collected from citrus orchards in Ishaqi district during January for the 2021 season. The infection was determined on the basis of the scars of laying eggs on the fruit, then the infected fruits were transferred to the Entomology Laboratory at the College of Agriculture, Tikrit University,

These infected fruits were placed in plastic cans with a layer of mixture soil thickness of 2 cm for the transforming the larvae into the pupal stage after they emerge from the fruits and covered with the boring cloth, tied with a rubber band, and placed in the incubator at a temperature of 27 ° C with a humidity of 50% until the exit of the adult (male and female) insects, for complete its life cycle. It was daily monitoring to follow the exit of the adult insects. After 21 days, the adults appeared, then they were taken out of the incubator and fed on a 75% sugar solution. After that, the adult insects were pulled out of the plastic cans using an air suction cup, placed in glass tubes Scrow cup and transferred to the molecular biology laboratory for the extracting the DNA of them.

Distribution of the Jackson Pheromone Trap in the Orchard

The Jackson trap was used, a pyramidal trap with a rectangular base, dimensions of 20 x 12.5 cm and a height of 8 cm. It is made of red cardboard. An adhesive substance was placed in the base of the trap that works to catch insects, then an industrial pheromone capsule QLure-CEC produced by Russell Company. IPM Ltd,UK was put in the middle trap to attract males of the insect as in Figure (1). The trap was suspended on the tree using a metal wire as in Figure (2).

Figure(1)shows the synthetic pheromone capsule placed in the middle of the adhesive.

Figure (2) Shows the Jackson pheromone trap hanging on the tree

Effect of the Jackson pheromone trap on attracting C. capitata males in the citrus orchard.

Jackson traps (with industrial pheromone capsule) were distributed on Citrus aurantium, orange and tangerine trees, where it was placed on the northern, southern, eastern, western and middle sides of the orchard at a height of 1 m for each type of these trees with three replicates for each side. The data were collected every 10 days for the period from 14 /12/2020 to 8/3/2021 by calculating the number of males of the caught insect as in Figure (3) and after each reading, the adhesive and pheromone are changed with a new one.

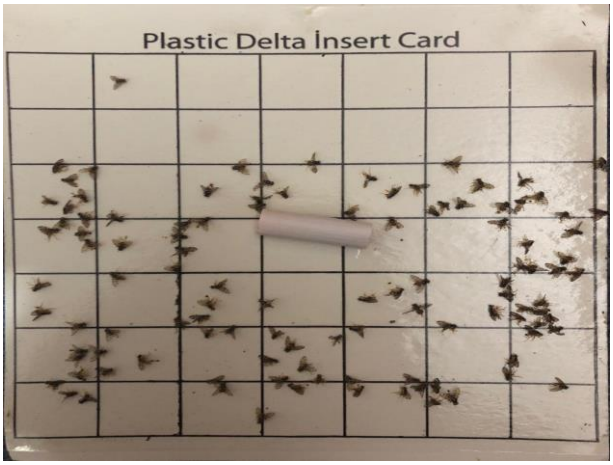

Figure (3) shows the caught male C. capitata fly

DNA extraction

G- spin DNA extraction kit, intron biotechnology, cat.no. 17045

3. Kit Contents

Label	Contents 50 Columns
Buffer CL	25 ml
Buffer BL	25 ml
Buffer WA	40 ml
Buffer WB	10 ml
Buffer CE	20 ml
Spin Column / Collection Tube	50 ea
RNase A (Lyophilized powder)	3 mg x 1 vial
Proteinase K (Lyophilized powder)	g x 1 vial

Protocol

1. Measure 25 mg of ground tissue sample, and then transfer into 1.5 ml tube using a spatula .
2. Add 200 µl Buffer CL, 20 µl Proteinase K and 5 µl RNase A Solution into sample tube and mix by vortexing vigorously.
3. Incubate the lysate at 56℃ (preheated heat block or water bath) for 10 ~ 30 min.
4. After lysis completely, add 200 µl of Buffer BL into upper sample tube and mix thoroughly. Then incubate the mixture at 70℃ for 5min.
5. Centrifuge the sample tube at 13,000 rpm for 5 min to remove un-lysed tissue particles. Then carefully transfer 350 ~ 400 µl of the supernatant into a new 1.5 ml tube (not provided).
6. Add 200 µl of absolute ethanol into the lysate, and mix well by gently inverting 5 - 6 times or by pipetting. DO NOT vortex. After mixing, briefly centrifuge the 1.5 ml tube to remove drops from inside of the lid.
7. Carefully apply the mixture from step 6 to the Spin Column (in a 2 ml Collection Tube) without wetting the rim, close the cap, and centrifuge at 13,000 rpm for 1 min. Discard the filtrate and place the Spin Column in a 2 ml Collection Tube (reuse).
8. Add 700 µl of Buffer WA to the Spin Column without wetting the rim, and centrifuge for 1 min at 13,000 rpm. Discard the flow-through and reuse the Collection Tube.
9. Add 700 µl of Buffer WB to the Spin Column without wetting the rim, and centrifuge for 1 min at 13,000 rpm. Discard the flow-through and place the Column into a 2.0 ml Collection Tube (reuse), Then again centrifuge for additionally 1 min to dry the membrane. Discard the flow-through and Collection Tube altogether.
10. Place the Spin Column into a new 1.5 ml tube (not supplied), and 30 - 100 µl of Buffer CE directly onto the membrane. Incubate for 1 min at room temperature and then centrifuge for 1 min at 13,000 rpm to elute.

Prepare of the Agarose gel

According to Sambrook et al. [8], the agarose gel has been made in 1.5% condensation by melting 1.5 g of agarose in 100 ml of previously made TBE buffer. Agarose has been heated to boil then left to cool down at (45-50ºC). The gel has been poured in the pour plate in which the plate of agarose support has been prepared after fixing the comb to make holes that would hold the samples. The gel has been poured gently not to make air bubbles and left 30 minutes to cool down. The comb has been removed gently of the solid agarose. The plate has been fixed to its stand in the Electrophoresis horizontal unit represented by the tank used in the Electrophoresis. The tank has been filled with TBE buffer in which it covers the gel surface

The primers used in the interaction

The primers were lyophilized, they dissolved in the free ddH2O to give a final concentration of 100 pmol/µl as stock solution and keep a stock at -20 to prepare 10 pmol/µl concentration as work primer suspended, 10 µl of the stock solution in 90 µl of the free ddH2O water to reach a final volume 100 µl, was investigated by IDT (Integrated DNA Technologies company, Canada).

The specific primer COX of gene

Primer	Sequence	Product size
Forward	5'- GGTCAACAAATCATAAAGATATTG - 3'	720 base pair
Reverse	5'- TAAACTTCAGGGTGACCAAAAAATCA - 3'	

Table: The Components of the Maxime PCR PreMix kit (i-Taq)	
Material	**Concetration**
i-Taq DNA Polymerase	5U/ µl
DNTPs	2.5mM
Reaction buffer (10X)	1X
Gel loading buffer	1X

Method of the reaction mixture for the PCR technology for the COX 1 gene

1. The reaction mixture was prepared by mixing the reaction components in a PreMix kit (i-Taq) tube for PCR technology, as in the following table:

Components	Concentration
Taq PCR PreMix	5µl
Forward primer	10 picomols/µl (1 µl)
Reverse primer	10 picomols/µl (1 µl)
DNA	1.5µl
Distill water	16.5 µl
Final volume	25µl

1. The mixture was centrifuged in a Microfuge for 4 seconds, then transferred to a thermocycler, a PCR technology device, and the following program was applied:
2. One cycle of 5 minutes at a temperature of 95º C for the initial denaturation of the DNA strand, then followed by 35 replication cycles. Each cycle included 45 seconds at a temperature of 95º C for the double strand denaturation and 45 seconds at a temperature of 58 º C, due to the binding of the primer to the template DNA and 45 seconds at 72 °C in order to elongate the primer, finally a final cycle for 7 minutes at 72 °C in order to complete the elongation phase [9].

3. The tubes were removed from the device after the reaction time had ended, 5 microliters were withdrawn from the tubes and loaded into the wells of the previously prepared 2% agarose gel. The samples were migrated for 59 minutes on the electrophoresis device.
4. The results were recorded by exposing the gel to a UV source device and then photographed using a digital camera.

4. Statistical analysis

For field experiment, randomized complete blocks Design (R.C.B.D) was used for data statistical analysis by Excel program using the computer

5. Results and discussion

DNA was extracted from the adults of the fly *C. capitata*, as the DNA was electrophoresed in 1.5% agarose gel extracted from the adults of the fly in two stages, the first for 10 minutes and the second for 45 minutes, as shown in Figure 4., then the PCR product was electrophoresed into a 2% agarose gel and a 720 bp band was obtained as shown in Figure (5). After that, the nitrogenous base sequences of the PCR product of the COX1 gene of *C capitata* were determined. The sequences obtained from the Korean company Bioneer were analyzed using the National Center Biotechnology Information (NCBI) world website under the Blast sub-window, then the secondary sub-window of the nucleotide blast was selected, finally the alignment and matching with the strains registered in the World Genetic Bank.

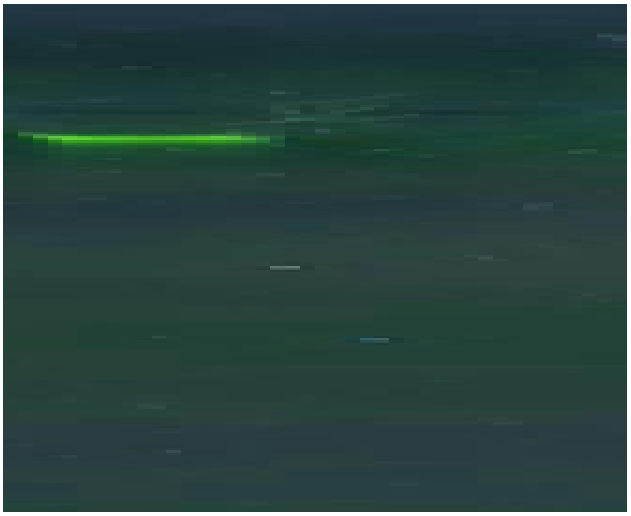

Figure (4:) Gel electrophoresis of genomic DNA extraction from Ceratitis capitata, 1.5% agarose gel

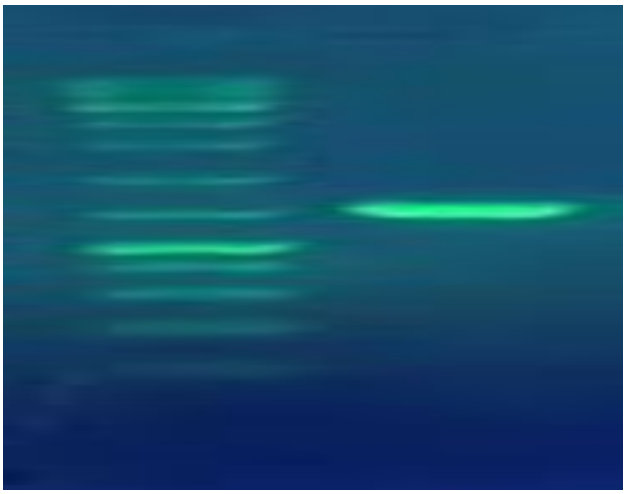

Figure (5) PCR product the band size 720 bp. The product was electrophoresis on 2% agarose N: DNA ladder (100).

C. capitata is considered one of the most economically destructive agricultural pests in the world, the knowledge of the molecular variances between members of the insect of the same species found in multiple geographic areas, therefore, to study the biological differences between them is useful [10-12]. The cytochrome oxidase COX1 gene is important for distinguishing closely related species [13].

The results confirmed that the Iraqi isolate showed 100% identical with the American strain *Ceratitis capitata* isolate 030220-11 TNE11 under the accession number JN705021.1 and with a percentage of 99.17 identical with the American strain *Ceratitis caetrata* isolate 030221-04 TNE12 under the accession number JN705054.1 and with a percentage of 99.17 identical with the Belgian strain *Ceratitis caetrata* voucher RMCA:1245 under the accession number GQ154186.1 and 99.00% identical to American strain *Ceratitis caetrata* isolate 071102-3 TNE13 under accession number JN705049.1 and 99.00 identical to American strain *Ceratitis caetrata* isolate 030221-03 TNE12 under accession number JN705053.1. The results of this study are consistent with findings of Güler et al. [14] in their study of the genetic structure of the Mediterranean fruit fly population in Turkey using mitochondrial DNA, which showed that the Mediterranean fruit fly was characterized by low levels of genetic diversity after comparing this sequence with nucleotide sequences in the world (USA, Pacific region, Australia), indicates that the Mediterranean basin played a more important role in the colonization of the New World. This study also agreed with similar studies in other insects, including the study of Firake et al. [15] on the invasive leaf miner insect of tomato crop in northeastern India through the mitochondrial DNA of the COX 1 gene, where the results showed similar sequences with similar data in the gene bank. Then, the genetic tree was drawn to show the relationship of the *C.capitata* with other registered strains, as in Figure (6).

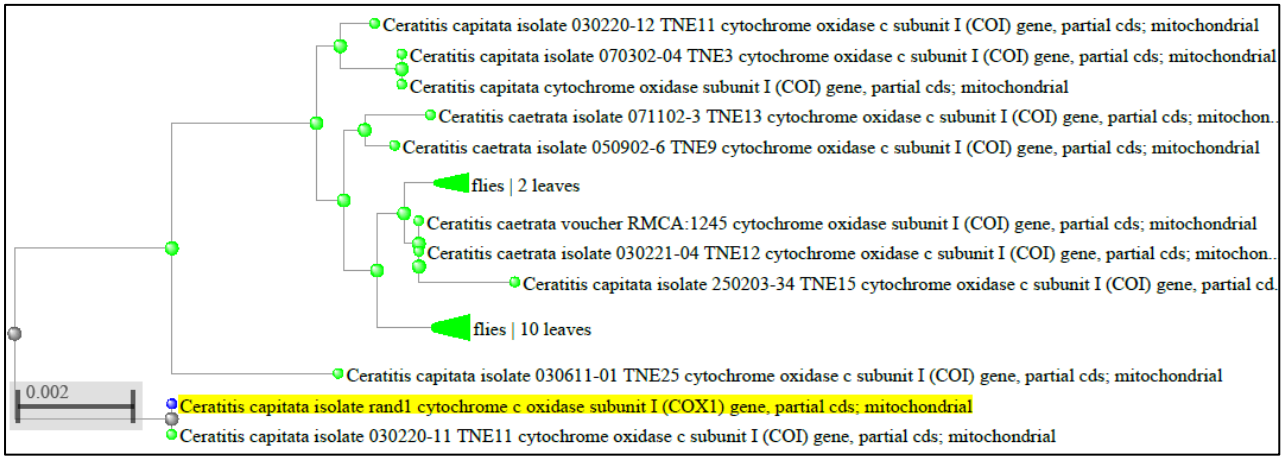

Figure (6) Genetic tree to show the relationship of the Ceratitis capitata with other registered strains.

This tree showed the genetic kinship of the Iraqi isolate *Ceratitis capitata* rand1 with the most identical insect strains, where the Iraqi isolate appeared 100% identical with the American *Ceratitis capitata* isolate 030220-11 TNE11 under the accession number JN705021.1, and the value of the genetic dimension appeared between them 0.002, this indicates a great similarity in the genetic material between them. The identification of this insect and its conformity with world insects helps to apply a effect on attracting males of the apple fruit worm insect, where it was found in his study that the traps in the northern and western sides of the orchard outperformed the rest of the southeastern and central orchard sides, as they gave the highest catch of the insect.

The results also agreed with study of Szyniszewska et al. [16] in which he showed that the trap in the middle of the pomegranate orchard achieved the highest rate of hunted males of the pomegranate fruit worm and superior on the other regions. The reason for this may be attributed to the concentration of trees infected with this insect *C. capitata* in the northern and western sides and the middle of the orchard more than the other directions.

unified global strategy in an integrated management to control it, then the Iraqi isolate *Ceratitis capitata* isolate rand1 was recorded in the world gene bank under the accession number MW889894.1

Effect of direction factor on the population density of male C. capitata caught in Jackson pheromone trap in citrus orchard.

The results of Figure (7) showed that the total number of the highest number males caught in the middle of the orchard for orange trees was 904 males, the results of Figure (8) showed that the western side achieved the highest total densities of the insect males on orange trees, which reached to 710 hunted males, while the results of Figure (9), showed that the north side achieved the highest total number of densities male caught on the trees of the tangerine which was 162 hunted males. These results did not agree with what was found by Szyniszewska et al. [16] in his study of *C. capitata,* which outperformed the trap located on the eastern side of the orchard than the rest of the traps, but agreed with Vincent et al. [17] in his study in the pheromone trap site

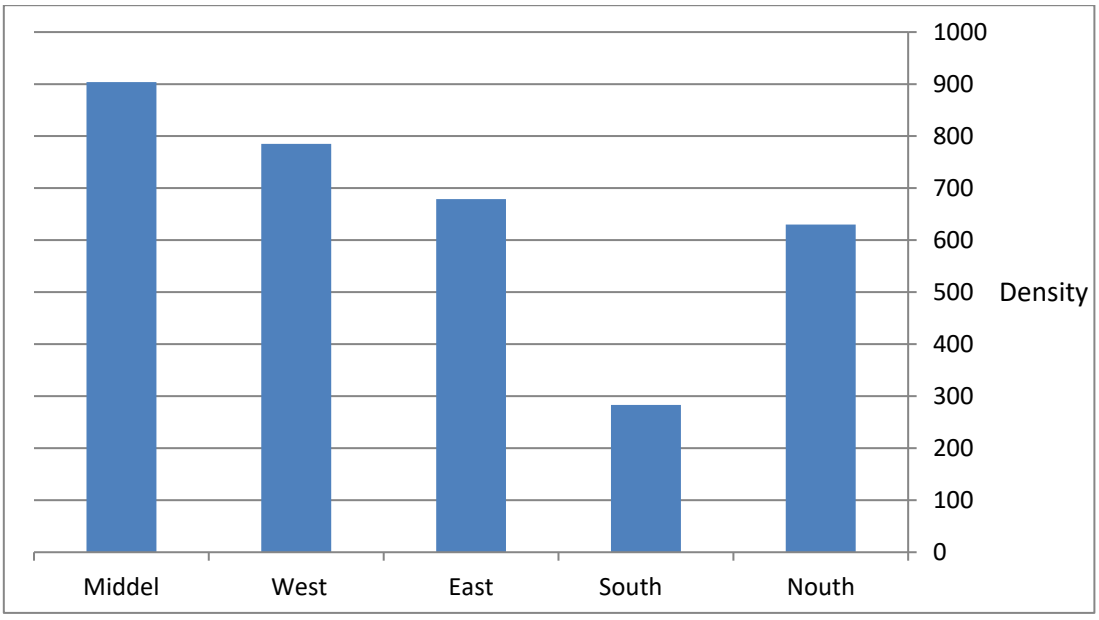

Figure (7) Population density of the C. capitata within the four directions and the middle of the orchard of Citrus aurantium trees for the pheromone trap.

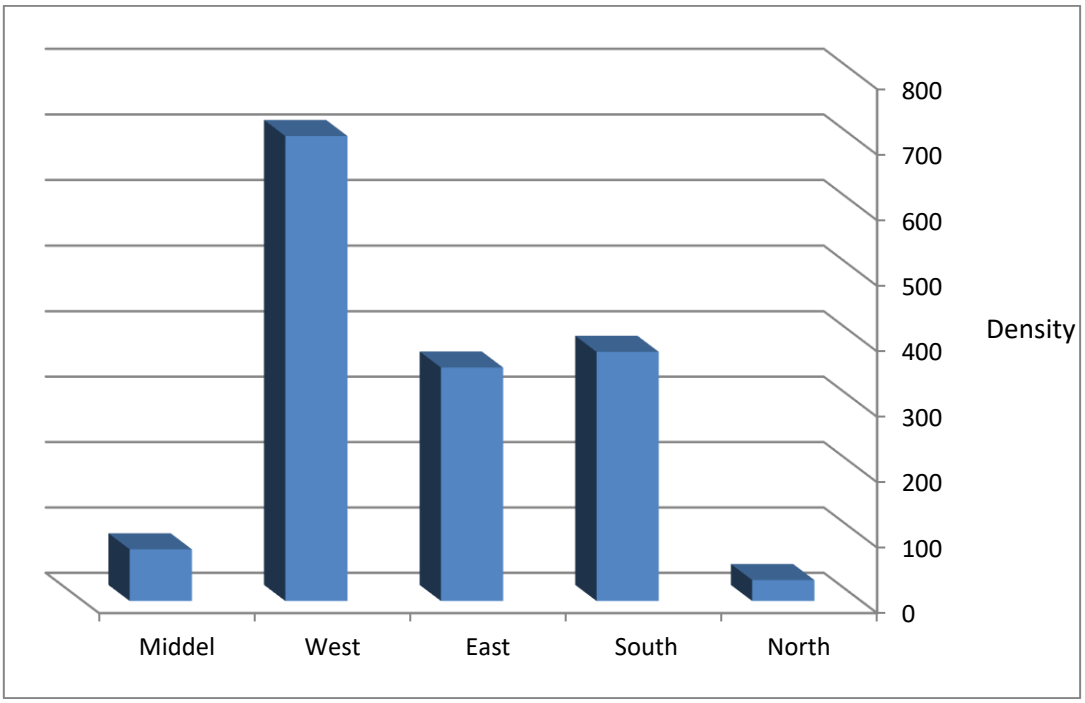

Figure (8) Population density of the C. capitata within the four directions and the middle of the orchard of orange trees for the pheromone trap.

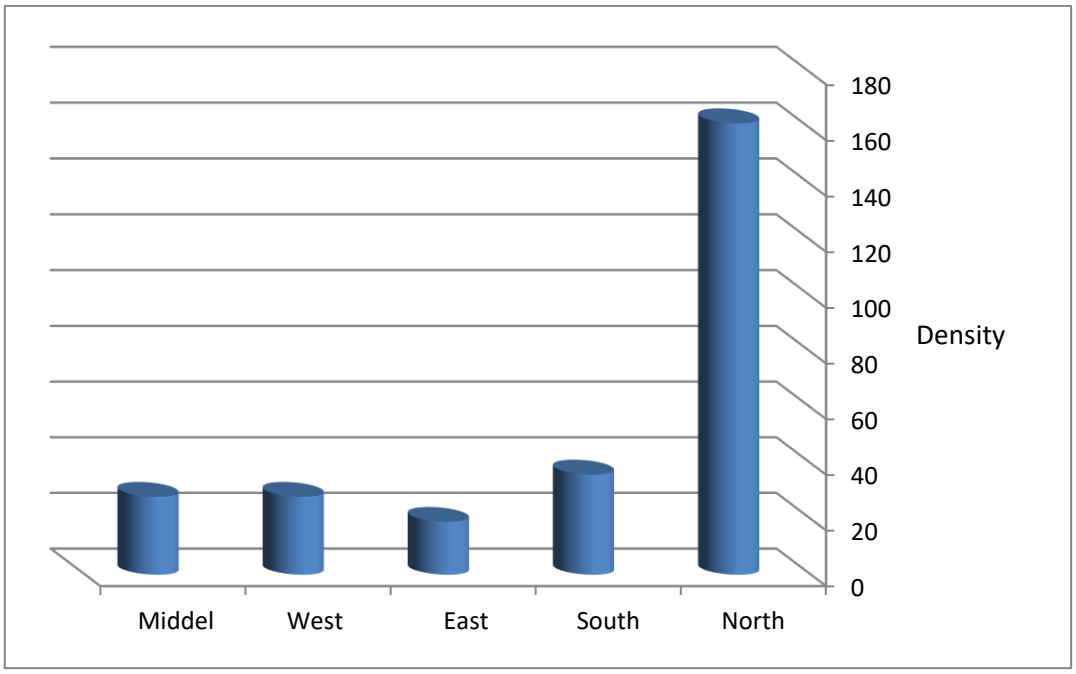

Figure (9) Population density of the C. capitata within the four directions and the middle of the orchard of the tangerine trees of the pheromone trap.

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