# INTEGRATED MANAGEMENT OF ROOT-KNOT NEMATODE (*MELOIDOGYNE* SPP.) IN CUCUMBER (*CUCUMIS SATIVUS* L.) AND ITS EFFECT ON NEMATODE POPULATION DENSITY, PLANT GROWTH AND YIELD IN SULAIMANI GOVERNORATE, KURDISTAN, IRAQ

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**Abstract.** Samples of cucumber plants (Cucumis sativus L.) were collected from different locations of Bakrajo/Sulaimani governorate, Kurdistan region, Iraq. The collected plants were by stunting and yellowing while the roots with obvious root-knots. For nematode identification two methods were used for nematode identification, Perennial Patterns and PCR, with species-specific primers, showing that there were two species; Meloidogyne incognita (Kofoid & White) Chitwood, and M. javanica (Treub) Chitwood. An experiment was designed, different methods have been used for controlling RKN, The results showed that after application of the above treatments whether alone or in combination, the minimum numbers of juvenile 2 (J2) were as well as in Nemakey, Humic acid and Chitosan combination treatment to be (66.33) recorded the maximum number after 30 days of application. Plant receiving combined treatments (Nemakey, Humic acid and Chitosan) significantly reduced the number of total nematode population at ($p \leq 0.05$) in comparison to controls. The minimum number of gall formation on the cucumber root system and galling index with the highest percentage of gall decreasing (23.76, 3.1 and 77.00%) were revealed in case of combined treatments (Nemakey, Humic acid and Chitosan). A significant decrease ($p \leq 0.05$) of disease severity percentage was recorded under Humic acid and Chitosan treatment (66%). The combination (Nemakey, Humic acid and Chitosan) also recorded vigorous growth and high yield. This paper is first scientific report about diagnosing and controlling the cucumber root-knot nematode in the region.
**Keywords:** *Meloidogyne, species-specific primers, PCR, Nemakey, Chitosan, Besto humic, Rugby*

## Introduction

Cucumber (*Cucumis sativus* L.) is an economic crop considered as one of the most popular vegetables which ranked the fourth most important vegetable after tomato, cabbage and onion in the world, and belongs to the Cucurbitaceae family (Tatlioglu, 1993; Wehner and Maynard, 2003; Weng and Sun, 2011). It contributes in human health and it has an important role in antioxidant, metabolism processes. It contains lariciresinol, pinoresinol and secoisolariciresinol – 3 lignans which have a strong history of research in connection in reducing risks of several types of cancer including colon, breast, prostate cancer, uterine and ovarian cancers (Maheshwari et al., 2014).

In Iraq, cucumber is an important economical crop to the farmers and is widely cultivated in Summer depending on irrigation throughout the year (Soppe and Saleh, 2012). Estimates cultivated area with cucumber in Sulaimani governorate under protected cultivation is 602 ha.


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Plastic house are often used to obtain the highest production per unit area, which attains the most practical method of achieving the objectives of protected agriculture and is considered cropping technique where the environmental condition surrounding the plant is controlled partially/fully as plant need during their period of growth which target get maximum yield and resource saving (Nair and Barche, 2014).

Cucumber in plastic houses is attacked by many diseases that are caused by bacteria, fungi, viruses, and nematodes. But nematodes are the most important and causes yield loses (Agrios, 2005). Plant Parasitic Nematodes (PPNs) contribute limited production of vegetables, estimated annual losses caused by (PPN) more than US$100 billion worldwide (Bird et al., 2008).

Most of the (PPN) that attack the roots of plants are Root-Knot Nematode (RKN). The most economically important genus which obligates plant roots is the genus *Meloidogyne* distributed worldwide. This genus includes 98 described species (Moens et al., 2009; Jones et al., 2013), and infect more than 2000 to 3000 species including almost all cultivated plants; including vegetables, fruit trees, oil crops, fiber crops, grains crops and leguminous crops. The most well-known species for genus *Meloidogyne* include *Meloidogyne incognita M. incognita* (Kofoid & White) Chitwood, *M. javanica* (Treub,) Chitwood, *M. arenaria* (Neal), Chitwood and *M. hapla* Chitwood were of outstanding economic importance because they were responsible for at least 90% of all damage caused by root-knot nematodes (Castagnone-Sereno, 2002; Agrios, 2005).

The symptoms of infected plants by (RKN) appear in both aboveground and underground, aboveground shows poor growth, fewer and small pale green leaves that tend to wilt in warm weather, the quality and production always reduced result of fewer of blossoms and fruits. The underground symptoms appear by information galls on the roots which are two to several times as larger in diameter as the healthy root that impact of absorbing and translocating water and dissolved nutrients to the plants. The nematodes parasitize roots cause injuries, which facilitate entry by other soil-borne pathogens (Agrios, 2005).

Management of (RKN) is often difficult and requires multiple and intensive efforts because they live in the soil and complete their life cycle inter plant tissues (endo-parasitic nature). At the same time, they have wide host ranges, short generation times, and high reproductive rates, however, discourage uses nematicides because they are highly toxic and contribute risk to human and animal health. In additional, they have negative influences on the environment (Abawi and Widmer, 2000; Trudgill and Blok, 2001; Hildalgo-Diaz and Kerry, 2008). Depending on single process to manage (RKN) is not often gives satisfactory results. Thus, a combination of management various techniques (Mesiha, 2019), generally provide acceptable management of (RKNs) and at the same time economic and eco-friendly method e.g. bioagent methods (Dong et al., 2012; Huang, et al., 2016), it is necessary promote techniques that aimed at integrated management to minimize their populations below the economic threshold level (Mitkowski and Abawi, 2003).

Although spread and distribution information of Root-Knot Nematode (RKN) species in Kurdistan especially in Sulaimani area is very little, yet, little work has been done to manage this disease. This work comes into request of the general directorate of Sulaimani Agricultural and Water Resources Office.

Therefore, this study is aimed to identify and diagnose of the nematode(s) *Meloidogyne* species based on morphological characteristics and molecular method which cause root-knot diseases of cucumber plant and its management by using different modern methods.


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## Materials and methods

### *Identification and diagnosis of root-knot nematode (RKN)*

For identification of the isolated RKNs two different methods were used: morphological characteristics (*Perineal Patterns***)** according to (Hartman and Sasser, 1985)**.** Three to four perineal patterns from a single population positioned on the slide with outer side uppermost and a glass cover slide applied (Taylor and Netscher, 1974; Taylor and Sasser, 1978; Eisenback and Hunt, 2009). The second method was *molecular marker;* species-specific primers have been prepared by (Sinacion, Bioscience Co. Iran), for identifying species of *Meloidogyne* as shown in *Table 1*. The extraction of DNA for *Meloidogyne* spp. and DNA amplification have been conducted at the Graduate Studies Laboratory- Animal Science Department – College of Agricultural Sciences, Sulaimani University.

***Table 1.*** *Species-specific primers used for Meloidogyne identification*

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|
| **Primer name** | **OD (1000 µm)** | **MW** | **nmol** | **Water/tube (µl)** | **TM** | **Seq. (5¯ __ 3¯)** | **mer** |
| AS1F | 7 | 6029 | 38.31 | 383.15 | 55.9 | CTCTGCCCAATGAGCTGTCC | 20 |
| AS1R | 7 | 5410 | 42.70 | 426.99 | 50.3 | CTCTGCCCTCACATTAGG | 18 |
| AS2F | 7 | 5939 | 38.90 | 388.95 | 53.2 | TAGGCAGTAGGTTGTCGGG | 19 |
| AS2R | 7 | 6412 | 36.03 | 360.26 | 52.4 | CAGATATCTCTGCATTGGTGC | 21 |
| AS3F | 6 | 6188 | 32.00 | 319.97 | 51.8 | GGGATGTGTAAATGCTCCTG | 20 |
| AS3R | 7 | 5917 | 39.04 | 390.40 | 55.9 | CCCGCTACACCCTCAACTTC | 20 |
| AS4F | 7 | 6117 | 37.76 | 377.57 | 55.9 | GTGAGGATTCAGCTCCCCAG | 20 |
| AS4R | 7 | 6968 | 33.15 | 331.52 | 57.1 | ACGAGGAACATACTTCTCCGTCC | 23 |
| AS5F | 7 | 6663 | 34.67 | 346.69 | 53 | CCTTAATGTCAACACTAGAGCC | 22 |
| AS5R | 7 | 6110 | 37.81 | 378.07 | 49.7 | GGCCTTAACCGACAATTAGA | 20 |
| AS6F | 7 | 6198 | 37.27 | 372.70 | 55.9 | GGTGCGCGATTGAACTGAGC | 20 |
| AS6R | 7 | 7008 | 32.96 | 329.62 | 57.1 | CAGGCCCTTCAGTGGAACTATAC | 23 |
| AS7F | 7 | 6102 | 37.86 | 378.56 | 53.8 | ACGCTAGAATTCGACCCTGG | 20 |
| AS7R | 7 | 6136 | 37.65 | 376.47 | 55.9 | GGTACCAGAAGCAGCCATGC | 20 |

### *Molecular method*

In this study, species-specific primers have been prepared by (Sinacion, Bioscience Co. Iran), for identifying species of *Meloidogyne* as shown in *Table 1*. The extraction of DNA for *Meloidogyne* spp. and DNA amplification have been conducted at the Graduate Studies Laboratory- Animal Science Department – College of Agricultural Sciences, Sulaimani University.

### *DNA extraction*

DNA of J2s and adult males isolated from soil were extracted by extraction-tray model (Thomas, 1958; Whitehead and Hemming, 1965). The females have been


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isolated from root tissue with sharp needle under stereo microscope. Therefore, J2s, males and female were collected with enough water in a tube that obtained by using standard technique (1.5 ml tube), the tube was put in a centrifuge (226R Refrigerated Universal Microcentrifuge, Labnet International Inc.) in 10000 rpm for 3 min in order to precipitate nematodes down to the bottom of the tube, then excess water was removed from the upper part. An amount of 25 mg from the precipitated nematodes was transferred to a slide and 20 µL of ethanol was added, waited till dry then the precipitate nematodes were powdered by sharp needle under stereo microscope (ST-39 Series, Motic Co, United Kingdom). DNA of RKN was extracted utilizing AccuPrep® Genomic DNA Extraction Kit from (BIONEER) which involved isolation of DNA from mammalian tissue.

### *Polymerase chain reactions (PCR) amplification*

Different primers were used for DNA amplification. The polymerase chain reactions (PCR) were carried out using the extracted RKN DNA as a template. *Table 2* shows preparing different PCR reaction tube volume (25 µL and 50 µL) using the following ingredients.

The DNA samples were amplified using primers for two common species in separate PCR reactions. All PCR reactions were carried out in MUTIGENE OptiMAX machine, Labnet International Inc. (PCR Thermal Cycler). Similar PCR programs were used to identify *Meloidogyne* spp. (Powers and Harris, 1993; Zijlstra et al., 2000; Meng et al., 2004; Adam et al., 2007). The program has been changed and different annealing temperatures have been used for amplifying the DNA of the species according to the previous studies.

***Table 2.*** *Different ingredients amount used to complete (25 µL and 50 µL) of the PCR reaction tube volume*

| Ingredients | Amount | Unit | Details |
|---|---|---|---|
| Master Mix | 7.5, 12.5 and 25 | µL | Including of dATP, dCTP, dGTP, dTTP, Taq DNA polymerase and PCR buffer |
| Sterilized distilled water | 4.5, 7.5, 12.5 and 22 | µL | |
| Forward primer | 1 | µL | |
| Reverse primer | 1 | µL | |
| DNA extraction | 3, 6 and 1 | µL | |

### *Gel electrophoresis*

The gel used for electrophoresis (2.5%) was prepared by weighing out 2.5 g of agarose powder and dissolving in 100 ml of 1X TBE buffer (100 ml of 10X TBE was diluted in 900 ml of distilled water). The buffer was heated for 1 min in a microwave oven to dissolve the agarose powder and allowed to cool slightly (55 °C) before adding 6 µL ethidium bromide. The mixture was poured into the electrophoresis chamber and allowed to cool. Combs with 16 and 13 teeth per row, were placed in the liquid agarose to make 16 and 13 µL wells. Once the gel had solidified, the electrophoresis chamber was filled with sufficient 1X TBE buffer to cover the gel. Once the gel set, the combs were gently removed to avoid damaging the wells. The wells were loaded with 10 µL of


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the PCR products mixed well with 3 µL of 6X loading buffer and 6 µL of DNA ladder was loaded into the left and right corner wells. The electrophoresis was run at 90-95 V and 65 A for 90 min to allow the separation of the fragments. The gel was placed in a UV transilluminator (Enduro™ GDS Touch, Labnet International Inc.). The gel pictures were digitally recorded and named with the sample number. DNA ladder (100-3000 bp) was used to approximate the size of the fragments. The size of bands on the gel was compared to the expected band size of *Meloidogyne* spp.

### *Plastic house experiment*

#### *Site study*

The experiment was conducted in the plastic house during July- December 2015 in Bakrajo 2 km far from the College of Agricultural Sciences, Sulamani University. The plastic house is located on a latitude of 35° 32' 03.2" N and longitude 45° 21' 15.7" E. GPS map (76CS, Garmin Co, Taiwan) had been used for this purpose.

#### *Site description and experimental design*

This study was conducted in the plastic house during 25th July to 5th December 2015. The total area of the plastic house 450 $m^2$ (9 × 50 m). This experiment was carried out by using the Randomized Complete Block Design (RCBD) with three replicates, each replicate consisted of nine plots (experiment units) distributed to three rows, the area of each plot = 2.7 $m^2$ (0.85 m × 3.2 m), the distance between plots was 1 m within one replicate, while 2 m was used between replicates, four pathways extend parallel to an along plastic house, the width of each pathway was 0.70 m. The soil of the plastic house was naturally infected by RKN (*Meloidogyne* spp) and soil samples (1 kg) have been taken randomly prior the applying of the treatments for the chemical and physical analysis at the soil department laboratory/Sulaimani Agricultural Research Center. For watering the plants, dripping system was used. Another one kg of soil sample/plot was taken before and after applying treatments within three periods (10, 20 and 30 days of applying the treatments (100 g of soil was taken for each experimental unit (plot)). For preparing seedling of cucumber cv. Naseem F1, seeds were sown in a special place near the plastic house. After 10 days, the seedlings reached to appropriate length (2-3 leaves stage) were transplanted to the permanent place in the plastic house, all needed agricultural processes have been conducted for all plots and treatments.

#### *The physical and chemical properties of the greenhouse soil*

Physical and chemical soil analysis of the greenhouse soil prior treatments was conducted at the Sulaimani Applied Agricultural Research Center-Department of Soil and Water Resource/GDAWR, Ministry of Agriculture and Water Resources, KRG, Iraq (*Table 3*).

***Table 3.*** *Some physical and chemical properties of the greenhouse soil before applying the treatments*

| Soil texture | EC $dsm^{-1}$ | PH | Total N % | Available P (ppm) | Exch. $K^{+1}$ ppm | Exch. $Na^{+1}$ meq/L | Soluble $Ca^{+2}$ meq/L | Soluble $Mg^{+2}$ meq/L | $Cl^{-}$ meq/L | % O.M | % CaCO3 | HCO3 | $CO3^{=}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Clay loam | 0.18 | 7.5 | 0.11 | 9.25 | 333.100 | 74.93 | 1.8 | 0.7 | 0.2 | 1.3 | 20 | 1.9 | 0 |


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*The treatments*

In the plastic house, different chemicals and organic substrates were used alone and in combinations as follows:

1. Nemakey1.5 ml/m² (The soil sprayed thrice, 10 days interval was between the spraying 12-8-2015, 22-8-2015 and 1-9-2015, respectively).
2. Besto Humic (Humic acid15%) 1.5 ml/m² (the soil sprayed thrice, 10 days interval was between the spraying 12-8-2015, 22-8-2015 and 1-9-2015, respectively).
3. Chitosan (2%) 1 g/500 ml water $(C_6H_{12}O_4)n$ Assay 98% (The foliar sprayed twice, 10 days interval was between the spraying 26-8-2015 and 6-9-2015, respectively).
4. Nemakey1.5 ml/m² + Besto Humic (Humic acid15%) 1.5 ml/m².
5. Nemakey1.5 ml/m² + Chitosan (2%) 1 g/500 ml water.
6. Besto Humic (Humic acid15%) 1.5 ml/m² + Chitosan (2%) 1 g/500ml water.
7. Nemakey1.5 ml/m² + Besto Humic (Humic acid15%) 1.5 ml/m² + Chitosan (2%) 1 g/500ml water.
8. Rugby® 100 ME 1.6 ml/m² chemical nematicide (positive control) (reference treatment) (The soil sprayed thrice, 10 days interval was between the spraying12-8-2015, 22-8-2015 and 1-9-, respectively).

The manufacturers of the chemical as in *Table 4* (all the chemicals were purchased from the local market).

***RKN population density***

One kg soil at the depth of 20-30 cm was collected from each plot and treatment, and mixed thoroughly, 100 g of this soil was taken at 12-8-2015 in order to calculate the no. of nematodes (J2) after applying the treatments, the process repeated for 10, 20 and 30 days. In order to isolate nematodes from the subsample soil (100 g) modified extraction-tray method was used according to (Thomas, 1958; Whitehead and Hemming, 1965), which the soil was placed on a double layer of paper towel (kitchen paper) supported by a metal buckle and placed over the extraction tray. Water was gently added to the tray until the soil was completely wet and then left for 48 h so that the nematodes would be washed down. counting dish digital hand counter and light stereo microscope (ST-39 Series, Motic Co. United Kingdom) 4× magnifications have been used to calculate the nematodes (Atamian et al., 2012; Bonuke, 2013).

***Reproduction factor (Rf)***

The purpose of Reproduction factor (Rf) was to evaluate the effect of the different treatments on the population densities of the nematodes, if the recorded number was at least more than one, this means that the treatment had bad effect on the decreasing of the nematode population, while if the number was less than one, this means an excellent effect of the treatment was produced on the nematode densities. For calculating Rf the following equation was used (Timper et al., 2006; Lima et al., 2009).

$$\text{Reproduction factor (Rf)} = \frac{\text{Final population of the nematodes (Pf)}}{\text{Initial population (Pi)}} \qquad \text{(Eq.1)}$$


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***Table 4.*** *The organic and chemical substrates were used in plastic house experiment*

| No. | Trade name | Active ingredient | Manufacturer |
|---|---|---|---|
| 1. | Nemakey | Organic | Merkez Anadolu Kimya Sanayi Co., Turkey |
| 2. | Besto humic | Humic acid (15%) | Organik Tarim Market Co., Turkey |
| 3. | Chitosan (98%) | N- acetyl- glucosamine* | Xi'an Lyphar Biotechnology Co., Ltd., China |
| 4. | Rugby® 100 ME | Cadosaphos | FMC corporation, Agricultural Products Group, Philadelphia, Pennsylvania, USA |

*Root galling index (RGI)*

120 days after transplanting cucumber plant cv. Naseem F1 to permanent place, the plants were carefully uprooted from the soil in each plot and washed in running tap water to remove the adhering soil particles. After that the galls were removed from the root systems in each plant in order to calculate the number of the galls. From each plot 10 plants have been taken. For root gall index evaluating standard scale has been used as described by Taylor and Sasser (1978) shown in *Table 5*, for calculating disease severity and gall index the equations below has been used according to Zewain (2014), the percentage of gall decreasing also has been calculated equations (Kesba and Al-Shalaby, 2008).

$$\text{Disease severity (\%)} = \frac{\Sigma\,(\text{No.of galled plants} \times \text{its galling degree})}{\text{Total galled plant no.} \times \text{highest galling degree}} \times 100 \quad \text{(Eq.2)}$$

$$\text{Gall index} = \frac{\Sigma\,(\text{No.of galled plants} \times \text{its galling degree})}{\text{Total galled plant}} \quad \text{(Eq.3)}$$

$$\text{Gall reduction (\%)} = \frac{(\text{Control 2}) - \text{Treatment}}{(\text{Control 2})} \times 100 \quad \text{(Eq.4)}$$

***Table 5.*** *Standard scale of Root-knot index*

| Root-knot index | Number of galls/root system |
|---|---|
| 0 | 0 |
| 1 | 1-2 |
| 2 | 3-10 |
| 3 | 11-30 |
| 4 | 31-100 |
| 5 | > 100 |

***Plant growth and yield parameters***

For calculating each parameter 10 plants have been used from each experimental unit (plot), for vegetative system; plant height (cm), stem diameter (cm), no. of nodes/plant, no. of leaves/plant, fresh weight (gm/plant), % of dry weight of vegetative parts and leaf area ($cm^2$) have been measured. For root system; root length (cm), at the end of the seasons, the root system was extracted manually for the representative plants, then main root length (cm) was measured from the crown zone to its farthest point using the metric tape, and fresh and dry root weights (gm/plant) have been calculated. Leaf chlorophyll (9 leaves of each 10 plants randomly selected) (Romero et al., 2012), Leaf chlorophyll


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intensity (SPAD unit) (Konica-Minolta, Osaka, Japan) (Coste et al., 2010), and leaf NPK content also calculated. For yield parameters; no. of aborted flowers/plant, no. of setting flowers/plant, fresh weight of fruits (gm/kg), diameter and length of fruits and moisture content, vegetative ratio (w/w), and finally the % of increased yield has been calculated for each treatment. The followings are the formula of the measured and calculated growth and yield parameters.

$$\text{Dry vegetative parts weight (\%)} = \frac{\text{Dry weight (g)}}{\text{Fresh weight}} \times 100 \quad \text{(Van De Sande – Bakhuyzen, 1928)} \quad \text{(Eq.5)}$$

$$\text{Fruit moisture content (\%)} = \frac{\text{Fresh fruit weight} - \text{Dry fruit weight}}{\text{Fresh fruit weight}} \times 100 \quad \text{(FAO, 2013)} \quad \text{(Eq.6)}$$

$$\text{Vegetative/fruit ratio (w/w) (\%)} = \frac{\text{Fresh plant weight (vegetative part)}}{\text{Total harvested fruit (total yield)}} \times 100 \quad \text{(Eq.7)}$$

$$\text{Percentage of increased yield (\%)} = \frac{\text{Yield (weight) of a treatment} - \text{Yield (weight) of control}}{\text{Yield (weight) of control}} \times 100 \quad \text{(Hasabo and Noweer, 2005)} \quad \text{(Eq.8)}$$

### *Statistical analysis*

Randomized Complete Block Design (RCBD) was used in this study, the treatments repeated thrice (three replicates) (Crotty et al., 2009; Machado et al., 2014), the data were analyzed using XLSTAT program version 7.5 (XLSTAT, 2004). For the comparisons between means, Least Significant Difference (LSD) at the probability level of 5% was used (Williams and Abdi, 2010; Chapuis-Lardy et al., 2015).

## Results

### *Identification and descriptions of isolated Root-Knot Nematodes (RKNs)*

One of the most important purposes of this study was to identify and put light on RKN *Meloidogyne* species present in the galls of the infected cucumber cv. Naseem F1 roots which was grown in the plastic house Bakrajo region. During isolation process the females of RKN from infected cucumber roots under stereo microscope, two species have been recorded *Meloidogyne incognita* and *Meloidogyne javanica* (*Fig. 1A, B, C*). The mature females of *M. incognita* under stereo microscope have a spherical shaped body, short projecting neck and white color (*Fig. 1A, B*).

### *Morphological characteristics (perineal patterns)*

The perineal patterns observed under light microscope (*Fig. 2A*) very closely resembled to the descriptions of *M. incognita* which typically high with square-like dorsal arch. Lateral field weakly demarcated by breaks and forked striae. Striae were distinct and wavy. Dorsal striae smooth, closely placed wavy to zigzag that appear the dorsal and ventral striae are interrupted and forked at the lateral line.

### *Molecular identification*

Accurate identification of RKN was obtained using molecular characteristics. Specific primers of *M. incognita* and *M. javanica* were used to amplify DNA fragment for all sample tested including J2s, males and females of *Meloidogyne* obtained from


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plastic house. The extracted DNA was visualized using Agarose gel and then the concentration of DNA was tested by Nanodrop 2000C device.

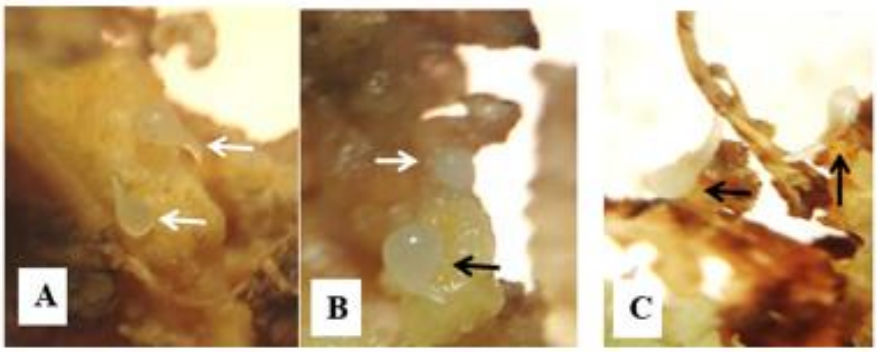


***Figure 1.*** *Stereo microscope photograph of female M. incognita (A), (B) and female of M. javanica (C) from infected cucumber root tissue (galls)*

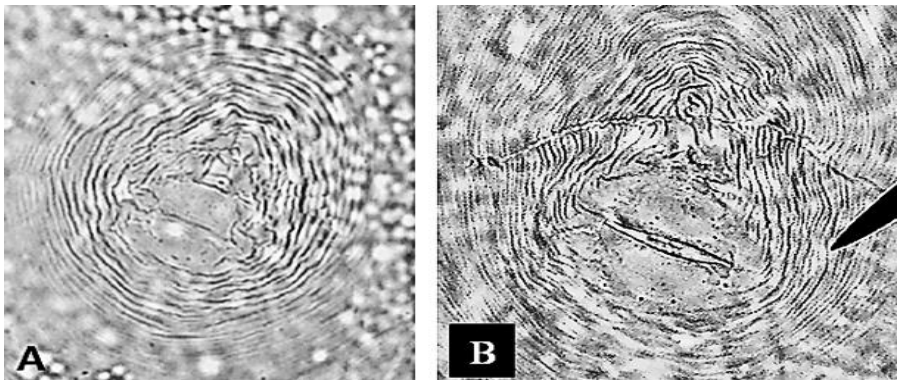


***Figure 2.*** *Perineal pattern of (A) M. ingonita and (B) M. javanica under light stereomicroscope (Binocular Digital Compound Microscope (X400))*

The procedure of DNA amplification has been performed six times and all pair primers were set up according to the annealing temperature for first and second times that mentioned by the manufacture company (Sinacion, Bioscience Co. Iran). The DNA samples were run again with all seven primers. They were set up according to the denaturation and annealing temperature conducted by previous studies. The ingredients amount of PCR reaction tube volume (25 µL and 50 µL) has been changed in every time.

Significant result of DNA amplification was able to be obtained using annealing temperature of 49 °C and 50 µL of the PCR reaction volume. Master mix, sterilized distilled water, forward primer, reverse primer and DNA extraction were 25, 22, 1, 1 and 1 µL in size respectively when tested on agarose gel (*Figs. 3A, B* and *4A, B*).

The fail in obtaining positive result for both species using, AS2F AS2R, AS4F AS4R, AS5F AS5R primers might be refer to the specificity of these two races of nematodes used in the current study, being different from those used for other investigations. The occurrence of genetic mutation and the variation due to mating system in the species might made the primers to misplacing the flanked region of target fragment on the species DNA.


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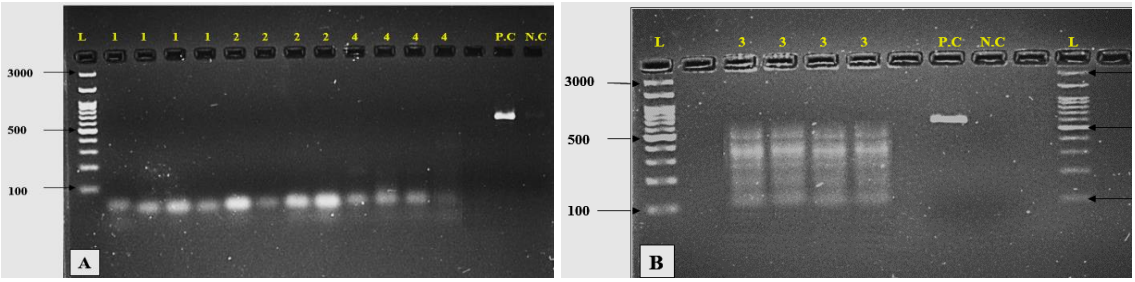


***Figure 3.*** *PCR products. (A) Extracts DNA amplified using species-specific primers name of primer 1 = AS1, 2 = AS2, 4 = AS4 (1200 bp, 1350 bp, and 955 bp) respectively for M. incognita, L = DNA ladder (100-3000 bp). P.C = Positive control, N.C = Negative control. (B) Extracts DNA amplified using species-specific primers 3 = AS3F AS3R (399 bp), for M. incognita. L = DNA ladder (100-3000 bp). P.C = Positive control, N.C = Negative control*

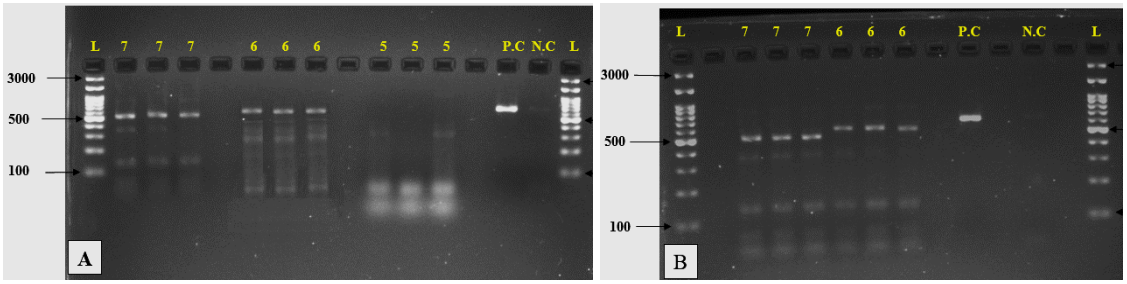


***Figure 4.*** *PCR products. (A) Extracts DNA amplified using species-specific primers name of primer 7 = AS7, 6 = AS6, 5 = AS5 (517 bp, 670 bp and 1650 bp) respectively for M. javanica, L = DNA ladder (100 bp-3000 bp), P.C = Positive control, N.C = Negative control. (B) Extracts DNA amplified using species-specific primers 7 = AS7, 6 = AS6 respectively for M. javanica, L = DNA ladder (100 bp-3000 bp), P.C = Positive control, N.C = Negative control*

## *Nematode population density*

*Population densities of root-knot nematode (Juveniles 2) in the soil before and after treatment application*

In the plastic house experiment, different treatments have been used in order to investigate their influence on controlling the population densities and numbers of juveniles 2 before applying the treatments and after 10, 20 and 30 days. *Table 6* shows that at ($p \leq 0.05$) the minimum numbers of juveniles 2 (J2s) were in using (Nemakey, Humic acid and Chitosan) which was (66.33), while maximum number of nematodes observed control (1 + 2). There were significant differences among the treatments ($P \leq 0.05$) with the control 2. On the other hand, there were no significant differences among the treatments and control after 10 and 20 days (*Table 6*).

The present results show an approach to control *M. incognita* and *M. javanica* in cucumber plant using modern methods and more active for controlling *Meloidogyne* which are safer than nematicides. These results should be considered during designing an integrated pest management program for RKN or other nematode pathogens in cucumber and other crops.


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***Table 6.*** *Effect of different treatments for controlling root-knot nematode (Meloidogyne spp.) on population densities (Juveniles 2)*

| Treatment sets | Population densities (Juveniles 2) / 100 g soil | | | |
|---|---|---|---|---|
| | One day before applying treatments (Pi) | After 10 days of application | After 20 days of application | After 30 days of application (Pf) |
| Nemakey | 163.00 a | 234.33 ab | 192.33 a | 151.66 c |
| Besto Humic | 152.00 a | 321.66 ab | 222.00 a | 140.00 cd |
| Chitosan | 123.66 a | 296.00 ab | 310.33 a | 301.66 b |
| Nemakey + Besto Humic | 180.66 a | 312.66 ab | 348.00 a | 125.33 cd |
| Nemakey + Chitosan | 208.66 a | 264.33 ab | 330.00 a | 133.00 cd |
| Besto Humic + Chitosan | 141.66 a | 235.33 ab | 447.00 a | 120.66 cd |
| Nemakey + Besto Humic + Chitosan | 133.33 a | 227.66 ab | 508.00 a | 66.33 d |
| Rugby® 100 ME (Control 1) | 148.33 a | 150.00 b | 197.00 a | 363.33 b |
| Water (Control 2) | 159.66 a | 592.66 a | 503.66 a | 587.33 a |
| LSD ($P \leq 0.05$) | 101.75 u | 403.02 u | 464.49 u | 80.87 u |

Data followed by the same letter in each column are not significantly different according to (LSD) test

*Reproduction factor (Rf)*

Data in *Figure 5* illustrate the impact of Nemakey, Humic acid and Chitosan alone or in combination comparing to Rugby® 100 ME as (Control 1) and spray water only as (control 2) on reducing *Meloidogyne incognita* and *Meloidogyne javanica* which infected cucumber plant (cv. Naseem F1) under plastic house condition. Results revealed that reproduction factor (Rf) was adversely affected by treatments whether alone or in combination. It has been considered the best significant treatment was the three combinations (Nemakey + Best Humic (Humic acid 15%) + Chitosan), the Rf value was less than one, although the others (Nemakey, Humic acid 15%), (Nemakey + Chitosan 2%), and (Humic acid15% + Chitosan 2%) treatments gave the same result, that mean the efficacy of the treatments in reducing the population density of root-knot nematodes (Juveniles 2).

*Root gall index (RGI)*

Data presented in *Table 7* show the influence of various treatments Nemakey, Humic acid and Chitosan whether alone or in combination in comparison to with controls (1 + 2) against RKN which infected cucumber crop (cv. Naseem F1) under plastic house condition.

Results indicated that all treatments obviously were reducing the number of gall index, disease severity percentage and percentage of gall decreasing (reduction). Among the treatments, combined treatments (Nemakey + Humic acid + Chitosan) recorded (23.76) of reducing the gall formation on the root system of the cucumber crop and achieved higher significant ($p \leq 0.05$) with the highest percentage of gall decreasing (77.00%) and found to be the most effective, since it reduced the number of galls more than those of the other treatment, followed by (Nemakey + Chitosan) (39.40 galls/plant, 61.86% reduction) and (Humic acid + Chitosan) (39.90 galls/plant, 61.38% reduction) as compared with controls (69.46, 103.33, 32.77% reduction) respectively. Minimum galling index (3.1) was recorded when the cucumber plant was treated with combined of (Nemakey, Humic acid and Chitosan) followed by (Humic acid and chitosan) (3.3) and (Nemakey and Humic acid) (3.6) as compared to controls (4.1 and 4.5) respectively.


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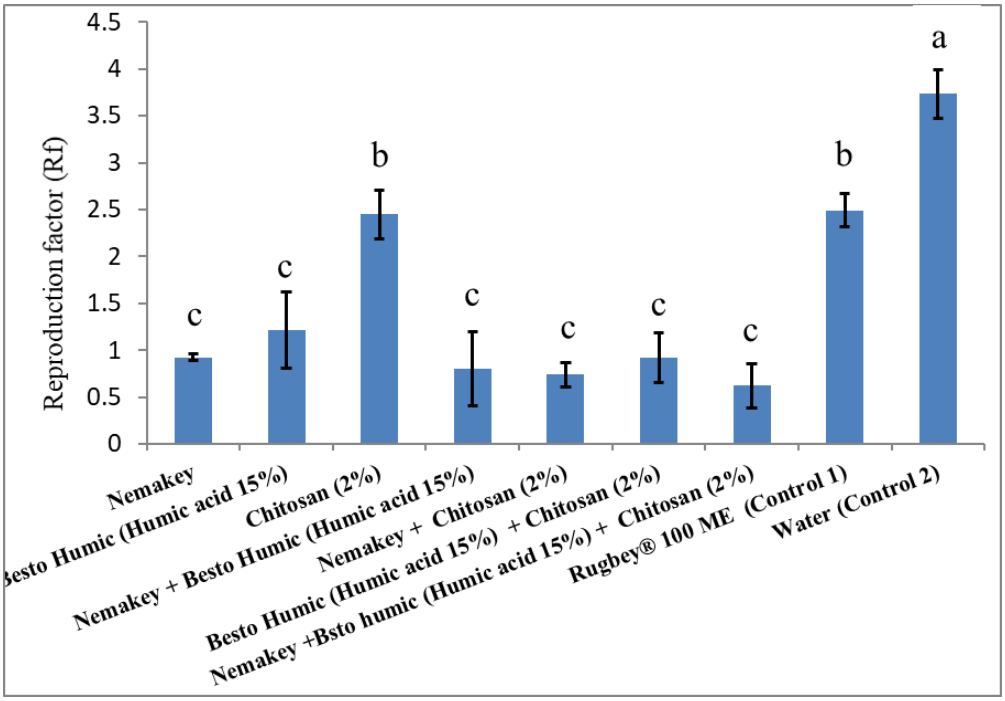


***Figure 5.*** *Effect of different methods of controlling on reproduction factor (Rf) after 30 days of calculating the no. of the nematodes (juveniles 2). The bars refer to the standard errors (SE) at ($P \leq 0.05$)*

***Table 7.*** *Effect of different treatments for controlling root-knot nematode (Meloidogyne spp.) on number of galls in the roots of cucumber (cv. Naseem F1)*

| Treatment sets | Means of number of the galls | | Gall index | Disease severity % | % of gall decreasing |
|---|---|---|---|---|---|
| | Average 0.05 | Average 0.01 | | | |
| Nemakey | 50.29 bc | 50.29 bc | 3.70 | 74.00 | 51.33 |
| Besto Humic | 50.86 bc | 50.86 bc | 3.90 | 97.50 | 50.77 |
| Chitosan | 54.23 bc | 54.23 bc | 3.90 | 78.00 | 47.51 |
| Nemakey + Besto Humic | 40.40 cd | 40.40 bc | 3.60 | 90.00 | 60.90 |
| Nemakey + Chitosan | 39.40 cd | 39.40 bc | 3.70 | 93.00 | 61.86 |
| Besto Humic + Chitosan | 39.90 cd | 39.90 bc | 3.30 | 66.00 | 61.38 |
| Nemakey + Besto Humic + Chitosan | 23.76 d | 23.76 c | 3.10 | 77.50 | 77.00 |
| Rugby® 100 ME (Control 1) | 69.46 b | 69.46 ab | 4.10 | 82.00 | 32.77 |
| Water (Control 2) | 103.33 a | 103.33 a | 4.50 | 90.00 | – |
| LSD ($P \leq 0.05$) | 25.83 | 35.58 | | | |

Data are means of three replicates. Data followed by the same letter in each column are not significantly different according to (LSD) test

Disease severity as shown in *Table 7* appeared that lowest disease severity percentage was recorded by (Humic acid and Chitosan) which achieved higher significant ($p \leq 0.05$) effect followed by Chitosan and (Nemakey, Humic acid and Chitosan) compared to controls.


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### *Plant growth characters*

*Vegetative system*

The combination treatments Nemakey, Humic acid and Chitosan significantly ($P \leq 0.05$) affected against RKN (*M. incognita* and *M. javanica*) infection as compared to controls treatment. The reduction in growth parameters was indirectly proportional with the number of root-galls, so all screened treatments obviously improved plant growth parameters with various degrees (*Table 8*). Among tested treatments, plant receiving combined treatments (Nemakey, Humic acid and Chitosan) improved significantly in plant height and stem diameter with percentage of the dry weight of the vegetable parts amounted to 2.97 cm, 8.46 cm and 11.67% respectively which exceeded all other treatments as compared to control 2 (1.61 cm, 6.17 cm, and 28.07%). On the other hand, it was also observed that there were significant differences among the treatments ($p \leq 0.05$) with control 2, while with Rugby® 100 ME (Conventional chemical control) or (Reference treatment), they were relatively had least effect. However, there were no significant differences among the treatments (with themselves).

***Table 8.*** *Effect of different treatments for controlling root-knot nematode (Meloidogyne spp.) on some plant growth parameters (vegetative parts) of cucumber (cv. Naseem F1)*

| Treatment sets | Plant height (cm) | Stem diameter (cm) | No. of nodes. plant $^{-1}$ | No. of leaves. plant $^{-1}$ | Fresh plant weight (vegetable parts) g plant $^{-1}$ | Leaf area. plant $^{-1}$ (cm²) | % of the dry weight of the vegetative parts |
|---|---|---|---|---|---|---|---|
| Nemakey | 2.40 bc | 7.68 bc | 40.66 de | 26.66 b | 293.30 cde | 250.68 ab | 20.87 |
| Besto Humic (Humic acid) | 2.38 c | 7.86 b | 39.66 e | 26.66 b | 271.66 de | 241.13 abc | 20.91 |
| Chitosan | 2.35 c | 7.14 c | 39.33 e | 26.66 b | 274.90 de | 215.07 cd | 16.10 |
| Nemakey + Besto Humic (Humic acid) | 2.63 b | 7.84 b | 48.66 b | 40.00 a | 424.90 ab | 261.52 ab | 13.96 |
| Nemakey + Chitosan | 2.46 bc | 7.67 bc | 42.66 cd | 40.00 a | 339.43 bcd | 231.68 bcd | 17.03 |
| Besto Humic (Humic acid) + Chitosan | 2.52 bc | 7.76 b | 44.66 c | 39.66 a | 386.33 abc | 239.30 abc | 14.67 |
| Nemakey + Besto Humic (Humic acid) + Chitosan | 2.97 a | 8.46 a | 50.33 ab | 42.33 a | 480.20 a | 266.99 a | 11.67 |
| Rugby® 100 ME (Control 1) | 2.07 d | 7.47 bc | 34.33 f | 26.66 b | 229.96 e | 231.12 bcd | 24.05 |
| Water (Control 2) | 1.61 e | 6.17 d | 27.66 g | 21.00 b | 201.30 e | 196.63 d | 28.07 |
| LSD ($P \leq 0.05$) | 0.24 u | 0.56 u | 2.17 u | 7.91 u | 96.75 u | 35.09 u | – |

Data are means of three replicates. Data followed by the same letter in each column are not significantly different according to (LSD) test

Results in *Table 8* show that other parameters of cucumber plant growth i.e. no. of nodes/plant and plant weight (vegetative parts) / plant (g) were significantly affected by applying these combined treatments (Nemakey, Humic acid and Chitosan) and (Nemakey and Humic acid) when compared to controls. On the other hand, there was less effect of the treatments on the no. of leaves. plant$^{-1}$ if compared to the controls (1 + 2).

*Root system*

There was a significant ($P \leq 0.05$) difference between the root length (cm), fresh root weight (g) and dried root weight (g) of the cucumber plants treated with different treatments of Nemakey, Humic acid, Chitosan and controls as shown in *Table 9*.

All the treatments were significantly ($P \leq 0.05$) different on the basis of root length. Maximum root length (32.08, 31.74 and 31.70 cm) was recorded in the plants treated with combined (Nemakey, Humic acid and Chitosan), (Nemakey and Humic acid) and


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(Nemakey and Chitosan) respectively, as compared to controls. Minimum root length (25.38 cm) was recorded in untreated control plants (control 2). It can also be noticed that (Nemakey + Humic acid and Chitosan), (Humic acid and Chitosan) and (Nemakey and Humic acid) were significantly showed reasonable improved fresh root weight and ranked the most active one among the other treatments for increasing fresh root weight of cucumber plant with 34.86, 30.60 and 30.76 g, respectively as compared to control 2, which was recorded 24.46 g. In the meanwhile, plants receiving combined treatments (Nemakey, Humic acid and Chitosan) and (Humic acid and Chitosan) gave significantly better dried root weight than all other treatments, followed by (Nemakey and, Humic acid) as compared to control.

***Table 9.*** *Effect of different treatments for controlling root-knot nematode (Meloidogyne spp.) on some plant growth (root parts) of cucumber (cv. Naseem F1)*

| Treatment sets | Root length (cm) | Fresh root weight (g) | Dried root weight (g) |
|---|---|---|---|
| Nemakey | 30.24 abc | 29.13 b | 11.31 d |
| Besto Humic (Humic acid) | 29.86 abc | 29.03 b | 12.91 cd |
| Chitosan | 27.19 bcd | 28.70 bc | 11.76 d |
| Nemakey + Besto Humic (Humic acid) | 31.74 a | 30.76 ab | 14.23 bc |
| Nemakey + Chitosan | 31.70 a | 29.43 b | 11.81 d |
| Besto Humic (Humic acid) + Chitosan | 31.58 ab | 30.60 ab | 15.07 ab |
| Nemakey + Besto Humic (Humic acid) + Chitosan | 32.08 a | 34.86 a | 16.46 a |
| Rugby® 100 ME (Conventional chemical control) (Control 1) | 27.10 cd | 27.50 bc | 8.22 e |
| Water (Control 2) | 25.38 d | 24.46 c | 6.06 f |
| LSD ($P \leq 0.05$) | 4.42 | 4.29 | 1.72 |

Data are means of three replicates. Data followed by the same letter in each column are not significantly different according to (LSD) test

*Leaf chlorophyll and NPK content*

Data as shown in *Table 10* summarize the description of the leaf chlorophyll intensity, the percentage of the Nitrogen (N), Phosphor (P) and potassium (K) content in the cucumber leaves as influenced by *M. incognita* and *M. javanica* infection and effect of applying Nemakey, Humic acid and Chitosan whether alone or in combination in comparison to Rugby® 100 ME (Conventional chemical control) (Reference treatment) or (control 1) and spray water only (control 2) in the greenhouse conditions.

Obviously, results in *Table 11* show that two of them significantly ($P \leq 0.05$) achieved high percentage increase the values of the leaf chlorophyll intensity and percentage of the Nitrogen (N) content in the leaf per (1 g) of dry weight under study. Among tested treatments, the combined treatments (Nemakey, Humic acid and Chitosan), (Nemakey and Humic acid) and (Humic acid and Chitosan) were superior in values of leaf chlorophyll (38.19, 35.35 and 35.10) respectively as compared to controls (28.77 and 24.79), whereas the combined treatments (Nemakey, Humic acid and Chitosan) and (Nemakey and Humic acid) ranked the first increase in percentage values of Nitrogen (N) content in the leaf per (1 g) of dry weight which were 4.86 and 4.83% respectively as compared to control 2 (4.56%). In addition, it is appeared that there were no significant differences among the treatments and controls on percentage of the


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Phosphor (P) and Potassium (K) content in the leaves (*Table 8*), and chlorosis of the leaves (pale green tended to yellowing).

***Table 10.*** *Effect of different treatments for controlling root-knot nematode (Meloidogyne spp.) on some plant content of cucumber (cv. Naseem F1)*

| Treatment sets | Leaf chlorophyll intensity (SPAD unit) | % of the nitrogen (N) content in the leaf dry weight | % of the phosphor (P) content in the leaf dry weight | % of the potassium (K) content in the leaf dry weight |
|---|---|---|---|---|
| Nemakey | 31.58 cd | 4.66 abc | 0.36 a | 3.08 a |
| Besto Humic (Humic acid) | 31.54 cd | 4.60 bcd | 0.35 a | 3.23 a |
| Chitosan | 31.50 cd | 4.40 d | 0.32 a | 3.22 a |
| Nemakey + Besto Humic (Humic acid) | 35.35 ab | 4.83 ab | 0.31 a | 3.43 a |
| Nemakey + Chitosan | 33.64 bc | 4.66 abc | 0.36 a | 3.46 a |
| Besto Humic (Humic acid) + Chitosan | 35.10 ab | 4.70 abc | 0.35 a | 3.22 a |
| Nemakey + Besto Humic (Humic acid) + Chitosan | 38.19 a | 4.86 a | 0.36 a | 3.23 a |
| Rugby® 100 ME (Control 1) | 28.77 d | 4.73 abc | 0.35 a | 3.24 a |
| Water (Control 2) | 24.79 e | 4.56 cd | 0.32 a | 3.14 a |
| LSD ($P \leq 0.05$) | 3.33 u | 0.25 u | 0.07 u | 0.51 u |

Data are means of three replicates. Data followed by the same letter in each column are not significantly different according to (LSD) test

***Table 11.*** *Effect of different treatments for controlling root-knot nematode (Meloidogyne spp.) of cucumber (cv. Naseem F1) on floral growth*

| Treatment sets | No. of flowers. plant $^{-1}$ | No. of aborted flowers. plant $^{-1}$ | No. of fruit. plant $^{-1}$ | Fresh weight of fruit. plant $^{-1}$ (g) | Diameter of fruits (cm) | Length of fruits (cm) | Fruit moisture content (%) | Vegetative/ fruit ratio (W/W) |
|---|---|---|---|---|---|---|---|---|
| Nemakey | 41.00 cd | 48.93 ab | 33.00 cd | 85.76 ab | 2.77 a | 13.70 ab | 97.59 a | 29.91 |
| Besto Humic (Humic acid) | 44.00 ab | 50.50 a | 34.66 bcd | 86.70 ab | 2.79 a | 13.27 bc | 97.52 a | 32.44 |
| Chitosan | 41.00 cd | 47.66 ab | 31.66 d | 84.75 ab | 2.68 a | 13.12 bc | 97.63 a | 32.05 |
| Nemakey + Besto Humic (Humic acid) | 44.66 a | 46.06 ab | 36.66 ab | 92.05 a | 2.78 a | 14.31 a | 97.41 a | 21.71 |
| Nemakey + Chitosan | 43.00 abc | 44.80 ab | 36.33 abc | 88.81 ab | 2.69 a | 13.39 b | 97.69 a | 27.91 |
| Besto Humic (Humic acid) + Chitosan | 42.00 bcd | 46.13 ab | 36.66 ab | 89.27 ab | 2.76 a | 13.51 ab | 97.66 a | 23.20 |
| Nemakey + Besto Humic (Humic acid) + Chitosan | 43.66 ab | 42.96 b | 38.66 a | 92.70 a | 2.75 a | 14.30 a | 97.47 a | 19.36 |
| Rugby® 100 ME (Control 1) | 39.66 de | 51.23 a | 27.00 e | 84.77 ab | 2.66 a | 13.52 ab | 97.49 a | 36.51 |
| Water (Control 2) | 38.00 e | 51.00 a | 24.00 e | 80.74 b | 1.70 b | 12.50 c | 96.15 b | 44.08 |
| LSD ($P \leq 0.05$) | 2.49 u | 7.21 u | 3.53 u | 10.67 u | 0.14 u | 0.85 u | 0.64 u | – |

Data are means of three replicates. Data followed by the same letter in each column are not significantly different according to (LSD) test

## *Plant yield parameters*

### *Floral growth*

Data in *Table 9* elicit the impact of Nemakey, Humic acid and Chitosan in comparison with Rugby® 100 ME (Conventional chemical control)(control 1) and water spray (control 2) on RKN, *M. incognita* and *M. javanica* infection and the consequent effect on plant yield parameters of cucumber plant under plastic house conditions. The highest and significant ($P \leq 0.05$) number of flowers per plant was recorded (44.66) when plant received the combined treatments (Nemakey and Humic acid) as compared to controls (39.66 and 38.00) respectively. Meanwhile, combined


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treatments (Nemakey, Humic acid and Chitosan) significantly were the highest effective on reduction number of aborted flowers per plant and increasing number of fruit. plant$^{-1}$ which recorded (42.96 and 38.66) respectively as compared to controls. In addition, the fresh weight of fruit (g) per plant and length of fruits (cm) in (Nemakey and Humic acid) and (Nemakey, Humic acid, Chitosan) combined treatments recorded significantly higher than those recorded in the other treatments as compared to controls. Whereas no significant differences were found in diameter of fruits (cm) and percentage of fruit moisture content among the treatments with themselves but at the same time there were significant effects among those treatments in comparison to control 2. In addition, the vegetative / Fruit ratio (W/W) was influenced by treatment with (Nemakey, Humic acid and Chitosan) that produced significantly result with least value than the other treatments and recorded (19.36), followed by (Nemakey, Humic acid) and (Humic acid and Chitosan) were (21.71 and 23.20) respectively as compared to control 1.

*Yield growth*

*Table 12* shows the effect of each of Nemakey, Humic acid and Chitosan alone or in combination on early yield, total yield and percentage of yield increasing of cucumber plant which influence by infection of RKN *M. incognita* and *M. javanica* under plastic house condition. In spite of no significant differences were found among treatments with controls in early yield, but among the three combined treatments, one of them recorded the highest value as compared to controls. Meanwhile, the higher and significant marketable total yields and percentage of yield increasing obtained in combination treatments (Nemakey, Humic acid and Chitosan) and (Nemakey, Humic acid) which recorded (6.36 and 6.29%) and (167.22 and 164.28%), respectively as compared to controls (4.19 and 2.38%).

***Table 12.*** *Effect of different treatments for controlling root-knot nematode (Meloidogyne spp.) of cucumber (cv. Naseem F1) on yield*

| Treatment sets | Early yield (kg. plant $^{-1}$) | Total yield (kg. plant $^{-1}$) | % of yield increasing |
|---|---|---|---|
| Nemakey | 0.70 ab | 4.76 bc | 100.00 |
| Besto Humic (Humic acid) | 0.72 ab | 4.87 abc | 104.62 |
| Chitosan | 0.87 ab | 4.48 c | 88.23 |
| Nemakey + Besto Humic (Humic acid) | 1.00 ab | 6.29 ab | 164.28 |
| Nemakey + Chitosan | 0.99 ab | 4.96 abc | 108.40 |
| Besto Humic (Humic acid) + Chitosan | 1.09 ab | 5.38 abc | 126.05 |
| Nemakey + Besto Humic (Humic acid) + Chitosan | 1.27 a | 6.36 a | 167.22 |
| Rugby® 100 ME (Control 1) | 0.89 ab | 4.19 c | 76.05 |
| Water (Control 2) | 0.42 b | 2.38 d | – |
| LSD ($P \leq 0.05$) | 0.79 u | 1.52 u | – |

Data are means of three replicates. Data followed by the same letter in each column are not significantly different according to (LSD) test

## Discussion

Depending on the morphological characteristic (perineal patterns**),** it has been revealed that there were two species of root-knot nematodes, described and recorded as *Meloidogyne incognita* and *M. javanica* (Whitehead, 1968; Esser et al., 1976). The two species usually accompanied with many vegetable and non-vegetable plant, previous


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studies showed the same results (Taylor and Sasser, 1978; Moens et al., 2009). On the other hand, these two species were found in a mixed population as mentioned by (Marahatta et al., 2012; Kayani et al., 2013). when explained perineal pattern of *M. incognita*. Whereas, the perineal patterns for *M. javanica* was typical with a rounded to flattened dorsal arch and conspicuous lateral lines that clearly separated the dorsal and ventral regions of the patterns (*Fig. 2A, B*). Same notation observed by Eisenback et al. (1985), Sen and Chatterjee (2007), Bohra (2011) For confirmation of the diagnosing and identification of the nematodes molecular markers have been used, by applying specific- species primers. The concentration and purity of DNA extraction for 25 mg of J2s, males and females of *M. incognita* and *M. javanica* were 69.4 ng/µL and 2.52 purity respectively when measured by using NanoDrop 2000C device. Different programs and annealing temperature were followed to amplify the specific DNA position of both species (*Meloidogyne incognita* and *Meloidogyne javanica*. The procedure of DNA amplification has been performed six times and all pair primers were set up according to the annealing temperature for first and second times that mentioned by the manufacture company (Sinacion, Bioscience Co. Iran). Our result was agreed with the outcome of other researchers) according to Zijlstra et al. (2000), Dong et al. (2001), Devran et al. (2009), Mwesige (2013), Toumi et al. (2014), Chanmalee (2014), Zhuran et al. (2014), Temple et al. (2015), Kemei et al. (2015), Ye et al. (2015), Agenbag (2016) and Aydinli and Mennan (2016) who used these species-specific primers for diagnosing both species of *Meloidogyne* by molecular characteristics.

In the plastic house experiment, different treatments have been used in order to investigate their influence on controlling the population densities and numbers of juveniles 2 before applying the treatments and after 10, 20 and 30 days. The present results show an approach to control *M. incognita* and *M. javanica* in cucumber plant using modern methods and more active for controlling *Meloidogyne* which are safer than nematicides. These results should be considered during designing an integrated pest management program for RKN or other nematode pathogens in cucumber and other crops.

The reduction of RKNs population in combination (Nemakey, humic acid and Chitosan) could be attributed to Nemakey extracts compounds (marigold, sesame, thyme, etc.), this extract contains Allelopathic compounds such as alpha-terthienyl which produced by plants as secondary metabolites that have significant effects on the activity of nematodes and were thought to be toxins and act as nematicidal action affected on reducing population densities. These finding completely agreed with the previous studies when they used these extracts in their researches (Visser and Vythftingam, 1959; Tsay et al., 2004; Ibrahim et al., 2006; Kong et al., 2007; Krueger et al., 2007; Elbadri and Yassin, 2010; Hooks et al., 2010). The effect of Nemakey was appeared after 30 days of application, this time agreed with the manufacturer product (Nemakey) who mentioned the effecting time of Nemakey is between 25-35 days. On the other hand, Humic acid also reduced nematode population due to improving the nutrient availability and impact on other important chemical, biological, and physical properties of soils, this conclusion in agreement with (Khaled and Fawy, 2011; Fahramand et al., 2014). It was also observed that the efficiency of Humic acid increased when it applied in combination, these outcomes were in comport with those investigation of (Saravanapriya and Subramanian, 2007; Gondal et al., 2014; El-Sherif et al., 2015). At the same time Chitosan also played its role during increasing cytosolic $Ca^{2}$, activation of MAP-kinases, oxidative burst, callus apposition, increase in pathogen-


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related proteins (PRP), phenolic acid synthesis, phytoalexin accumulation, hypersensitive response (HR) proteinase inhibitors and lignin synthesis subsequently enhance the defense of the cucumber plant to RKN infections and decreasing nematode population densities (Hadwiger, 2013; Malerba and Cerana, 2016). Results revealed that reproduction factor (Rf) was adversely affected by treatments whether alone or in combination. Plants which received combined treatments (Nemakey, Humic acid and Chitosan) and (Nemakey and Chitosan) significantly ($p \leq 0.05$) dominated other treatments in reducing number of total nematode population (pf) as compared to controls. It is worthy to observe that Nemakey's compound as a nematicide ranked first in diminishing nematode final population with Humic acid and Chitosan which they have the important role for the decreasing nematode population in the soil as shown in *Table 6* subsequently it has role of reducing the (Rf) value. This conclusion is approved by many previous studies when they used Humic acid or Chitosan whether alone or in a combination with other treatments (Elmiligy and Norton, 1973; Kesba and Al-Shalaby, 2008; Khalil and Badawy, 2012; Dina et al., 2013; El-Sayed and Mahdy, 2015; El-Sherif et al., 2015; Mota and dos Santos, 2016).

As the organic fertilizer and nematicide, Nemakey has been newly manufactured by the Turkish Merkez Anadolu Kimya Sanayi Company. In fact, during reconsideration the references, it could not be found any researches in this field whether applied it alone or in combination to control RKN, so we could not compare our results with the results of previous studies, but depend on its compounds (marigold, sesame, thyme, organic acid oils, free natural amino acids) we could compare our results with the results of other researchers who they used these components in their researches.

All the treatments had significantly reduced the root galls, gall index, disease severity and percentage of gall decrease as compared to controls, a great variability was observed in nematode development and reproduction. Good efficacy of Nemakey, Humic acid and plant protector Chitosan found to be the most effective in reducing the root galls, gall index and disease severity and percentage of gall decreasing endorsing the plant growth parameters. Chitosan show good effect in reducing RKN due to developed systemic acquired resistance (SAR) in cucumber plants. This result agrees with Hadwiger (2013), Malerba and Cerana (2016) and Mota and dos Santos (2016). Nemakey affected on RKN due to its potential killing nematodes at all of its stages of life. Nemakey destroys nematode eggs and creates an unfriendly environment in the soil, which complicate reproduction and proliferation of plant parasitic nematodes in addition to its activity as a bio-stimulant product to promote the production of main and secondary roots (http://www. merkezanadolu.com.tr/nemakey-e0f), the present results in agreement with other studies (Husain et al.,1984; Swamy et al., 1995; Krueger et al., 2007; Abd-Elgawad and Omer, 1995; Ibrahim et al., 2006; Natarajan et al., 2006). Humic acid has role in reducing number of galls. The present results were in agreement with the findings that reported by Kesba and Al-Shalaby (2008) and Saravanapriya and Subramanian (2005). It was observed that the chemical nematicide Rugby® 100 ME had lower effect on reducing number of galls, this outcome came to an agreement with another study (Safdar et al., 2013).

In the present study, effect of the essential oils and extract Nemakey's compounds which are mixture of different compound made more than one mechanism of action exist. Some of the recently proposed hypotheses concerning mechanisms of action of essential oils include denaturation of proteins, inhibition of enzyme action, and interference with electron flow in the respiratory chain, or with ADP phosphorylation


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(Abd-Elgawad and Omer, 1995). On the other hand, Humic acid has been reported to improve root development and plant growth (Adani et al., 1998). This finding is in agreement with earlier observations made by many scientists who confirmed that Nemakey's compound enhanced the growth parameters of tomato plant (Natarajan et al., 2006).

Using Humic acid through soil might be more helpful in enhancing the plant growth under RKN infection (Gondal et al., 2014), so applied Humic acid stimulation cucumber plant growth parameters through increasing cell division, as well as optimizing uptake of nutrients and water (El-Sherif et al., 2015) as compared to controls due to their effect on the reducing by its influences on the nematode infecundity (nematode population) subsequently decreasing the root galls as compared to controls.

The present results were in agreement with the findings that reported by Kesba and El-Beltagi (2012) in grape rootstocks, Gondal et al. (2014) in potato plant and Dina et al. (2013) improved plant growth parameters of sugar-beet against nematode infection. On the other hand Chitosan was very effective against the RKN under plastic house conditions (Malerba and Cerana, 2016) so it has been used in this experiment as a powerful elicitor than a direct antimicrobial or toxic agent, therefore it had important role to increase plant parameter due to promote elicitation and signaling to systemic acquired resistance (SAR) subsequently protective mechanisms activation in plant tissues inhibited the growth of RKN (Dörnenburg and Knorr, 1997; Heil, 1999; Neto et al., 2005; Strange, 2006; Choudhary et al., 2007; Badawy and Rabea, 2011; Zargar et al., 2015), these results in agreement with the results of others researcher who used chitosan in their studies (Zinov'eva et al., 1999; Kloepper et al., 2004; Khalil and Badawy, 2012; El-Sayed and Mahdy, 2015).

There was a correlation between root growth parameters and nematode infestations, Kankam and Adomako (2014) also pointed to the same fact. The present study showed that Nemakey compounds, Humic acid and Chitosan compounds enhanced the cucumber root growth to a different extent over the controls. This finding is in agreement with earlier observations made by many scientists who confirmed that Nemakey's compound, Humic acid and Chitosan in a combination stimulated the development of the growth in root system (Radwan et al., 2012); Dina et al., 2013; Gondal, et al., 2014; Khalil and Badawy, 2012; El-Sayed and Mahdy, 2015).

It is appeared that there were no significant differences among the treatments and controls on percentage of the Phosphor (P) and Potassium (K) content in the leaves, chlorosis (pale green tended to yellowing) this result also mentioned by Olsen (2011), Karssen and Moens (2006) and Mitkowski and Abawi (2003). In fact, leaves chlorosis not only caused by the low availability of mineral nutrient in soil but also to the impaired translocation of nutrients in plants infested by RKN that is able to damage and to block the vascular system of host plants similar results were obtained by Ogaraku (2007) on cowpea plants, therefore leaf chlorophyll intensity in cucumber plants was influenced by the higher and lower level of RKN as reported by Amin and Abd El-Wanis (2014). The leaf chlorophyll and Nitrogen (N) content have the important role for increasing process of photosynthesis and increasing crop yield, the present results were in agreement with the findings that reported by Hao and Papadopoulos (1999) and Yang et al. (2009) who recorded the same result in cucumber plant.

A positive correlation has noticed between treatments and cucumber floral parameters, therefore all screened treatments whether alone or in combination obviously improved plant floral growth due to effect on inhibition nematodes population directly


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or indirectly (*Tables 8* and *9*) and improved crop yield quality and quantity during increasing vegetative growth of cucumber, subsequently increasing number of flowers and decreasing number of aborted flowers per plant, this finding is in agreement with Polthanee and Yamazaki (1996), Elbadri and Yassin (2010), Arncon et al. (2006), Shafeek et al. (2016) and Malerba and Cerana (2016).

The early and total yield reductions were often directly related to the RKN levels in the soil table (4.2) and the environmental stresses imposed upon the plant during crop growth. In general, the more presence of root-knot suggests a potentially serious problem, so at very high levels the RKN significantly affect plant health especially the roots function; stabilization of the plant, ability of the absorption and root secretions (the healthy plant root usually secrets various compounds involved phenolic compounds, complex saguaros, simple saguaros and growth hormones) therefore it indirectly influence on significantly marketable yield as reported also by Noling (1999). So, treating cucumber plant with Nemakey, Humic acid and Chitosan improve total yield and percentage of yield increasing, as mentioned by many researchers in their studies (Arancon et al., 2006 on pepper plant; Saravanapriya and Subramanian, 2007; Amin and Abd El-Wanis, 2014; Shafeek et al., 2016.

## Conclusion

It is concluded from the results that based on the morphological and molecular characteristics in the current study, two species of Meloidogyne RKN were diagnosed as*; Meloidogyne incognita* (Kofoid & White) *Chitwood, and M. javanica* (Treub) Chitwood which are common in green or plastic houses at Bakrajo district. The combination of treatments (Nemakey, Humic acid and Chitosan) gave the best results in the majority of studied parameters followed by (Nemakey and Humic acid) and (Humic acid, Chitosan) which gave better results after the three combined treatment for controlling RKN. From this study revealed that these improved the health of cucumber plants and increased the nitrogen content in the leaves (*Tables 3* and *10*). More studies need to be conducted to obtain detail surveying and investigation about other species which might be accompanied with vegetable crops especially cucumber. We recommend using other methods like biological control as a complementary of integrated disease management (IDM). It was recommended studying some botanical nematicide like garlic, rosemary and mints.

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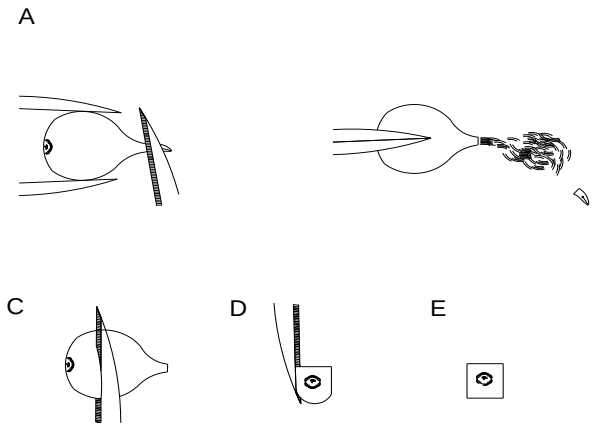
## APPENDICES

***Appendix 1.*** *How to cut perineal patterns. A, B: excised female with neck region removed and body contents gently expelled; C: posterior body with perineal pattern removed; D: trimming surplus cuticle around perineal pattern; E: trimmed perineal pattern ready for mounting*





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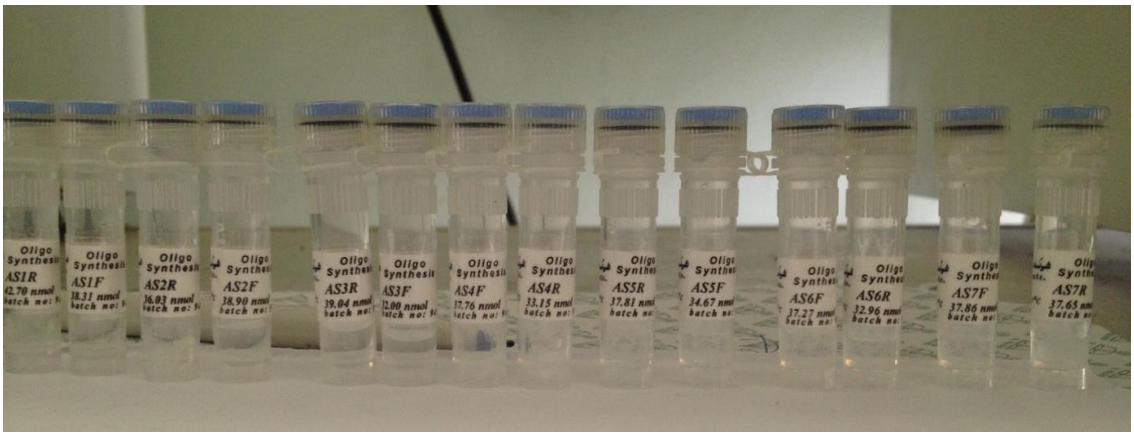
***Appendix 2.*** *Species specific primers prepared by Sinacion, Bioscience Co. Iran*




***Appendix 3.*** *MULTIGENE OptiMAX machine (PCR Thermal Cycler)*


***Appendix 4.*** *PCR programs*

| | Step 1 | Step 2 | Step 3 | Step4 | Step5 | Step 6 |
|---|---|---|---|---|---|---|
| | **Initial denaturation** | **Denaturation** | **Annealing temp. °C** | **Extension** | **Final extension** | **Hold** |
| Temp. | 94.0 and 95.0 | 94.0 and 95.0 | 49.0-64.0 | 72.0 | 72.0 | 4.00 |
| Time | 8:00 | 30-60 s | 1:00 | 2.00 | 7:00 | ∞ |
| | | Repeated for 35 cycles | | | | |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***Appendix 5.*** *Programs and different annealing temperatures were used for amplifying the DNA of the species*

| Species | Primers | Fragment bp | Denaturation temperature °C | Annealing temperature °C | References |
|---|---|---|---|---|---|
| ***M. incognita*** | AS1F AS1R | 1200 | 94 and 95 | 49, 50, 54 and 55 | Sinacion, Bioscience Co. Iran; Zijlstra et al., 2000; Mwesige, 2013; Toumi et al., 2014; Temple et al., 2015; Agenbag, 2016 |
| | AS2F AS2R | 1350 | 94 and 95 | 49, 50, 54 and 55 | Sinacion, Bioscience Co. Iran; Dong et al., 2001; Toumi et al., 2014; Temple et al., 2015 |
| | AS3F AS3R | 399 | 94 and 95 | 49, 50, 55, 60 and 64 | Sinacion, Bioscience Co. Iran; Devran et al., 2009; Toumi et al., 2014; Ye et al., 2015; Agenbag, 2016 |
| | AS4F AS4R | 955 | 94 and 95 | 49, 50, 55, 60, 61, 62 and 63 | Sinacion, Bioscience Co. Iran Devran et al., 2009; Toumi et al., 2014; Chanmalee, 2014; Zhuran et al., 2014; Kemei et al., 2015; Agenbag, 2016 |
| ***M. javanica*** | AS5F AS5R | 1650 | 94 and 95 | 49, 50, 52 and 55 | Sinacion, Bioscience Co. Iran; Dong et al., 2001; Devran et al., 2009; Toumi et al., 2014 |
| | AS6F AS6R | 670 | 94 and 95 | 49, 50, 55, 60, 63 and 64 | Sinacion, Bioscience Co. Iran; Zijlstra et al., 2000; Devran et al., 2009; Toumi et al., 2014; Chanmalee, 2014; Aydinli and Mennan, 2016 |
| | AS7F AS7R | 517 | 94 and 95 | 49, 50, 55, 61 and 62 | Sinacion, Bioscience Co. Iran; Zhuran et al., 2014; Toumi et al., 2014; Kemei et al., 2015 |

***Appendix 6.*** *Electrophoresis chamber (power supply and the tray)*



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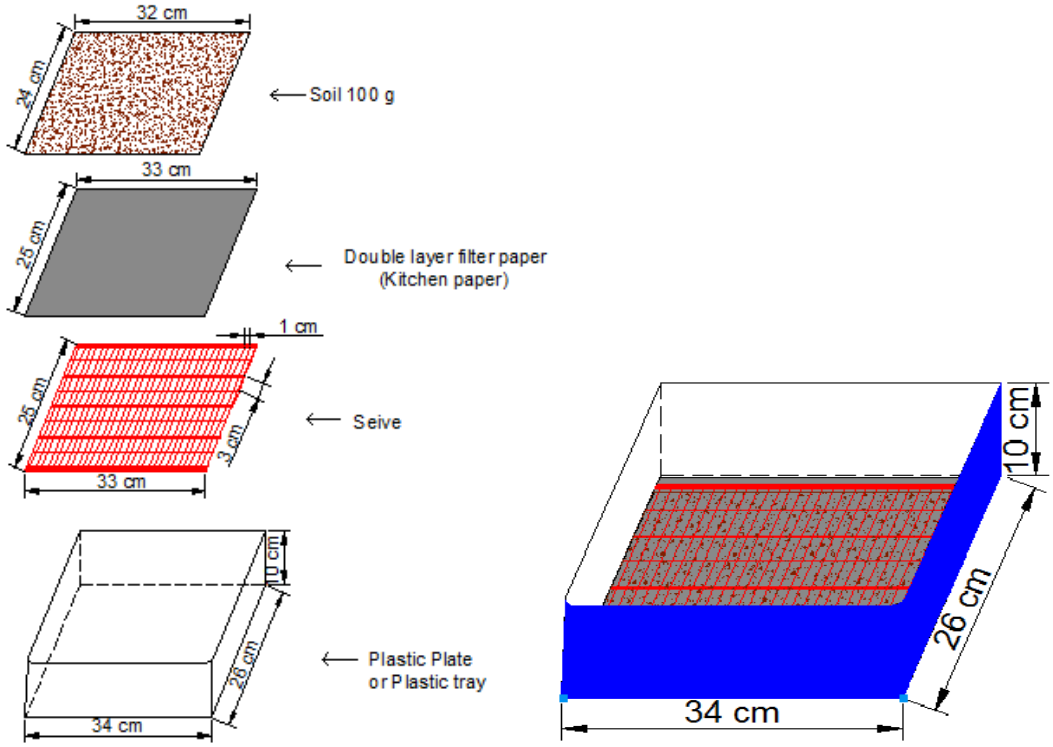
***Appendix 7.*** *UV transilluminator*


***Appendix 8.*** *Extraction-tray models for isolating the nematodes (Juveniles 2) (original)*





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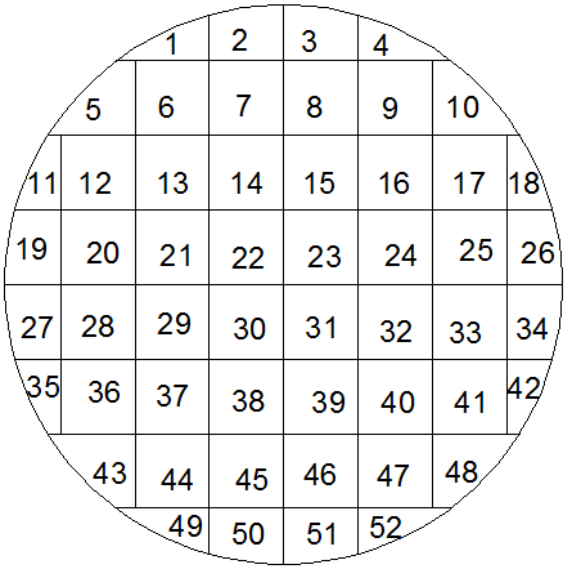
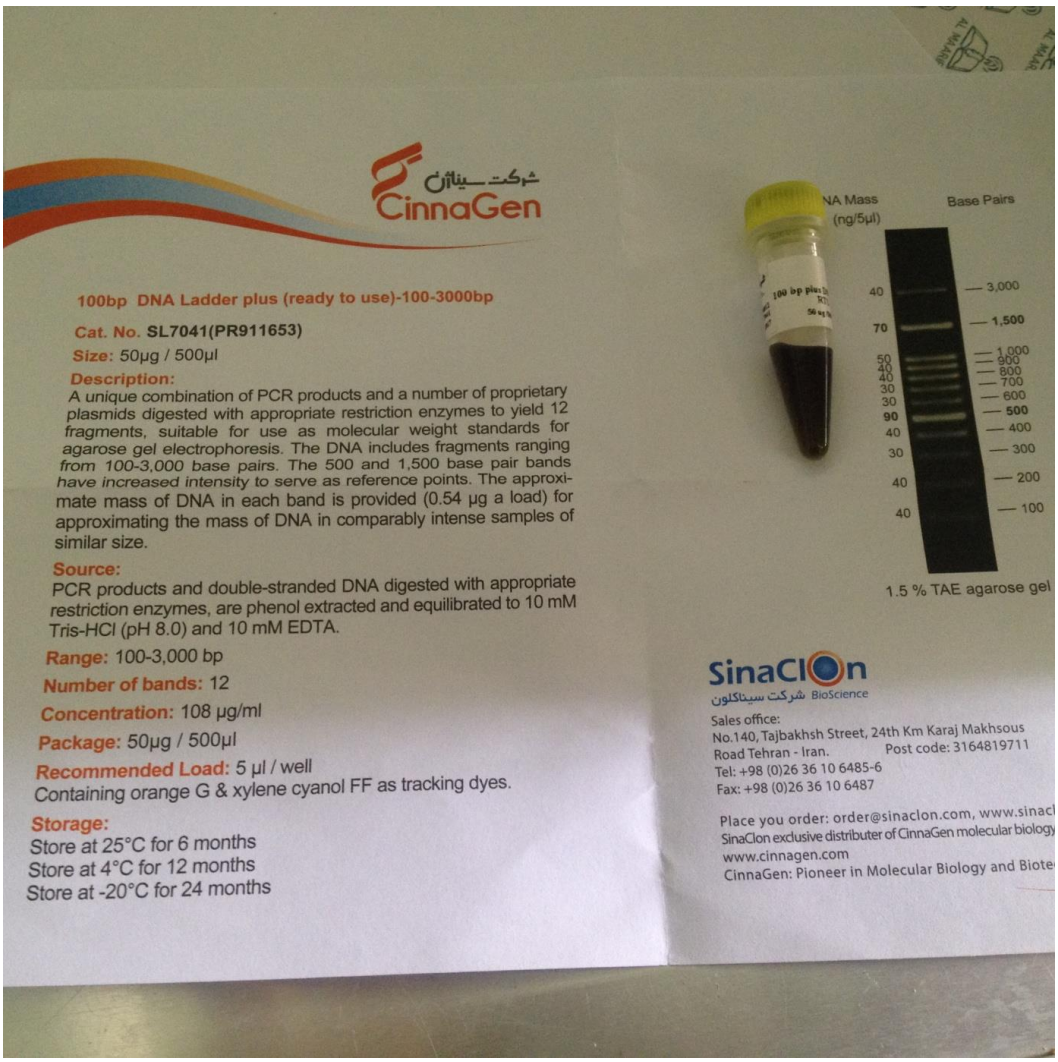
***Appendix 9.*** *Counting dish designed for calculating the nematodes (Juveniles 2) (original)*




***Appendix 10.*** *The tube of ladder and its information paper by Sinacion company-Iran*





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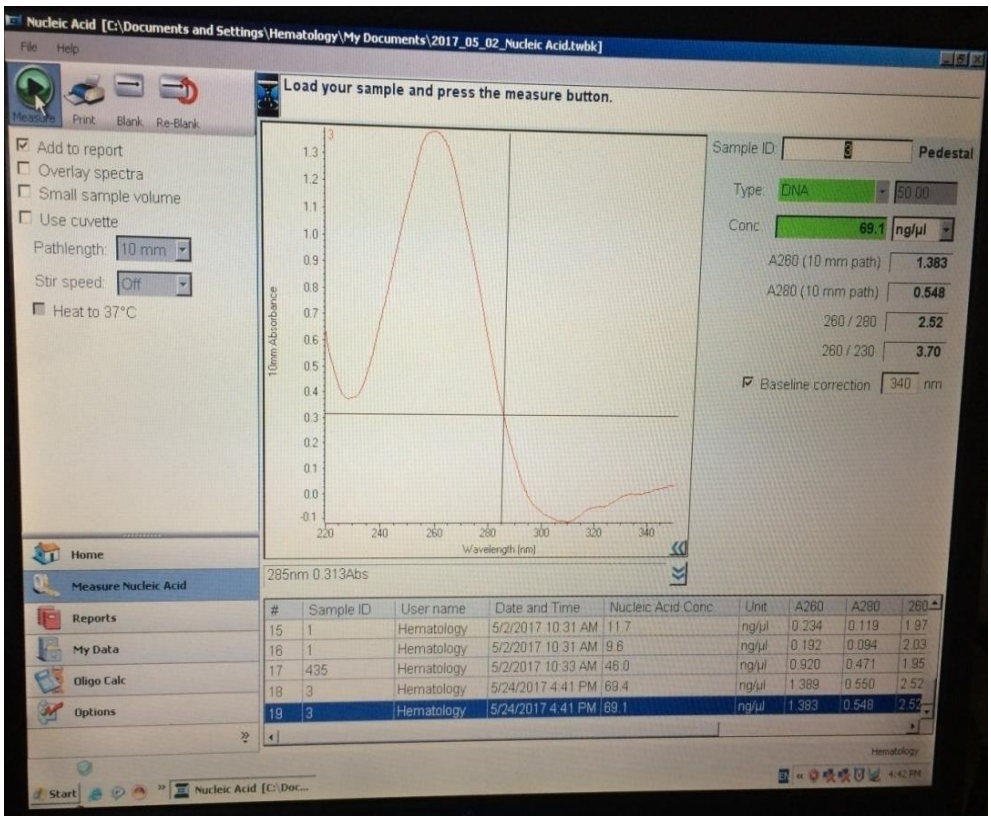
***Appendix 11.*** *(A) The device of thermo Scientific Nanodrop 2000C. (B) The concentration and purity diagram of Meloidogyne spp. DNA extraction as tested by Nanodrop 2000C device*


A




B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***Appendix 12.*** *Analysis of variance including degree of freedom (df) and mean squares of replication, treatments and error for Population densities (Juveniles 2) / 100 g soil and number of galls*

| Characters | Replications (d.f = 2) Mean square | Treatments (d.f = 8) Mean square | Error (d.f = 16) Mean square |
|---|---|---|---|
| One day before of applying treatments (Pi) | 13980.778 | 1979.500NS | 3455.944 |
| After 10 days of application | 51674.704 | 46246.315 NS | 54210.204 |
| After 20 days of application | 126899.593 | 46380.759 NS | 72008.676 |
| After 30 days of application (Pf) | 3831.148 | 83707.037** | 2182. 773 |
| Number of the galls ($P \leq 0.05$) | 771.797 | 1569.273** | 222.680 |
| Number of the galls ($P \leq 0.01$) | 771.797 | 1569.273* | 222.680 |

* indicates significant difference at $P \leq 0.05$, ** indicates high significant difference at $P \leq 0.05$ and Ns indicates non-significant difference

***Appendix 13.*** *Analysis of variance including degree of freedom (df) and mean squares of replication, treatments and error for some plant growth parameters (vegetative parts) of cucumber (cv. Naseem F1)*

| Characters | Replications (d.f = 2) Mean square | Treatments (d.f = 8) Mean square | Error (d.f = 16) Mean square |
|---|---|---|---|
| Plant height (cm) | 0.138 | 0.420** | 0.020 |
| Stem diameter (cm) | 0.658 | 1.186** | 0.108 |
| No. of nodes. plant$^{-1}$ | 21.333 | 164.833** | 1.583 |
| No. of leaves. plant$^{-1}$ | 11.704 | 198.009NS | 20.912 |
| Fresh plant weight (vegetable parts) g. plant$^{-1}$ | 10901.248 | 25751.528* | 3124.286 |
| Leaf area. plant$^{-1}$ (cm$^2$) | 3402.690 | 1456.129* | 411.055 |

* indicates significant difference at $P \leq 0.05$, ** indicates high significant difference at $P \leq 0.05$ and Ns indicates non-significant difference

***Appendix 14.*** *Analysis of variance including degree of freedom (df) and mean squares of replication, treatments and error for some plant growth (root parts) of cucumber (cv. Naseem F1)*

| Characters | Replications (d.f = 2) Mean square | Treatments (d.f = 8) Mean square | Error (d.f = 16) Mean square |
|---|---|---|---|
| Root length (cm) | 11.576 | 18.532* | 6.530 |
| Fresh root weight (g) | 78.390 | 23.188* | 6.146 |
| Dried root weight (g) | 13.863 | 31.973** | 0.988 |

* indicates significant difference at $P \leq 0.05$, ** indicates high significant difference at $P \leq 0.05$ and Ns indicates non-significant difference


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***Appendix 15.*** *Analysis of variance including degree of freedom (df) and mean squares of replication, treatments and error for some plant content of cucumber (cv. Naseem F1)*

| Characters | Replications (d.f = 2) | Treatments (d.f = 8) | Error (d.f = 16) |
|---|---|---|---|
| | Mean square | Mean square | Mean square |
| Intensity of leaf chlorophyll (SPAD unit) | 18.498 | 46.605** | 3.707 |
| % of the Nitrogen (N) content in the leaf dry weight | 0.034 | 0.060* | 0.022 |
| % of the Phosphor (P) content in the leaf dry weight | 0.011 | 0.001 NS | 0.002 |
| % of the Potassium (K) content in the leaf dry weight | 0.097 | 0.046NS | 0.087 |

* indicates significant difference at $P \leq 0.05$, ** indicates high significant difference at $P \leq 0.05$ and Ns indicates non-significant difference

***Appendix 16.*** *Analysis of variance including degree of freedom (df) and mean squares of replication, treatments and error for floral growth*

| Characters | Replications (d.f = 2) | Treatments (d.f = 8) | Error (d.f = 16) |
|---|---|---|---|
| | Mean square | Mean square | Mean square |
| No. of flowers. plant$^{-1}$ | 132.333 | 14.333* | 2.083 |
| No. of aborted flowers. plant$^{-1}$ | 330.581 | 25.753 NS | 17.356 |
| No. of fruit. plant$^{-1}$ | 179.593 | 71.759* | 4.176 |
| Fresh weight of fruit. plant$^{-1}$ (g) | 179.505 | 43.718 NS | 38.041 |
| Diameter of fruits. plant$^{-1}$ (cm) | 0.032 | 0.365 NS | 0.007 |
| Length of fruits (cm) | 0.955 | 0.979 NS | 0.243 |
| Fruit moisture content (%) | 0.593 | 0.690NS | 0.141 |

* indicates significant difference at $P \leq 0.05$, ** indicates high significant difference at $P \leq 0.05$ and Ns indicates non-significant difference

***Appendix 17.*** *Analysis of variance including degree of freedom (df) and mean squares of replication, treatments and error for yield*

| Characters | Replications (d.f = 2) | Treatments (d.f = 8) | Error (d.f = 16) |
|---|---|---|---|
| | Mean square | Mean square | Mean square |
| Early yield (kg. plant$^{-1}$) | 0.019 | 0.182 NS | 0.211 |
| Total yield (kg. plant$^{-1}$) | 4.972 | 4.234* | 0.781 |

* indicates significant difference at $P \leq 0.05$, ** indicates high significant difference at $P \leq 0.05$ and Ns indicates non-significant difference


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