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# SCREENING OF VINEYARDS ROOTSTOCK AND CULTIVARS FOR RESISTANCE TO ROOT-KNOT NEMATODE (*Meloidogyne incognita)*

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## ABSTRACT

Three hosts of grape (*Vitis vinifera* L), Flame seedless, Thompson seedless, Superior" and two resistant rootstocks "Harmony and Freedom" were inoculated with 3000 second stage juveniles per plant. After two months from inoculation for evaluation of nematode reproduction, Flame seedless was highly susceptible to *M. incognita*, while Thompson seedless and Superior were moderate susceptible. The difference in protein banding patterns among healthy and infected samples proved they were differences in buffer soluble protein extracted from the leaves of five tested cultivars as the response to infection of *M. incognita*. ISJ-PCR was used to measure the degree of similarity among grape varieties and rootstock and to calculate the genetic distance between these varieties. Polymorphic fragment reveled the difference and establish systemic relationships among the tested varieties may be referred the resistance in the rootstocks.

**Keywords**:- Grape varieties , resistance, PCR, *Meloidogyne incognita*

## INTRODUCTION

Plant parasitic nematodes are major pest of grape of the worldwide. Root–knot nematodes alone cause a 20% economic loss. In many vineyards root-knot nematodes, *Meloidogyne* spp., and other species gradually increase on the newly developing root systems (Ferris and Mckenry1975, Mckenry *et al*. 2001). Vineyards commonly are infested with *Meloidgyne incognita* that is widely distributes in sandy soils. Resistant grape rootstocks are used to limit the damage caused by *Meloidgyne* spp. Harmony grape rootstock displays resistance to several *Meloidogyne* spp. but that resistance is not durable in commercial vineyard settings (Mckenry and Anwar 2007). Anwar *et al.* (2000) compared the reproductive variability of four field populations of *Meloidgyne* spp. were found to be virulent to grape hosts on two resistant *Vitis* rootstocks "Harmony and Freedom" are normally non-hosts to a virulent nematodes. Melakeberhan *et al*., (1990) measured the effect of *M. incognit*a on growth of Thompson seedless (moderate resistant) *Vitis vinifera* cultivars.

The difference in protein banding patterns among healthy and infected samples relation to infection with the root-knot nematode *M.incognita*, proved the difference in response of those cvs. to infection. (El-Moflehi 2001, Baklawa 2004, Hassan 2007). Diversity analysis of the grape (*Vitis vinifera* L.), varieties and root stock based on molecular markers. ISJ-

PCR was used to measure the degree of similarity among grape varieties and rootstock and to calculate the genetic distance between these varieties. Vidal *et al*., (2000), Fujita *et al*.,(2009) and Alizadah and Singh (2009). There are several techniques for determining genetic variation among grape cultivars. Many investigators employed RAPD-PCR to assay genetic fingerprinting and diversity in wide range of plants. Xu *et al*., (1995) and Faraj *et al*., (2000) used RAPD markers to identify grape (*Vitis vinifera* L.), rootstocks and accessions of grapevine germplasm. They used primers for analysis based on the polymorphism bands. The results indicated that some of the studied varieties are synonyms, while some of the other have a high similarity. The use of the RAPD technique for identification of grapevine material was recommended. Also, Zhen *et al*., (1996) used RAPD markers to screen eighteen peach rootstock cultivars, most of (*Prunus persica* L.). The first major bifurcation in the dendrogram divided these rootstock cultivars into two groups according to their resistance or susceptibility to root-knot nematodes (*M. incognita*) and (*M. javanica*).

This study aimed to evaluate grape rootstocks and cultivars against root-knot nematode *Meloidgyne incognita*. And knowledge of the genetics distances among the different cultivars is very useful and successful for genetic improvement.

## MATERIALS AND METHODS

**1- Evaluation of vineyards cultivars for infection with *M. incognita***

Root–knot nematode *M.incognita* was isolated from common grape roots which were reared on tomato plants in greenhouse and used to inoculate Plantlet of five grape cultivars viz **"**Flame **seedless, Thompson seedless, Superior, Harmony and Freedom ".** This pure culture was used for all further studies in this work. Plantlet from each cultivar (50 Plantlet) were sown in 50 clay pots 25 cm diameter, filled with steamed sterilized sandy clay soil to one seedling in each pot. Five Plantlet of each cultivar were inoculated with root-knot nematode using 3000 second stage juveniles per plant. They introduced in water suspension and pipette onto three holes around the root system of each Plantlet. The rest of pots were served as check (un treated) without nematode inoculation. All pots were kept in the greenhouse at 25±2°C, watered as required with tap water and horticultural treated the same. After two months from inoculation, fresh and dry weights of shoot and root system were recorded and parameters for nematode (no. of eggs, no. of larvae, no. of egg-masses, G.I. and E I.).

**The percentage of reduction in plant fresh weight (R %) was calculated using the formula**

$$R\% = \frac{\text{Control plants weight – infected plants weight}}{\text{Control plants weight}} \times 100$$

Roots of the harvested plants were washed carefully by running tap water and stained for 15-20 min in an aqueous solution of phloxin B (0.15g/ 1L distilled water ) to emphasize egg mass (Hartman, 1983). A volume of 250cm soil was used to extract nematode using sieving and Baermann pan technique (Barker, 1985). The extracted juveniles were counted by using 1ml counting slide under stereoscopic microscope.

Numbers of root galls and nematode egg masses were counted and plants were rated on root gall index (G.I.)and egg masses index (E.I.), according to (Sasser *et al*., 1984), where G.I and E.I were determined as follows: 0: no galls, 1: 1-2, 2: 3-10, 3: 11-30, 4: 31-100, 5: +100 galls or egg masses per plant . Statically analysis, A Costat computer program was then used to analyze the obtained data statistically and mean separation according to Duncan's multiple range test ($p<0.5$).

**2- Electrophoresis Protein analysis (SDS-PAGE):-**

The difference in buffer soluble protein extracted from the healthy and infected plants of the five tested grape cultivars (Flame seedless, Thompson seedless, Superior, Harmony and Freedom) were shown by Electrophoresis protein analysis [Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)] procedure was used according to laemmli, (1970).

**2-1-SDS- Polyacrylamide Gel Electrophoresis (SDS-PAGE) procedure**

One –dimensional SDS-PAGE was performed with Mini-protean apparatus (Bio Rad) using a 12 %( w/v) separating (resolving) gel and 4% (w/v) stacking. Protein extracted samples were mixed with equal volumes of protein sample buffer, denatured at 80-90 °C in water bath for 3-5 minutes and followed by immediate cooling on ice then 25 µl of each protein samples was loaded on each lane. Electrophoresis was carried out with current of 100 volt (Bio –Rad power supply, Model 300) per the 2 gels until the BPB marker reached the bottom of the gel. The gels were stained with 100 ml protein staining solution for overnight then washed twice with water. The gels were destined with destaining solution for several times. The protein molecular marker used was Bio RAD protein molecular weight standard marker.

**2-2-Analysis of SDS-PAGE Products:-**

The products of SDS_PAGE procedure were analyzed using protein Gel Documentation System (P.G.D.S) program according to El-Araby, (2001).

**3- Genetic diversity analysis of the grape** (*Vitis vinifera* L.)**, varieties and root stock based on molecular markers.**

**3.1. Intron- exon splice junctions (ISJ) Protocols:**

**3.1.1. DNA isolation and quantification:**

DNA of five grape varieties was isolated using CTAB (Cetyltetramethyl ammonium bromide) method, (Murray and Thompson 1988).

For DNA isolation, one hundred mg of fresh seedling leaves were homogenized in a chilled pestle and mortar using liquid nitrogen. 700 *µl* of 2X CTAB extraction buffer were added and homogenized well. The samples were transferred to Eppendorf tubes and incubated at 65 °C for 30-60 min with occasional gentile swirling. 700 *µl* of Chloroform Isoamyle alcohol (24:1)

were add and mixed by inverting the tube several times. Sample was centrifuged at 15000 rpm for 15 min at 4°C. The aqueous was transferred to a fresh centrifuge tube with a wide bore tips to avoid DNA shearing. Then, 0.6 volume of chilled isopropanol was added and followed by quick and gentle inversion and incubated at -20°C for 30 minutes. DNA pellet was precipitated at 10000 rpm for 10 min at 4 °C. Pellet was washed three times with 70 % ethanol, well dried and dissolved in 100 *µl* TE. DNA was quantified using gel quantification method in which the samples were loaded on 0.8 % agarose gel in 0.5X TAE running buffer and using known concentrations of λ uncut genomic DNA as standard. After some cycles of dilutions, the concentration of DNA was approximately adjusted to 15 ng*/ µl,* and this concentration is suitable for PCR reaction.

**3.1.2. ISJ protocol**

Five ISJ primers *i.e.* ISJ-5, ISJ-6, ISJ-7, ISJ-8 and ISJ-9 were used to study the selected genotypes. The nucleotide sequences of these primers are as follows:

| primer | sequence | oligonucleotide |
|---|---|---|
| **ISJ-5** | 5'-CAG GGT CCC ACC TGC-3' | 15 mer |
| **ISJ-6** | 5'-ACT TAC CTG AGC CAG CGA-3' | 18 mer |
| **ISJ-7** | 5'-TGC AGG TCA GGA CCC T-3' | 16 mer |
| **ISJ-8** | 5'-GAC CGCTTG CAG GTA AGT-3' | 18 mer |
| **ISJ-9** | 5'-AGG TGA CCG ACC TGC A-3' | 16 mer |

PCR reactions for ISJ primers were carried out in 10 *µl* volume containing:

| | |
|---|---|
| **Total genomic DNA** (15 ng/ *µl*) | **1.00 *µl*** |
| **d.d.$H_2O$** | **4.35 *µl*** |
| **10 X PCR buffer** | **1.00 *µl*** |
| **$MgCl_2$** (25 mM) | **0.80 *µl*** |
| **dNTPs** (1mM) | **0.10 *µl*** |
| **Taq DNA polymerase** (5 U/ *µl*) | **0.25 *µl*** |
| **ISJ primer (**30 ng/ *µl*) | **2.50 *µl*** |
| **Total** | **10.00 *µl*** |

Using profile suggested by El-Moghazy (2007): initial denaturation at 94°C for 3 min, 45 cycles of amplification under the following parameters; template denaturation at 94°C for 1 min, primer annealing at 48°C for 1 min and extension at 72°C for 2.30 min by the end of the 45$^{th}$ cycle, final extension at 72°C for 7 min followed by storage at 4°C.

**3.1.3. Electrophoresis, staining and analysis**

DNA amplified fragments were loaded in 1.2 % agarose gel containing ethidium promide (2 *µl*/100 ml). The 0.5X TAE was used as a running buffer and 50 and 100 bp DNA ladders (0.5 µg / *µl*, fermentas) as molecular weight markers. Electrophoresis was conducted at 70 Volts, 50 mA for 3 hours. Then, gels were photographed and analyzed using BioDoc Analysis software (Biometra, Germany).

**3.1.4. Phylogenetic tree construction**

The presence/absence matrix for amplified DNA fragments of the five ISJ markers was used to study the phylogenic relationships among the

studied genotypes. The statistical software NTSYS pc2.0 (Rohlf, 2000) was used to estimate the genetic relationships among the tested genotypes. Employing the computer package NTSYS pc2.0, Nei and Lei's similarity coefficients (Nei and Lei, 1979) were calculated and used to establish genetic relationships among the genotypes based on un-weighted pair group method of arithmetic means (UPGMA) and sequential agglomerative hierarchical nested (SAHN) clustering**.**

## RESULTS AND DISCUSSION

### 1- Evaluation of vineyards cultivars for infection with *M. incognita*

Data in table (1) indicated that generally nematode was developed and reproduced well with significantly differences on three tested cultivars (Flame seedless, Thompson seedless, Superior). Flame seedless (common grape cultivar) was highly susceptible to infect by *M. incognita* with significant differences between all tested cultivars. While Thompson seedless and superior seedless were moderately infected with *M. incognita* whereas, Harmony and Freedom were resistant cultivars, to due the resistant cultivars represented a diversity of nematode defense mechanisms; one mechanism is a hypersensitive response operating at root tip to half or reduce penetration by second- stage juveniles and at vascular bundles to limit female development and reproduction (Anwar and Mckenry, 2002, Anwar and Mckenry 2000, Anwar *et al*. 2000). Moreover, resistance cultivars had greater numbers of roots, an ability to compensate root damage, and enhanced capability to take water and nutrients).

**Table (1): Evaluation of vineyards cultivars for infection with *M. incognita***

| Cultivars | No. of larvae/250ml.soil | No. of galls/plant | No. of egg-masses/plant | E. I. | G. I. |
|---|---|---|---|---|---|
| Thompson seedless | **1000**[b] | **95**[b] | **48**[b] | **4** | **4** |
| Flame seedless | **1200**[a] | **172**[a] | **71**[a] | **5** | **5** |
| Superior | **1000**[b] | **58**[b] | **31**[b] | **4** | **4** |
| Harmony | **300**[c] | **0**[c] | **0**[c] | **0** | **0** |
| Freedom | **200**[c] | **0**[c] | **0**[c] | **0** | **0** |

**E.I=Egg Masses Index G.I= Galled Index**

**Means in each column followed by the same letter (s) are not significantly different according to Duncan's multiple range tests (p≤0.05).**

### 2- Effect of infection with *M. incognita* on plant growth parameters of vineyards cultivars.

Our results in Table (2) indicated that the reduction of plant growth (shoot fresh weight, root fresh weight) as a result of the nematodes infection ranged between 20-36%, 16-33%, respectively. Thompson seedless and Superior were more the most affected cultivars than Flame cultivar while Harmony and Freedom were the lowest effect that results were agreement with (Anwer *et al*. 2002, Mckenry, *et al*. 2001) pointed to Harmony and Freedom rootstocks have been commercially acceptable for their resistance to root-knot

nematode. Plant parasitic nematodes affect their hosts by removing cell contents (energy demand) and inducing pathogenic effects. Each effect varies with the nematode's feeding habit, pathogenicity and population density (Atkinson, 1985, Ferris, *et al*., 1984, Melakeberhan, *et al*, 1985). Depending on the nature of the host –parasite interaction, the combined effect of these effects influences host physiological processes such as photosynthesis and crop yield (Melakeberhan, *et al*, 1985).

**Table (2): Effect of infection with *M. incognita* on plant growth parameters of vineyards cultivars.**

| Cultivars | Fresh Shoot | | | | | | Dry weight shoot | | |
|---|---|---|---|---|---|---|---|---|---|
| | Weight (gm) | | | Length (cm) | | | Dry weight (gm) | | |
| | C. P. | I. P. | R% | C. P. | I. P. | R% | C. P. | I. P. | R% |
| Thompson seedless | 39$^{b}$ | 25$^{a}$ | 36 | 52$^{b}$ | 34$^{c}$ | 35 | 13$^{b}$ | 11$^{a}$ | 15 |
| Flame seedless | 41$^{b}$ | 27$^{a}$ | 34 | 59$^{b}$ | 47$^{bc}$ | 20 | 21$^{a}$ | 12$^{a}$ | 43 |
| Superior | 40$^{b}$ | 32$^{a}$ | 20 | 79$^{b}$ | 60$^{ab}$ | 24 | 13$^{b}$ | 11$^{a}$ | 15 |
| Harmony | 44$^{b}$ | 35$^{a}$ | 20 | 81$^{b}$ | 47 $^{bc}$ | 43 | 19$^{ab}$ | 15$^{a}$ | 21 |
| Freedom | 63$^{a}$ | 45$^{a}$ | 28 | 117$^{a}$ | 80$^{a}$ | 31 | 19$^{ab}$ | 15$^{a}$ | 21 |

| Cultivars | Fresh Root | | | | | | Dry weight Root | | |
|---|---|---|---|---|---|---|---|---|---|
| | Weight (gm) | | | Length (cm) | | | Dry weight (gm) | | |
| | C. P. | I. P. | R% | C. P. | I. P. | R% | C. P. | I. P. | R% |
| Thompson seedless | 19$^{ab}$ | 13$^{a}$ | 32 | 41$^{c}$ | 37$^{a}$ | 10 | 8$^{b}$ | 6$^{b}$ | 25 |
| Flame seedless | 19$^{ab}$ | 14$^{a}$ | 26 | 45$^{bc}$ | 33$^{a}$ | 27 | 9$^{b}$ | 4$^{b}$ | 56 |
| Superior | 15$^{b}$ | 10$^{a}$ | 33 | 54$^{bc}$ | 47$^{a}$ | 13 | 7$^{b}$ | 6$^{b}$ | 14 |
| Harmony | 26$^{a}$ | 16$^{a}$ | 16 | 60$^{b}$ | 33$^{a}$ | 45 | 16$^{a}$ | 11$^{a}$ | 31 |
| Freedom | 19$^{ab}$ | 16$^{a}$ | 16 | 87$^{a}$ | 47$^{a}$ | 46 | 9$^{b}$ | 6$^{b}$ | 33 |

**C.P =check plants, I.P = infected plants.**

**R% = percentage of reduction =cp-ip/cpX100**

**Means followed by the same letter (s) are not significantly different according to Duncan's multiple range tests (p≤0.05). Means followed by the different letter (s) are significantly different according to Duncan's multiple range tests (p≤0.05).**

### 3- Electrophoresis analysis of Protein (SDS-PAGE):

The buffer soluble proteins extracted from the leaves of the five-tested grape cultivars (Flame seedless, Thompson seedless, Superior, Harmony and Freedom) were used as a criterion of reaction to infection with the root-knot nematode *M. incognita*. SDS-PAGE products were illustrated in Fig (1), and the protein gel documentation system (P.G.D.S) program analysis was recorded. Data revealed the presence of protein bands in healthy and infected plants with distinguishable differences in the number, size, molecular weights and Amt% between the five tested grape cultivars. Data can be summarized as follows:-

**Table (3): Molecular weight (kDa) of protein bands in five grape cultivars healthy and infected plants with *M. incognita*.**

| Pk/ln# | M | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 260 | 410 | 368 | 421 | 389 | 399 | 399 | 378 | 368 | 314 | 297 |
| 2 | 135 | 274 | 252 | 245 | 260 | 245 | 282 | 289 | 245 | 194 | 213 |
| 3 | 95 | 171 | 177 | 177 | 177 | 171 | 194 | 188 | 183 | 132 | 140 |
| 4 | 72 | 116 | 118 | 118 | 120 | 120 | 125 | 122 | 125 | 72 | 70 |
| 5 | 52 | 74 | 76 | 76 | 76 | 86 | 83 | 85 | 83 | 44 | 46 |
| 6 | 42 | 55 | 60 | 56 | 55 | 65 | 35 | 35 | 70 | 38 | 39 |
| 7 | 34 | 47 | 47 | 47 | 46 | 47 | 30 | 29 | 44 | 30 | 31 |
| 8 | 26 | 31 | - | 31 | - | 34 | 24 | 22 | 36 | 20 | 24 |
| 9 | 17 | 26 | 26 | 27 | - | 29 | - | - | 30 | - | - |
| 10 | - | 21 | - | 21 | - | 21 | - | - | 22 | - | - |

**M: protein Marker**

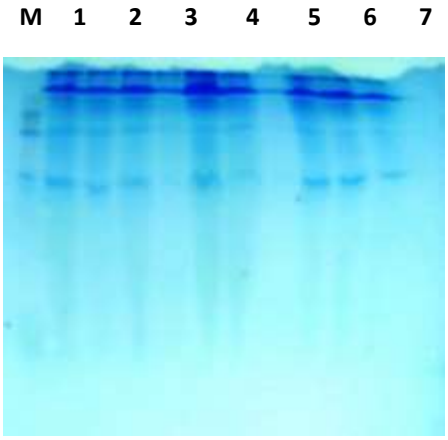


**Fig. (1) SDS-PAGE of five tested grape cultivars leaves.**

**M: protein Marker**
**Lane 1: leaves of healthy Thompson seedless**
**Lane 2: leaves of infected Thompson seedless**
**Lane 3: leaves of healthy Flame seedless**
**Lane 4: leaves of infected Flame seedless**
**Lane 5: leaves of healthy Superior**
**Lane 6: leaves of infected Superior**
**Lane 7: leaves of healthy Harmony**
**Lane 8: leaves of infected Harmony**
**Lane 9: leaves of healthy Freedom**
**Lane 10: leaves of infected Freedom**

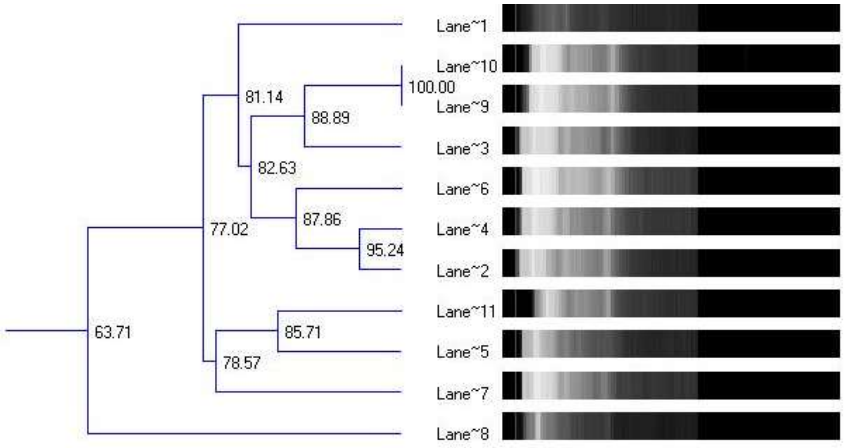


**Figure (2): Cluster analysis showing the similarity polymorphism of protein banding patterns obtained by SDS-PAGE**

The data in Fig (2) showed that the similarity between proteins banding pattern of five cultivars infected with *M. incognita* and the control. The similarity between Lane (9) and Lane (10) was 100%, Lane (2) and Lane (4) was 95.24%, Lane (5) and Lane (11) was 85.71%.

Results indicated differences in buffer soluble protein extracted from the leaves of five tested grape cultivars in relation to infection with the root-knot nematode *M. incognita*, these differences in protein banding pattern due to infection by *M.incognita* may be attributed to the response of the host plant to infection. The differences in protein banding profile are expectable due to the differences in genetic composition on these cultivars. Moreover, the difference in protein banding patterns among healthy and infected samples proved the difference in response of those cvs., to infection.

**4- Genetic diversity Analysis of the grape (*Vitis vinifera* L.), varieties and root stock based on molecular markers.**

Variability and identification of the available germplasm are essential for varieties improvement. Knowledge of the genetic distances among the different varieties is very useful and successful for genetic improvement (Ceron and Angel, 2001) ISJ-PCR was used to measure the degree of similarity among grape varieties and rootstock and to calculate the genetic distance between these varieties. Five grape cultivars were screened for ISJ-PCR markers using single to measure the degree of similarity among grape cultivars and rootstock and to calculate the genetic distance between these varieties. Although most primers produced banding patterns that were scored in Fig ( ) these bands were scored and used for fingerprinting. Cluster analysis of five grape cultivars using five ISJ-PCR markers produced a dendrogram of genetic relatedness in good agreement with their putative pedigrees. So the main bifurcation in the dendrogram divided these cultivars into two gropes according to their resistant or susceptibility of *M.incognita*.

**Isj 8**

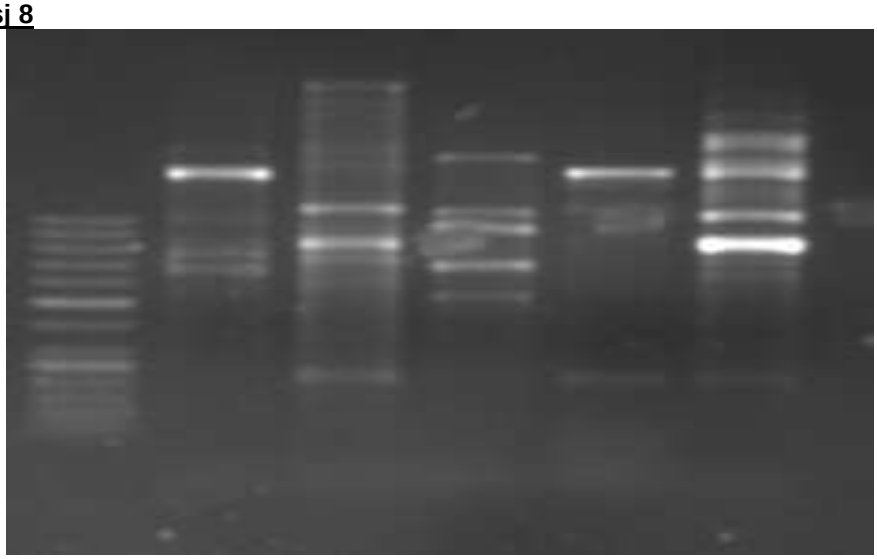

**Table ( ). Survey of the ISJ generated fragments in five grape varieties.**

| M | **1** | **2** | **3** | **4** | **5** |
|---|---|---|---|---|---|
| - | 0 | 1* | 0 | 0 | 0 |
| - | 0 | 1* | 0 | 0 | 1* |
| - | 0 | 1* | 0 | 0 | 1* |
| - | 0 | 1* | 0 | 0 | 1* |
| 1000 | 0 | 0 | 0 | 1 | 1* |
| 912 | 0 | 1 | 1 | 1 | 1 |
| 800 | 1 | 0 | 1 | 0 | 0 |
| 700 | 0 | 1* | 0 | 0 | 1* |
| 600 | 1 | 1 | 1 | 0 | 1 |
| 500 | 1 | 0 | 0 | 0 | 1 |
| 408 | 0 | 0 | 1 | 0 | 0 |
| 250 | 0 | 1* | 0 | 1 | 1* |

*** Unique bands.**

**Fig ( ) Agarose gel electrophoresis of ISJ markers in five grape (M) Marker, (1) Flame seedless, (2) Freedom, (3) Thompson seedless, (4) Superior, (5) Harmony.**

The monomorphic fragments are constant and cannot be used to study the diversity while polymorphic fragments revealed differences and could be used examine and establish systemic relationships among the genotypes (Hadrys *et al*., 1992). Results in Table (3) showed that the total number of polymorphic fragments was 27 bands, out of them 12were unique. The presence of unique fragment for a given genotype is referred as positive marker in two resistant rootstocks Freedom and Harmony.

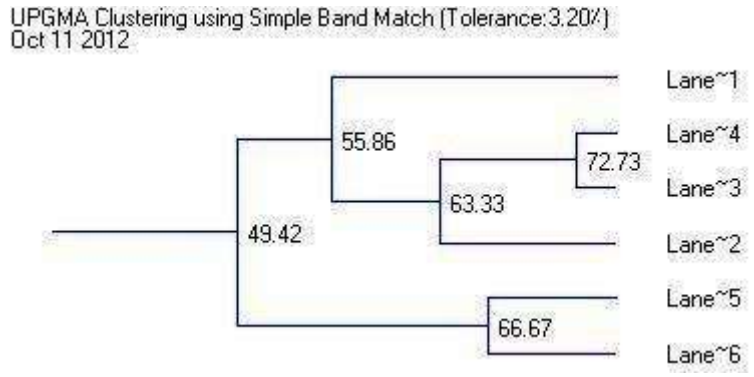


**Fig (4). Dendrogram of five grape varieties based on ISJ analysis.**

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## تقييم بعض اصناف العنب لمقاومة نيماتودا تعقد الجذور (ميليدوجيني انكوجنيتا)

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تم اضافة العدوي بثلاثة الاف يرقة من الطور اليرقي الثاني لنيماتودا تعقد الجذور "ميليدوجين انكوجنيتا" الي كل نبات من ثلاثة اصناف عنب هي – Flame seedless – superior Thompson seedless واثنان مقاومان هما – Harmony Freedom لهدف تقييم تكاثر النيماتودا عليهم منفردا ، وبعد مرور شهران من اضافة العدوي كان صنف Flame seedless قابل للاصابة بشدة لنيماتودا تعقد الجذور "ميليدوجيني انكوجنيتا" بينما صنف Thompson seedless وsuperior كانا متوسطا الاصابة ، واثبتت الاختلاف في بروتين العينات المصابة والسليمة انها كانت مختلفة من البروتين السائل المستخلص من الاوراق للخمس اصناف المختبرة كما كان رد الفعل للاصابة بنيماتودا تعقد الجذور " ميليدوجيني انكوجنيتا" واستعمل ISJ-PCR لقياس درجات التشابة ضمن اصناف العنب وجذوره وحساب المسافة الوراثية بين الاصناف وتعكس التحليل الجنيني الاختلاف وينشأ العلاقة الجهازية ضمن الاصناف المختبرة الذي ربما يرجع ذلك المقاومة في الجذور

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