MOLECULAR SCREENING AND INHERITANCE OF THE ROOT-KNOT NEMATODE RESISTANCE IN TOMATO (*L. esculentum* Mill.)

***Refaat, M.H.**

**Department of Genetics, Fac. of Agric. Moshtohor, Benha University, Egypt.*

ABSTRACT

Root-knot nematodes are major pests of field and vegetable crops in Egypt and worldwide. Cause damage to many economically important horticultural crops like potato, cotton and tomato. In the 1940s the root-knot nematode resistance gene (*Mi*) was introgressed into the cultivated tomato from the wild species *Lycopersicon peruvianum*. Today, many commercial tomato varieties carry the *Mi* gene, which has been mapped. This gene confers resistance to *Meloidogyne incognita*, *M. javanica* and *M. arenaria*. The short arm of chromosome 6 and many markers linked to *Mi* have also been identified. The *Mi* gene has been isolated, cloned and sequenced. In this study, plants were infected with *M. incognita* race 2 for determined genotypes of resistant and susceptible. According to nematode resistance assays, the root-gall index was determined as > 2 and ≤ 2 for susceptible and resistant F_1, F_2 and BC_1 plants, and reproduction factors were 0 and > 1 for resistant and susceptible F_1, F_2 and BC_1 plants, respectively. In conjunction with traditional screening *Mi* gene specific primers were used to differentiate between resistant and susceptible plants with a1.5 to 1.6 kb DNA band being detected in resistant plants but absent in susceptible plants. The data showed a clear correlation between traditional screening and the use of markers and support the possibilities of using marker assisted selection for *M. incognita* resistance breeding.

INTRODUCTION

Root-knot nematodes, *Meloidogyne* spp., are obligate, Chit wood and *M. incognita* Chit wood. They cause serious sedentary endoparasites of many plant species. Their damage to tomato crops **(Lamberti, 1979),** especially in potential host range encompasses more than 3000 plant tropical, sub-tropical and warm climates. In Egypt, the most important and widely spread in different soil types and locations are the root knot nematodes which are in some cases they consider as limiting factors of tomato production **(Houssny and Oteifa, 1956)**

Host-plant resistant to root-knot nematodes is a powerful tool for crop protection, and it is destined to play a more important role

than ever before in managing nematode problems in sustainable agriculture. The most effective nematicides have been restricted in agriculture because of the high risk to human health and the environment **(Veremis and Roberts, 1996).**

Many commercial tomato varieties carry a single, dominant gene called *Mi* which confers resistance to 3 of the most damaging species of root-knot nematodes (*Meloidogyne incognita, M. javanicaand M. arenaria*). This resistance is associated with the localized death of host tissue near the invading nematode in the root tips. The *Mi* gene was discovered 50 years ago in an accession (P.I. 128657) of *Lycopersicon peruvianum* (Mill.), a wild relative of the edible tomato (*Lycopersicon esculentum* Mill.) that was grown in the western coastal region of South America **(Cap** *et al.,* **1991).**

This resistance was transferred and expressed in F1 plants derived from a cross between *L. peruvianum* P.I. 128657 and *L. esculentum* 'Michigan State Forcing' made by (**Smith 1944** and **Baird** *et al***.,1996).**

The *Mi* gene is located on the short arm of chromosome 6, this chromosome has been mapped in considerable detail, and multiple markers for other traits linked to *Mi gene* have been identified **(Messequer** *et al***., 1991; Williamson** *et al***., 1994)**. Recently, the *Mi* gene was isolated by a positional cloning approach **(Kaloshian** *et al***., 1998; Milligan** *et al***., 1998)** and DNA sequence analysis was carried out to identify *Mi* candidates. Sequencing revealed 2 genes, *Mi-1.1* and *Mi 1.2*, those were 95% identical to each other, and encoded proteins with a high similarity to previously cloned plant resistance genes. Complementation analysis showed that the introduction of *Mi-1.2,* but not of *Mi 1.1* to susceptible tomato plants was sufficient to confer a nematode-resistant plant phenotype with the same spectrum resistance as that of *Mi* **(Milligan** *et al***., 1998).**

The objective of this study was to inheritance and screening F_1, F2 and BC_1 plants' resistance to *M. incognita* race 2 derived from a cross of Ronita (P_3) and Anahu (P_4) (Resistant) with Edkawi (P_1) and Super Marmande (P_2) (Susceptible) by PCR.

MATERIALS AND METHODS

1. **Plant Materials**

This research was conducted at the Agricultural Experimental Station of the Horticulture Department, Faculty of Agriculture at Moshtohor, Benha University, during three summer seasons of 2003 to 2005. Seeds of these cultivars were obtained from the National Germplasm Resources Laboratory, Beltsville, U.S.A. and Germplasm Preservation Laboratory, Faculty of Agriculture at Moshtohor, Benha University, Egypt. The cultivars Ronita (P3) and Anahu (P4), which were found to be Resistance to the Root-Knot Nematode and the local cultivar Edkawi (P1) and Super Marmande (P2), which expressed susceptible to root-knot nematode, were selected to study the inheritance and Screening of the root-knot nematode resistance in tomato (*L. esculentum* Mill.).

On March 22nd, 2003, seeds of all selected cultivars were sown in the field. The following crosses were made between the parental genotypes: (Edkawi X Ronita), (Edkawi X Anahu), (Super Marmande X Ronita) and (Super Marmande X Anahu). Seeds of the F1's were harvested separately and kept for the next season.

On March 17th, 2004, hybrid seeds of each cross and seeds of the parental genotypes were planted in the field. Crosses between the parental genotypes were repeated and F1 plants were selfed to obtain F2 seeds. Backcrosses populations were obtained by crossing each F1 hybrid with its respective parents

2. **Nematode cultures**

M. incognita race 2 was described based on perineal patterns and the host range by **Barker** *et al.* **(1985).** *M. incognita* race 2 culture was maintained on susceptible tomato in flower pots containing a sterile, moist loamy soil (80% sand, 15% silt, and 5% clay) in a growth chamber for 6 weeks at 22-26^{0}C on a 16 h of light regime.

3. **Screening Tests**

The following methods were used for nematode inoculation: (1) two seeds were sown in a 15 cm diameter flowerpot containing a sterile, moist loamy soil (80% sand, 15% silt, and5% clay), (2) plants were thinned to one per pot at the second true leaf stage, (3)

M .incognita race 2 inoculum was produced in the growth chamber on susceptible tomato, (4) nematode eggs were extracted from roots, and (5) each plant was inoculated at the second-fourth true leaf stage with 1000 J2 of the *M. incognita* race 2. Plants were harvested 8 weeks after the inoculation and evaluated according to the method of **Barker** *et al.* **(1985)**. Plants were classified as resistant (≤ 2 root gall index) or susceptible (> 2 root gall index) based upon the distribution of F1, F2 and BC1 plants infected with *M. incognita* race 2. In addition, reproduction factors were calculated as the final level of J2 in soil / 1000 J2 (the amount of J2 in each flowerpot that was initially inoculated with *M. incognita* race 2) and statistical analysis was detected according to **Mather (1963).**

4. DNA isolation

Total DNA was extracted by the method of **Doyle and Doyle (1987)** with minor modifications. Fresh leaf tissue was frozen in liquid nitrogen and ground using an Eppendorf tube and glass bar. The homogenate of the leaf tissue was placed in a tube together with 500 µl of DNA extraction buffer [2% (w/v) CTAB (hexadecyltrimethylammoniumbromide), 1.4 M NaCl, 0.2% (v/v) 2-mercaptoethanol, 20 mM EDTA, and 100 mM Tris-HCl, pH 8.0], and incubated in a water bath at 60^0 C for 30 min with occasional swirling. After incubation, the lysate was extracted once with chloroform: phenol (1:1, v/v). The aqueous phase was mixed with a two-thirds volume of cold isopropanol. The precipitated DNA was resuspended in TE (10 mM Tris pH 8.0, and 0.1 mM EDTA).

5. PCR analysis

PCR amplification was carried out in a 25 µl solution containing 10 ng of DNA, 2 mM MgCI2, 200 µM dNTP, PCR Buffer, 0.4 mM each of the primers C1/2 (5'-cagtgaagtggaagtgatga-3') and C2/S4 (5'-ctaagaggaatctcatcacagg-3') (**Milligan** *et al.*, **1998),** 1 unit of Taq DNA polymerase and deionised water.

PCR was conducted with a Thermal Cycler using the following cycling profile: one cycle of 60 s at 94^0C, 30 s at 65^0C, and 60 s at 72^0C, followed by 11 cycles of 1^0C lower annealing temperature each and 24 cycles of 30 s at 94^0C, 30 s at 56^0C, 60 s at72^0 C. After completion of the PCR, 5 µl of loading dye (0.25%

bromophenol blue, 0.25% xylene cyanol FF, and 40% sucrose) was added to each reaction tube. The samples were electrophoresed in 1.5% agarose gel in TAE buffer and stained with 0.5 µg/µl of ethidium bromide. The gel was illuminated by ultraviolet light, and photographed **(Figure 1A, B and C).**

RESULTS AND DISCUSSION

Root-knot nematodes of the genus Meloidogyne are economically important pathogens of a wide range of crops **(Sasser and Carter, 1985).** Infective second-stage juveniles of these obligate endoparasites penetrate the roots of the host and migrate intercellularly to the vascular cylinder **(Williamson and Hussey, 1996).** The primary symptom of root-knot nematode infection is the formation of typical root galls on the roots of susceptible host plants. Their invasion of the root system of host plants results in a shallow, knotted root system and susceptibility to other pathogens. Nutrient and water uptake are substantially reduced because of the damaged root system, resulting in weak and poor yielding plants **(Abad *et al*., 2003).**

Recently, foreign firms have developed fresh market tomato varieties with resistance to root-knot nematode, and these have become commercially available as an option for nematode management. In these resistant varieties, nematodes fail to develop and reproduce normally within the tomato root tissues, allowing plants to grow and produce fruit even though nematode infection of roots occurs.

1. Inheritance of the Root-Knot Nematode Resistance:

The frequency distribution for plant reaction to root- knot nematode resistant in the F_2, BC_1 and BC_2 populations of the crosses Edkawi (P_1) X Ronita (P_3), Edkawi (P_1) X Anahu (P_4), Super Marmande (P_2) X Ronita (P_3) and Super Marmande (P_2) X Anahu (P_4), **Table (1),** indicated that this character was inherited quantitatively. The plant reaction to root- knot nematode was resistant in F1 families from crosses of (P_1 X P_3), (P_1 X P_4), (P_2 X P_4) and (P_2 X P_4) and F_2 families from these crosses segregated in a 3 resistant: 1 susceptible ratio; the backcross of F_1 (P_1 X P_3), F_1 (P_1 X P_4) to Edkawi and F_1 (P_2 X P_3), F_1 (P_2 X P_4) to Super Marmande

segregated 1 resistant: 1 susceptible ratio. These segregations clearly indicate that susceptible gene in Edkawi and Super Marmande parents is controlled by a single recessive gene and allelic to *Mi- 1.1* or *Mi-1.2* reported in cultivars (**Milligan** *et al*., **1998**).

Table (1): Frequency distributions for plant reaction to Root-Knot Nematode Resistance in parents, F_1, F_2, Bc_1 and Bc_2 segregations derived from some Tomato crosses.

Cross	Population	Scale of root gall index under Nematode infection					Total No. of plants	#Response to root-knot Nematode		X^2
		1	2	3	4	5		R	S	
Edkawi X Ronita	P_2^S	–	–	–	10	20	30	–	30	
	$Bc_1(F_1XP_2)$	26	11	3	11	9	60	37	23	*3. 266***
	F_1	3	27	–	–	–	30	30	–	
	F_2	38	45	4	14	19	120	83	37	*2. 177***
	$Bc_2(F_1XP_3)$	24	22	–	6	8	60	46	14	
	P_3^R	30	–	–	–	–	30	30	–	
Edkawi X Anahu	P_2^S	–	–	–	10	20	30	–	30	
	$Bc_1(F_1XP_2)$	20	16	8	11	5	60	36	24	*2. 400***
	F_1	6	24	–	–	–	30	30	–	
	F_2	50	47	6	5	12	120	97	23	*2. 177***
	$Bc_2(F_1XP_4)$	16	26	5	2	11	60	42	18	
	P_4^R	30	–	–	–	–	30	30	–	
Super-Marmande X Ronita	P_2^S	–	–	5	10	15	30	–	30	
	$Bc_1(F_1XP_2)$	24	10	5	11	10	60	34	26	*1. 066***
	F_1	5. 0	25	–	–	–	30	30	–	
	F_2	41	50	4	13	12	120	91	29	*0. 044**
	$Bc_2(F_1XP_3)$	34	19	–	6	1	60	53	7. 0	
	P_3^R	30	–	–	–	–	30	30	–	
Super-Marmande X Anahu	P_2^S	–	–	5	10	15	30	–	30	
	$Bc_1(F_1XP_2)$	18	14	5	14	9	60	32	28	*0. 266***
	F_1	7	23	–	–	–	30	30	–	
	F_2	42	45	3	12	18	120	87	33	*0. 400***
	$Bc_2(F_1XP_4)$	20	25	1	7	7	60	45	15	
	P_4^R	30	–	–	–	–	30	30	–	

#R: Resistant (≤ 2 root gall index), S: Susceptible (> 2 root gall index).

Inheritance of the root–knot nematode resistance in the crosses segregation ratio suggested a single dominant gene for the high level of resistant; although the data on disease expression were recorded on a 1 to 5 scale, the results and subsequent observation fitted a single gene model for resistance better than a quantitative pattern.

2. **Molecular Screening of the Root-Knot Nematode Resistance:**

In this study, the homozygous resistant parents (P_3) and (P_4) carrying the *Mi* gene, the homozygous susceptible parents (P_1) and (P_2) and F_1 plants, F_2 families and BC_1 families from a crossing of (P_1) , (P_2) and (P_3) and (P_4) were used to test resistance to *M. javanica* race 2. Fallowing bioassay, resistant and susceptible strains were determined in Table (1).

The resistant parents and selected F_2 plants and BC_1 plants had a mean gall index value of 1 and 2. The susceptible parents and F_2 plants and BC_1 plants had a gall index value that averaged 3 to 5 (Table1). This is in agreement with similar studies under greenhouse conditions **(Cap** *et al***., 1991; Yaghoobi** *et al.,* **1995).**

In molecular screening, parents and F_2 individuals' families and BC_1 family were examined with a C1/2 and C2S4 primer combination. The DNA banding patterns of PCR amplification products correlated well with the known resistant or susceptible phenotypes, which were previously evaluated in an inheritance study under greenhouse conditions. A 1.5 to 1.6 kb amplification which corresponded to a portion of the 3' region of the gene was detected in parents (P_3, P_4) and F_2 families and BC_1 families containing the *Mi-1.2* gene (Figure 1A, B and C) by PCR. However, the 1.6 kb PCR product was absent in susceptible parents (P_1, P_2) and F_2 families and BC_1 families. This is in agreement with the results obtained by **(Williamson** *et al.,* **1994; Milligan** *et al.***, 1998** and **Rossi** *et al.,* **1998).**

In this study, *Mi* gene specific primers (C1/2 and C2S4 primers) were used and resistant and susceptible plants were

distinguished from each other whereas resistant heterozygote and homozygote individuals were not distinguishable.

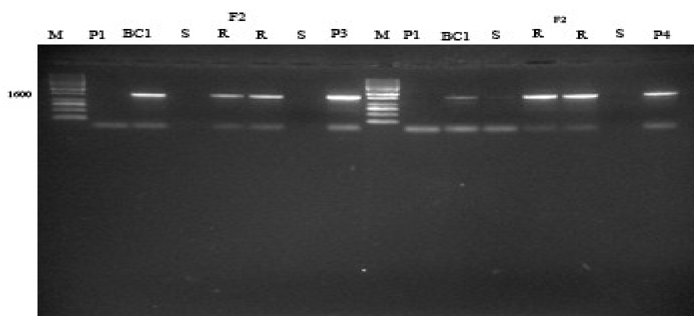
In breeding, the identification of the root-knot nematode resistance gene Mi in tomato plants depends on the traditional screening assay for resistance. If many recombinants are to be screened, this is time consuming, tedious and labour intensive. However, this situation can be overcome by using molecular markers in a marker assisted selection (MAS) programme.

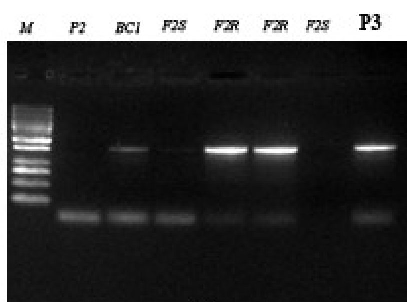
MAS' dictates that the selection of one or more traits of interest be conducted indirectly by selecting for markers linked to the trait(s) (**Melchinger, 1990).**

MAS are most efficient when selection for the marker is convenient and there is tight linkage between the marker and the trait of interest **(Kelly, 1995).** The major advantage of DNA molecular markers is that they are not influenced by environmental effects, have low negative selection pressure in populations and are developmentally stable. Nematode resistant genes, *Gro1* for *Globodera rostochiensis* (Woll.) in potato **(Ballvora** *et al.,* **1995),** *Cre3* and *Cre1* for *Heterodera avenae* in wheat **(Eastwood** *et al.,* **1994; Ogbonnaya** *et al.,* **2001)** and Mij for *M. javanicaand* and *M. incognitain* peach **(Lu** *et al.,* **1999),** are routinely used in breeding programmes to integrate these resistances.

This study clearly demonstrates the tools of markers to incorporate key resistances of interest. Currently many tomato hybrids have the *Mi* gene incorporated using MAS belonging to foreign seed firms and there is limited application of MAS capacity in local seed firms.

This paper clearly demonstrates the use of the *Mi* marker for incorporating *Mi* resistance that is reliable, timely and effective and supports the application hybrid of this tool for commercial tomato breeding companies.





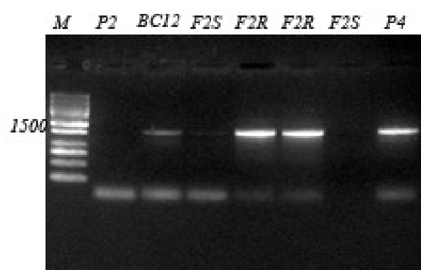


Figure (1): Molecular screening of the root-knot nematode resistance *Mi* gene amplification products obtained using C1/2 and C2S4 primers in Tomato Crosses.

(A): P1,P3,P4,F2(Susceptible families),F2(Resistance families),BC1(Resistance families).

(B): P2,P3,F2(Susceptible families),F2(Resistance families),BC1(Resistance families).

(C): P2,P3,F2(Susceptible families),F2(Resistance families),BC1(Resistance families).

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المسح الجزيئى و وراثة المقاومة لنيماتودا تعقد الجذور فى الطماطم

محمد حسن رفعت

قسم الوراثة– كلية الزراعة بمشتهر – جامعة بنها

الملخّص العربى

يعتبر مرض تعقد الجذور النيماتودية من الافات الرئيسيةَ لمحاصيل الحقل و الخضرِ في مصر والعالم و تلحق العديد من الاضرار للمحاصيلِ البستانيةِ المهمةِ إقتصادياً مثل البطاطس و القطن و الطماطمَ . عام ١٩٤٠تم ادخال جين مقاومةَ مرض تعقد الجذور النيماتودية جين مقاومةِ (*Mi*) عن طريق التهجين النوعى بين الطماطمِ المنَزْرعة مِنْ النوعِ البرى *Lycopersicon peruvianum*. اليوم، يوجد العديد مِنْ اصناف الطماطم التجاريةِ تحمل جينِ المقاومة للنيماتودا *Mi*، حيث يَعطى هذا الجينِ المقاومةَ ضد انواع النيماتودا المختلفة مَثل:

***Meloidogyne incognita, M. javanica, M. arenaria* .**

وجد ان الذراع القصير فى الكروموسوم رقم ٦ و العديد من المعلمات الجزيئية ترتبط مع جين المقاومة للنيماتودا و تميزه وقد تم عزل هذا الجين و اجراء له عملية كلونة و دراسة التتابعات الوراثية له.

فى هذه الدراسة اجراء عدوى صناعية بواسطة النيماتودا *M. incognita* السلالة رقم ٢ للتحديد المقاومة و الحساسية لمجموعة من هجن الطماطم المختلفة من حيث المقاومة لمرض تعقد الجذور النيماتودا وفقا لمعاير دليل قياس المقاومة لنيماتودا عندما تكون القمة اكبر من ٢ و اقل او تساوى ٢ تعتبر حساسى للاصابة و مقاومة للاصابة على التوالى و قد تم تحديد و قياس ذلك على عائلات الجيل الاول و الجيل الثانى و التهجين الرجعى الاول.

و امكن استخدام التقيم و المسح الجزيئى بواسطة استخدام بادى متخصص لكشف عن وجود جين المقاومة للنيماتودا *Mi* باستخدام تكنيك PCR بالاشترك مع طرق التقيم التلقيدية و قد امكن تحديد حزمة واحدة من المادة الوراثية دانا وزنها الجزيئى حوالى ١.٥ الى ١.٦ كيلو زوج من القواعد و هذه الحزمة كانت موجودة فى النباتات المقاومة فقط للمرض تعقد الجذور النيماتودا و لكن كانت غائبة فى النباتات الحساسة للاصابة بمرض.

فقد اظهرت البيانات و النتائج المتحصل عليها ان هناك ارتباط قوى واضح بين طرق التقيم و المسح التقليدية و الغير تقليدية باستخدام المعلمات الجزيئية لمقاومة مرض تعقد الجذور النيماتودا فى هجن الطماطم المختلفة و هذا يعطى امكانية للاستخدام المعلمات الجزيئية المتخصصة على نطاق واسع فى المساعدة فى القيم و الانتخاب فى برامج التربية للمقاومة النيماتودا.