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Application of Tissue Culture Technique for Improving Tomato's Tolerance to Root-Knot Nematode Infection

[1]Abdel-Kafie, Omaima; [2,3]A. H. Nour El-Deen; [4,5]Hadeer Y. Darwesh; [1]W. Al-Saadi and [1]M. Abdel-Baset

[1]*Vegetables and Floriculture Dept., Faculty of Agriculture, Mansoura University, Egypt.*
[2]*Nematology Research Unit, Agricultural Zoology Dept., Faculty of Agriculture, Mansoura University, Egypt.*
[3]*Biology Dept., Faculty of Science, Taif University, Saudi Arabia.*
[4]*Medicinal and Aromatic Plants Research Dept., Horticulture Institute, Agricultural Research Center, Egypt.*
[5]*Biotechnology Dept., Faculty of Science, Taif University, Saudi Arabia.*

Abstract: *The influence of three medicinal plants extracts i.e. Neem (Azadirachta indica), Solanum (Solanum nigrum L), and Indian ginseng or Ashwagandha (Withania somnifera L) that were used for tomato seed germination c.v. Fatema and TY727 (F1) before transplanting in pots with sterilized soil on Meloidogyne incognita infection was studied under greenhouse conditions 30±5°C. Results indicated that all of the tested extracts protected and improved tomato plant growth of either Fatema or TY to a certain extent. Among all tested materials, neem extract at 5 % gave the highest increment in fresh weight of the whole plant with values of 144.9 % and 183.1 %, respectively. The same trend was observed with respect to shoot dry weight and number of leaves of either tomato cultivars. The highest reduction percentage in number of galls and egg masses were obtained from pots received neem extract treatments which amounted to 100 % on each variety. The results of RAPD on Fatema tomato cultivar indicated the presence of genetic variability as a result of present or absent of bands among the studied treatments. Of the 95 amplified bands, 86 were present in untreated plants, whereas, 84 were present in treated ones. Nine bands only were absent of untreated plant, but 11 bands were absent of treated plants with neem extract.*

Keywords: *Aseptic culture, tomato, botanicals, Meloidogyne incognita.*

I. Introduction

Tomato (*Lycopersicon esculentum* L.) is the world's largest vegetable crop and known as productive as well as protective food. Moreover, tomato is one of the most important commercial and dietary vegetable crops all over the world as well as in Egypt. In 2012, China was the largest producer of tomatoes but Egypt ranked fifth among the five top producers of tomatoes in the world which cultivated on 515020 feddans producing 8625219 tones according to FAOSTAT.One of the serious pathogens affecting tomato production is plant parasitic nematodes. Worldwide, crop loss attributed to these pests could be estimated by 20.6% (Sasser and Freckman, 1987). The root-knot nematodes, *Meloidogyne* spp. are an economically important parasite of plants; *M. arenaria, M. hapla, M. javanica* and *M. incognita* are considered the most popular species which caused more than 90% of the estimated damages.

The practice of plant tissue culture has contributed towards the propagation of large number of plant from small pieces of stock plants in relatively short period of time (Daniel, 1998). Compared to conventional planting material, tissue culture plants give higher yield; and earlier and more vigorous sucker production. Tissue culture plants are uniform and available all year round as: important criteria for commercial farming. Rapid and easy mass production also allows for improvement of selections of plant with enhanced stress or pest resistance. However, during the early transplanting stages, tomato tissue culture plantlets need higher level of care and attention than conventional plants. So, there is a need for producing healthful seedlings that tolerant the infection of plant parasitic nematodes by applied certain components in media.

Plant parasitic nematodes are controlled by cultural practices, chemical nematicides and the use of resistant cultivars. However, nematicides do not provide long-term suppression of nematodes, and environmental and human health concerns are resulting in increased restrictions on their use. Some safe procedures for nematode control have been developed passed on biological control agents and organic amendments; however, there is still a need for alternative, friendly methods or compounds for effective nematode control to be developed (Noling and Becker, 1994). One way of searching for such nematicidal compounds is to screen naturally occurring components in certain plants. Consequently, a large number of plants/ plant parts have been screened for their nematicidal activities (Nour El-Deen and Darwish, 2011 and Nour El-Deen et al., 2013). Many compounds with nematicidal activity have been found in plants including alkaloids, diterpenes, fatty acids, glucosinolates, isothiocyanates, phenols, polyacetylenes, sesquiterpenes and thienyls (Chitwood, 2002 and Taba et al., 2008).

Extracts of certain plants and/or their components have been tested for nematicidal activity in vitro and in soil by several workers (Oka et al., 2000; Pandey et al., 2000; Duschatzky et al., 2004; Pavaraj et al., 2012; Nour El-Deen et al., 2013 and and Sardaria et al., 2015). Results of most previous papers were greatly affected by the percentage of organic matter and clay contents within the soil media of such experiments, since the influence of the extracts of *Azadirachta indica*, *Withania somnifera*, *Taxodium* spp. callus and *Euphorbia geniculata* at 2000 mg/l on root galling of eggplant was more pounced in sandy loam soil (Elbadria et al., 2008; Nour El-Deen and Darwish, 2011).

So an attempt was made to avoid the application of plant extracts or their products directly to the soil by mixing the tested concentration of such materials with the artificial media where germination of plant seed taken place and plant roots naturally grow under aseptic condition before transplanting to soil. Therefore, the present investigation was carried out to determine the nematicidal activity of extracts of three medicinal plant leaves as protectants added to Murashige and Skoog (MS) medium on growth of tomato seedlings that finally transplanted to pots infected with *Meloidogyne incognita* under greenhouse conditions.

II. Materials and Methods

Plantlet induction:

Tomato seeds c.v. Fatema as tolerant cultivar and TY727 (F1) as susceptible one (unpublished data) were shacked for 10 minutes in sterilized distilled water provided with some drops of soap, then surface sterilized by immersing in 30% NaOCl solution plus a drop of tween20 for 12 min. Thereafter, these soaked seeds were rinsed three times in sterilized distilled water, and cultivated on autoclaved watered-cotton as a liquid medium in 250 ml jars under aseptic conditions. Ten seeds were distributed randomly in each jar and kept in growth chamber at 25°C with 16 h. light/ 8 h. dark cycle. Seeds were then germinated after three days.

Preparation of the media:

Three-fourth strength Murashige and Skoog medium (3.3 g/l.) were prepared. The media were solidified with 8 g/l. agar and sucrose at 30 g/l. The media were distributed into 375 ml clean jars, contained 300 ml of nutrient media each. The pH was then adjusted to 5.7 before autoclaved process.

Preparation of Extracts:

Three medicinal plant extracts, i.e. Neem, Solanum and Indian ginseng were used in this study. Dried leaves of plants were powdered and soaked overnight in sterilized distilled water (v/v), then the extract was screened through a piece of cloth. Resulted extract was stored in vials at 4°C until used.

Nematode inoculum:

Fresh hatching second-stage juveniles of the root-knot nematode *M. incognita* (J_2) were obtained from a pure culture established from single egg mass of *M. incognita* that previously identified according to the characteristics of its perineal pattern (Taylor and Sasser, 1978) and reared on coleus plants, *Coleus blumei* in the greenhouse of Nematology Research Unit, Faculty of Agriculture, Mansoura University, Egypt.

Impact of certain plant extracts on tomato seedlings growth and nematode infection:

The concentrations of 5, 10 and 15% of each extract under study was added to the medium with a drop of Tween20, then shaking thoroughly to determine its effect on the seedlings of two cultivars growth against nematode development. Twenty jars with extracts were served as control. The media were distributed into 250 ml sterile jars, contained 30 ml of medium supplemented with extracts under a sterilized environment within a laminar airflow cabinet. One week old sterilized tomato seedlings from each cultivar were cultured in previously prepared media. Each treatment of the extract tested has ten jars as replicates (each contained 3 explants), kept inside the growth chamber at 25°C under the system of 2000 Lux. fluorescent lamps for 16 h. light and 8 h. dark cycle for three weeks.

Afterwards, one month old tomato seedlings were transferred to 7-cm-d. plastic pots (one seedling/pot) filled with steam-sterilized mixture of peatmoos and sand (1:1, v:v) for acclimatization. Glass jar was placed on the top of each seedling and kept in a growth chamber as previously mentioned. One week later, these tomato seedlings were transferred to 14- cm-d. plastic pots (one seedling/pot) filled with 900 g steam-sterilized sandy loam soil (1:1). One week later, forty five seedlings (45 day old) of each tomato cultivar were separately inoculated with 1500 fresh hatching second-stage juveniles of *M. incognita*. Three untreated with any of the extract and inoculated seedlings were served as control. Each treatment was replicated five times and all pots were randomly arranged on a greenhouse bench at 30±5°C. Plants were watered regularly as needed.

After 45 days from nematode inoculation, plants were harvested. Data dealing with length of shoot and root, and fresh weights of shoot and root as well as shoot dry weight were determined and recorded. Infected tomato roots were stained in 0.01 hot lactic acid fuchsin and examined for the numbers of galls and egg masses.

DNA extraction:

Genomic DNA for tomato roots of Fatema cultivar produced from the best treatment of neem extract (5 %) as well as untreated ones (control) was isolated using protocol of Anna et al. (2001).

RAPD amplification:

The following components were added to a sterile eppendorf tube placed on ice during pipetting as followed: 2.5 µl 25 mM MgCl2; 0.5 µl 40 mM dNTPs; 1 µl Taq DNA polymerase (1 unit/µl); 2 µl 0.4 uM 10-mer primer (manufactured by Bioneer, New technology certification from ATS Korea). The volume was completed to 25 µl dsH2O. Thirty nanogram from each DNA extracted sample were used for amplification reaction 5 µl of the 10-mers random primer (15 ng /ml) were added to Gene pack PCR tubes kits. The total volume was completed to 25 µl using sterile dsH2O water. The amplification protocol was carried out as follows using PCR Programe (Biometra):

A. Denaturation at 94ºC for 1 min.
B. 35 cycles each consists of the following steps:
1. Denaturation at 94ºC for 30 sec.
2. Annealing at 45ºC for 1 min.
3. Extension at 72ºC for 1 min.
C. Final extension at 72ºC for 5 min.

Data analysis:

Statistically, the obtained data were subjected to analysis of variance (ANOVA) (Gomez and Gomez, 1984) followed by Duncan's multiple range to compare means (Duncan, 1955). The RAPD reproducible bands were scored as present (1) or absent (0), each of which was treated as independent locus regardless of its intensity. By comparing the banding patterns of treated and untreated plants for a specific primer, treatment - specific bands were identified. Faint or unclear bands were not considered. Band size was estimated by comparing with 1 kb ladder (Invitrogen, USA) using Total lab, TL120 1D v2009 (nonlinear Dynamics Ltd, USA).

III. Results and Discussion

Data in table (1) document the plant growth and root galling of tomato seedlings c.v. Fatema previously reared on MS medium provided with three plant extracts, i.e. Neem, Solanum and Indian ginseng before planting in 900 g sterilized soil (1:1)/ pot and then infected with *M. incognita* under greenhouse conditions at 30±5°C. Results indicated that all of the tested materials were found to be effective in protecting and improving tomato plant growth infected with nematode to certain extent. It is clear that all of the tested extracts significantly improved growth of the infected tomato plant parameters as compared with those of untreated ones (Table 1). Neem extracts at all concentrations significantly increased plant lengths and fresh weights and shoot dry weight as well as number of leaves when compared with the other treatments tested or nematode alone. Among all tested materials, neem extract at concentration of 5 % showed the highest increment in fresh weight of the whole plant, shoot dry weight and number of leaves with values of 144.9 %, 169.2 % and 87.4 %, respectively.

Moreover, all levels of indian ginseng achieved the second values to neem extract treatment in increase percentage of both fresh weight of whole plant as well as shoot dry weight, whereas, solanum extract ranked lowest values of the same plant growth parameters. Concerning root galling, a significant reduction in number of galls on tomato plant root was achieved with reduction percentages ranged from 74.6 to 100 % (Table 1). All tested extracts significantly decreased number of galls on tomato roots with reduction percentage of 100 % except those received solanum extract at 5 % which gave 74.6 % reduction in galls number. Regarding egg masses numbers, *M. incognita* did not clearly reproduce on plants treated with all of the tested materials. Data in table (2) reveal the plant growth and root galling of tomato seedlings c.v. TY727 previously reared on MS medium provided with the previous materials, then transplanted to sterilized soil and inoculated with *M. incognita* under greenhouse conditions at 30±5°C.

Results indicated that all of the tested materials at all concentrations were found to be effective in protecting and improving tomato plant growth infected with nematode to certain extent. It is clear that all of the tested extracts significantly improved growth of the infected tomato plant parameters as compared with those of untreated ones (Table 2). Among the tested materials, neem extract treatment at the level of 5 % also accomplished the best results in improving tomato plant growth where the percentage increase values of the whole plant fresh weight, shoot dry weight and number of leaves averaged to 183.1, 180.0 and 146.6 %, respectively. Indian ginseng treatment at the same concentration appeared to have the second rank to neem in values of percentage increase of the same previous measurements (84.6, 100.0 and 91.8 %), respectively.

A considerable percentage increase in the previous plant growth characters was recorded by solanum extract treatment at the same concentration with values of 21.5, 40.0 and 50.7 %, respectively. It is worthy to observe that solanum extract treatment at 10 and 15 % gave the lowest values for plant growth criteria which amounted to 9.2, 20.0 and 23.3 %; and 6.2, 20.0 and 16.4 %, respectively (Table 2).

Table (1): Effect of three medicinal plant extracts added to MS medium on the growth of tomato c.v. Fatema, root galling and reproduction of *Meloidogyne incognita* under greenhouse conditions.

Treatments	Conc.	*Plant growth response										Galling and reproduction			
		Length (cm)		Fresh weight (g)		Fresh wt. of the whole plant (g)	Increase %	Shoot dry weight (g)	Increase %	Number of leaves	Increase %	Number of galls	Reduction %	Number of Egg-masses	Reduction %
		Shoot	Root	Shoot	Root										
Neem	5	75.8 a	27.8 a	19.5 a	9.5 a	28.9 a	144.9	3.5 a	169.2	19.3 a	87.4	0.0 c	100	0.0 b	100
	10	68.0 b	24.0 b	17.3 b	8.8 ab	26.b	120.3	2.9 b	123.1	17.8 ab	72.8	0.0 c	100	0.0 b	100
	15	65.3 bc	20.8 cd	16.7 b	8.2 bc	24.9 b	103.3	2.7 b	107.7	17.0 b	65	0.0 c	100	0.0 b	100
Solanum	5	51.5 e	20.0 d	12.1 d	3.3 f	15.4 e	30.5	1.8 ef	38.5	12.8 d	24.3	8.0 b	74.6	0.0 b	100
	10	49.8 ef	12.5 f	9.0 e	5.5 de	14.5 e	22.9	1.7 cd	30.8	12.5 d	21.4	0.0 c	100	0.0 b	100
	15	46.8 fg	9.0 gh	7.9 f	6.5 e	14.4 e	22.03	1.4 de	7.7	12.3 de	19.4	0.0 c	100	0.0 b	100
Indian ginseng	5	63.0 c	22.5 bc	14.5 c	8.2 bc	22.7 c	92.4	2.6 b	100	16.8 b	63.1	0.0 c	100	0.0 b	100
	10	57.5 d	16.8 e	13.6 c	5.8 d	19.4 d	64.4	2.1 c	61.5	14.5 c	40.8	0.0 c	100	0.0 b	100
	15	50.5 e	10.8 fg	11.1 d	7.5 c	18.6 d	57.6	2.0 c	53.8	13.8 cd	34	0.0 c	100	0.0 b	100
Ck		41.5 h	7.0 h	6.6 g	5.2 g	11.8 f	--	1.3 ef	--	10.3 e	--	31.5 a	--	16.3 a	--

*Each value is the mean of five replicates.
Means in each column followed by the same letter (s) did not differ at P< 0.05 according to Duncan multiple-range test.

Data presented in table (2) revealed that number of *M. incognita* galls on tomato roots was significantly affected by all tested components as compared to nematode alone. Obviously, application of neem extract treatment at 5 % achieved the highest reduction percentage with value of 100 %, followed by 10 % (85.0 %) and 15 % (79.3 %). Plants receiving indian ginseng at the same concentrations ranked second to neem for reduction percentage of root galls with values of 75.8, 62.8 and 61.7 %, respectively. Egg-masses were significantly suppressed with egg-mass reduction percentages ranged from 37.2 to 100 % as compared with that of the control. The highest reduction percentage (100 %) was obtained from plants receiving neem extract at 5 and 10 % as well as indian ginseng at 5 %.

Results from the present inplanta experiment indicated that tested extracts directly affect nematode reproduction as recorded by other phytochemical compounds (Perez et al., 2003) who reported that the essential oil of *Chrysanthemum coronarium* and organic amendments from Asteraceae species may serve as nematicides. The present results are in accordance with those reported by Nour El-Deen et al (2007) who mentioned that egg masses numbers of *M. incognita* did not clearly reproduce on tomato plants treated with neem oil in tissue culture media as well as improving plant growth. These results may possibly be attributed to its contents of fatty acids such as linoleic acid 21%, palmitic acids 18.5%, stearic acids 0.2% and oleic acid 58.5%; arachidic acid 1.5%; vitamin E and other essential amino acids (Hossain, 2005).

It is clear to see that solanum extract application did not act as strong nematicide on nematodes, since it was found to be the least effective to enhance plant growth parameters as well as nematode development. In 2011, Nour El-Deen and Darwish mentioned that *W. somnifera* extract was the best treatment among tested extracts and callus tissue for both ameliorating eggplant growth parameters and controlling root-knot nematode. The nematicidal activity of such extract could be attributed to the richness of several secondary metabolites such as steroidal alkaloids, triterpenoid, saturated fatty acids as myristic acid and unsaturated fatty acids as oleic and linoleic acids.

In the present study, it is worth to note that low concentrations of all tested extracts were the best in improving the two tomato varieties growth and suppressing root-knot nematode reproduction than high concentration did.Regarding genetic analysis, high level of differences was observed among treated or untreated tomato Fatema variety (Table 3 and Fig. 1). A total of 15 ten (10) mer arbitrary oligonucleotide primers were initially used to establish RAPD-PCR fingerprints of untreated and treated Fatema tomato variety. Only six primers were successfully generated reproducible polymorphic products (Fig. 1). The six primers used for RAPD analysis detected a total of 95 fragments, with an average of 15.8 fragments per primer. Of the 95 amplified bands, 86 were present in untreated plants with an average of 14.3 bands per primer, whereas, 84 were present in treated ones with an average of 14 bands per primer. Nine bands only were absent of untreated plant, but 11 bands were absent of treated plants with neem extract. The number of bands detected in each primer depended on primer, sequence and the extent of variation in two treatments. The number of amplified fragments varied from 11 (Primer 3 and 6) to 21 (Primer 1) and the amplicon size ranged from 80 bp (Primer 1) to 2500 bp (Primer 5).

Table (2): Effect of three medicinal plant extracts added to MS medium on the growth of tomato c.v. TY727, root galling and reproduction of *Meloidogyne incognita* under greenhouse conditions.

Treatments	Conc.	*Plant growth response										Galling and reproduction			
		Length (cm)		Fresh weight (g)		Fresh wt. of the whole plant (g)	Increase %	Shoot dry weight (g)	Increase %	Number of leaves	Increase %	Number of galls	Reduction %	Number of Egg-masses	Reduction %
		Shoot	Root	Shoot	Root										
Neem	5	56.3 a	27.0 a	16.8 a	1.6 a	18.4 a	183.1	1.4 a	180	18.0 a	146.6	0.0 e	100	0.0 f	100
	10	52.0 ab	20.3 b	14.5 ab	0.9 b	15.4 b	136.9	1.3 ab	160	15.0 b	105.5	9.8 cde	85.0	0.0 f	100
	15	44.0 bc	19.0 bcd	12.4 bc	0.8 bc	13.2 bc	103.1	1.3 ab	160	14.8 bc	102.7	13.5 cd	79.3	1.8 ef	90
Solanum	5	44.8 bc	16.3 cde	7.2 d	0.7 bcd	7.9 de	21.5	0.7 d	40	11.0 e	50.7	27.8 bc	57.4	7.8 cd	56.7
	10	30.5 e	12.5 ef	6.8 ef	0.3 e	7.1 e	9.2	0.6 de	20	9.0 f	23.3	27.8 bc	57.4	9.3 c	48.3
	15	20.3 f	10.0 fg	6.3 e	0.6 cd	6.9 e	6.2	0.6 de	20	8.5 f	16.4	34.5 b	47.2	11.3 bc	37.2
Indian ginseng	5	42.5 cd	19.8 bc	11.2 c	0.8 bcd	12.0 c	84.6	1.0 bc	100	14.0 bc	91.8	15.8 cd	75.8	0.0 f	100
	10	34.8 de	15.8 de	10.2 c	0.5 de	10.7 c	64.6	0.9 cd	80	13.0 cd	78.1	24.3 bcd	62.8	3.3 def	81.7
	15	28.0 ef	11.8 fg	7.4 de	0.3 ef	9.7 d	49.2	0.8 d	60	12.0 de	64.4	25.0 bc	61.7	7.0 cd	61.1
Ck		10.5 g	8.5 g	6.3 ef	0.2 ef	6.5 ef	--	0.5 e	--	7.3 g	--	65.3 a	--	18.0 a	--

*Each value is the mean of five replicates.

Means in each column followed by the same letter (s) did not differ at P< 0.05 according to Duncan multiple-range test.

Table (3): List of RAPD primers, the number of amplified products and the number of present and absent bands obtained by analyzing Fatema variety either untreated or treated with neem extract at 5 %.

S/N	Primer sequence (5' 3')	Mol. wt Range(bp)	Total number of bands	Treatment	Number of present bands	Number of absent bands
1	AAC GCG CAA C	80-920	21	Untreated	19	2
				Treated	21	0
2	CCC GTC AGC A	241-920	12	Untreated	12	0
				Treated	11	1
3	CCA CAG CAG T	180-807	11	Untreated	10	1
				Treated	11	0
4	AAG CCC GAG G	200-1700	20	Untreated	17	3
				Treated	14	6
5	GGA CGG CGT T	90-2500	20	Untreated	20	0
				Treated	18	2
6	GAC CAA TGC C	405-1810	11	Untreated	8	3
				Treated	9	2

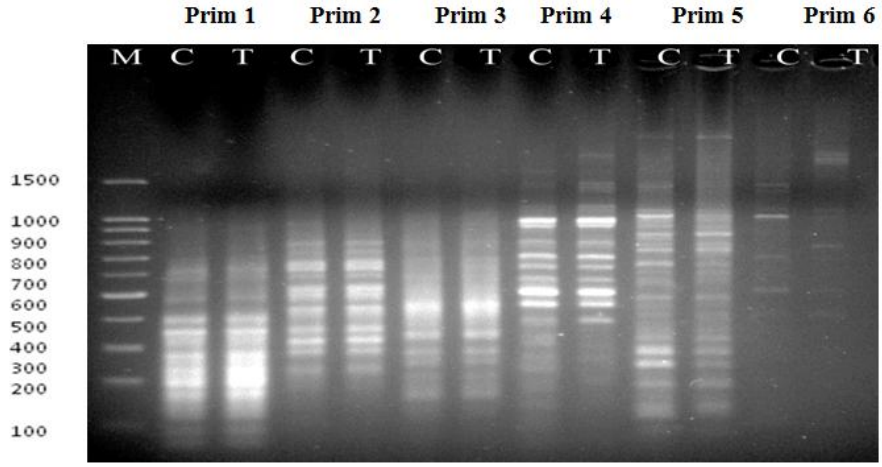

Fig. (1): RAPD-PCR amplification products of Fatema tomato variety produced by six primers. Lane M is 1 kb ladder and lanes C and T represent untreated or treated plants with neem extract at 5 % as listed in table (3).

Generally, results of this experiment indicated that neem extract application at any of concentrations in MS medium for each tomato cultivars seedlings induction considered as superior method in protecting and improving plant growth against *M. incognita* infection as well as keep nematode population at low level in order to increase crop yield. It was clear from the present results that the percentage increase of fresh weights of whole plant, shoot dry weights and number of leaves as well as genetic variation in the infected Fatema tomato variety over control was resulted due to the exist tolerance of tomato plants to nematode infection that expressed by the presence of neem extract at 5 % within the artificial medium.

Variations in DNA sequences lead to polymorphism which is indicative of genetic diversity. The results indicated the presence of wide genetic variability as a result of the high polymorphism among the studied treatments. Based on RAPD marker data, the percentage of present and absent fragments were 90.5 and 9.5 for

untreated plants; and 88.4 and 11.6 for treated ones. This high level of variation could be attributed to the location of those plants in different regions and/or their pedigree information. Also, higher numbers of bands for each primer indicates the existence of larger genetic diversity among the two treatments under investigation (Agrama and Tuinstra, 2003).

A possible explanation for the difference in resolution of RAPDs is that this marker technique target different portions of the genome which are subjected to different mechanisms generating genetic variation (Lalhruaitluanga and Prasad, 2009). Bands of a given RAPD primers may bind to many parts of the genome, so each primer may give information on the polymorphism of several chromosome regions which may affected by neem extract added to media. Some studies have shown that RAPD markers are found throughout the genome and may be associated with functionally important loci (Penner, 1996) that may reverse the tolerance expression in tomato roots.

Apparently, the importance of using plant tissue culture technology in this paper due to the need of propagation to large number of susceptible tomato plant as TY variety or tolerant one as Fatema in a short time, the addition of extract traces to such artificial medium where seeds germination took place in order to produce healthful tolerant seedlings against the infection of *M. incognita* under greenhouse conditions. Obviously, results of this investigation indicated the possible use of these traces at low cost to MS medium of plant tissue culture will avoid the pollution of 60% of the cultivated Egyptian soil (heavy soil) which prevent the nematicidal activities of such plant extracts or products when added directly to such soil against *M. incognita* infecting such economic plant.

IV. Conclusion

In conclusion, we can suggest that addition of neem extract at low concentration in MS medium for susceptible tomato cultivar induction could be the best method for producing healthful seedlings those tolerant *M. incognita* infection as well as increasing crop yield.

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