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Research Article

Evaluation of biological Control potential for different *Trichoderma* strains against Root-Knot Nematode *Meloidogyne javanica*

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Abstract: Twenty strains of four *Trichoderma* species (*Trichoderma harzianum, Trichoderma viride, Trichoderma koningii* and *Trichoderma asperellum*) were evaluated for its potential to control the root-knot nematode *Meloidogyne javanica*. Culture filtrates from *Trichoderma* strains were tested in 24-well tissue culture plates for effects on *Meloidogyne javanica*. Chitwood egg hatch and mobility of hatched second-stage juveniles (J2) were evaluated, all the twenty *Trichoderma* strains showed the ability to colonize *M. javanica* separated eggs and second-stage juveniles (J2) in sterile *in vitro* assays. *T. asperellum* possess the strongest egg-parasitic ability and very effective against 2nd stage larvae of *M. javanica*.
In this investigation, randomly amplified polymorphic DNA (RAPD) markers was used to estimate the genetic variations between four strains of *Trichoderma asperellum* (KC898190, KC898191, KC898192 and KC898193) which were previously isolated from the rhizospheres of different plants growing in Fayoum Governorate, Egypt as a new strain of *T. asperellum* in Egypt**.** RAPD assay using 6 random primers identified *T. asperellum* strains with 5 specific unique markers.

Keywords: *Trichoderma* spp., Biocontrol agent, *Meloidogyne* and RAPD marker.

1. Introduction

The free-living soil fungus *Trichoderma* spp. is a potential biological control agent of plant-parasitic nematodes [1,2]. Biocontrol of the root-knot nematodes (*Meloidogyne* spp.) by different species of *Trichoderma* has been reported by several studies [3,4,5,6,7,8, 9,10,11].

Although Trichoderma species are sometimes found associated with *Meloidogyne* spp. in field soils and can penetrate their eggs and adult females, their successful deployment as a biocontrol agent against nematodes may depend on a thorough understanding of this fungus. Compatibility between the fungal isolate, host cultivar and soil substrate may, therefore, play an important role in the proliferation and persistence of *Trichoderma* spp. in soil. It is important that biocontrol strains are able to compete and persist in the environment, rapidly colonize and efficiently proliferate on newly formed roots [12] and provide continued benefits over the duration of annual crops [13]. Several articles have been published on *Trichoderma* spp. against *Meloidogyne* spp. with good results [6,7,14,15].

However, some important factors that are required for proper evaluation were sometimes neglected, especially the parasitic potential of the fungus in relation to its inoculum densities.

To fully evaluate the potential of a biological control agent, a dose-response relationship between the concentration of the applied antagonist and the reduction of plant damage needs to be established. However, the inoculum density of the antagonist is difficult to determine in the kind and amount necessary for optimal activities. Different studies on antagonist dose–plant disease response relationships in biological control systems have been reported [16,17]. Some studies on the effects of different inoculum densities of *Trichoderma* against *Meloidogyne* spp. have demonstrated an increase in their efficacy at increasing inoculum density but up to certain levels [14,15].

The purpose of this study was to evaluate the effects of four Trichoderma species (*Trichoderma harzianum, Trichoderma viride, Trichoderma koningii,*

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Trichoderma asperellum) on their biocontrol efficacy against root-knot nematode *Meloidogyne javanica.*

2. Material and Methods

2.1 Nematode extraction

Tomato roots (*Lycopersicon esculentum* Mill cv. Castle Rock) infected with the root-knot nematodes, *Meloidogyne* spp. were collected from Fayoum Governorate. Root samples were obtained by lifting the plants carefully with a trowel and samples were directly sent to the laboratory for nematode extraction and identification.

Root samples were carefully washed with tap water and cut into small pieces in a Petri dish with 10ml water. Roots were then teased with dissecting needles to collect mature females necessary for species identification and pure culture propagation.

2.2 Propagation of *Meloidogyne* species in pure culture

The infected roots were cultured on roots of tomato seedlings (*Lycopersicon esculentum* Mill cv. Castle Rock). The collected roots, infected with the root-knot nematode were washed to remove the adhering soil particles and cut into small pieces in a Petri dish under stereoscopic microscope, where individual egg–masses were removed from their females. The collected single egg-mass was used to inoculate a single tomato seedling planted in 20cm diameter plastic pots filled with steamed sterilized sandy clay soil. Plants were kept in a greenhouse on 25-28°C for 8 weeks. Infected roots were used as a source of inoculation for other series of pots. Identification of each isolate was made based on the perineal pattern of the adult females.

2.3 Isolate identification by perineal pattern technique

Species of *Meloidogyne* were identified on the basis of perineal pattern technique. Adult females were separated from galled roots and examination of the prepared posterior cuticular patterns of such females was achieved according to the procedure of Hartman and Sasser [18]. The slides were labeled and examined under the microscope by using 40X power. Identification of *Meloidogyne* species was established after referring to the morphological characteristics given by Taylor and Sasser [19].

2.4 Preparation of culture filtrates

Trichoderma strains were previously isolated from the rhizospheres of different plants growing in Fayoum Governorate, Egypt [20]. *Trichoderma* strains were incubated until it reached the stationary phase (5 days at 25ºC and 250 rpm) in PDB medium. Cultures were filtered through cheesecloth, centrifuged at 10 000g for 10 min, and the supernatant passed through a 0.2µm filter. All culture filtrates were stored at -20ºC until used.

2.5 *In vitro* inhibition assays

Culture filtrates from *Trichoderma* strains were tested in 24-well tissue culture plates for effects on *Meloidogyne javanica*. Chitwood egg hatch and mobility of hatched second-stage juveniles (J2), were evaluated as described by Nitao *et al.*, [21]. *M. javanica* egg masses were collected from roots of glasshouse-grown tomato plants and the eggs subsequently separated and sterilized with 0.525% sodium hypochlorite. In each well, 0.1ml sterile water containing eggs was combined with 0.9ml of either a culture filtrate or a control. Treatment with water and culture filtrates from *Trichoderma* strains grown in PDB. 225 eggs were added per well. Each of the treatments was placed in five wells. Counts were made of total J2 and of mobile J2 in each well after 3-4 days and again after 14 days.

Percentages of immobile J2 were calculated as mean number of immobile J2 / (mean number of mobile + immobile J2) according to Meyer *et al.*, [22].

Final population densities of nematodes were determined and the reproduction factor (RF) was calculated according to Oostenbrink [23].

2.6 Statistical analysis

Data were statistically analyzed using analysis of variance (ANOVA), and treatment means were separated by Fisher's least significant difference (LSD) using SAS (SAS, 2013).

2.7 Randomly Amplified Polymorphic DNA (RAPD)

The RAPD-PCR method can be applied to detect polymorphisms in a wide variety of organisms using variety of different primers.

2.8 Genomic DNA extraction

Mycelium of four *Trichoderma asperellum* strains KC898190, KC898191, KC898192 and KC898193 which were previously isolated as a new isolates of *T. asperellum* in Egypt [20] collected from 7 days old culture plates and 250mg mycelium were used to extract DNA using the Wizard® Genomic DNA Purification Kit (Promega, USA) according to manufacturer's instruction.

2.9 RAPD-PCR reaction

The concentration of DNA of *T. asperellum* samples was different. The optimal results were obtained by using 50ng of DNA template. Six random, 10-mer primers (Table 1) were used in the detection of polymorphism among the four *T. asperellum* strains. PCR reactions were conducted according to Williams *et al.*, [24]. The amplification reaction was carried out in

25µl reaction volume containing 1x PCR buffer, 1.5mM $MgCl_2$, 0.2mM dNTPs, 0.5µM primers, 2µ/ 25µl Taq DNA polymerase and 100ng template DNA. Twenty-five µl of light mineral oil were layered on top of reaction mixture for each of genomic DNA samples. Each cycle consisted of a denaturation step at 94°C for 1 min, an annealing step at 36°C for 1 min and an elongation step at 72°C for 2 min. The primer extension segment was extended to 7 min at 72°C in the final cycle, then 4°C. The PCR products were checked by electrophoresis on 1.2% agarose gel and stained with ethidium bromide for visualization under UV light. One kb ladder DNA molecular weight marker (250-10000 bp) was used.

Table 1. Sequence of the six arbitrary primers used in RAPD-PCR.

S. No.	Primer code	Sequence 5' to 3'
1	**OPA-01**	CAGGCCCTTC
2	**OPA-03**	AGTCAGCCAC
3	**OPA-05**	AGGGGTCTTG
4	**OPA-06**	GGTCCCTGAC
5	**OPA-09**	GGGTAACGCC
6	**OPA-10**	GTGATCGCAG

2.10 Statistical analysis of RAPD data

The NTSYS-pc (Numerical Taxonomy and Multivariate Analysis System, Applied Biostatistics Inc., Setauket, New York) computer program was used for data analysis. The banding patterns generated by RAPD-PCR analyses were compared to determine the genetic relatedness of *T. asperellum* strains. The presence of an amplified band (amplicon) in each position was recorded as 1 and the absence as Sneath and Sokal, 1973. The dendrograms was constructed by The UPGMA (Unweighted Pair-Group Method Using Arithmetic Averages).

3. Results and Discussion

In order to detect the efficacy of the nematicidal activity of *Trichoderma*, twenty strains of four nematode-trapping fungi were tested *in vitro* against root-knot nematode *Meloidogyne javanica*.

3.1 The effect of *Trichoderma* spp. filtrates on eggs of *M. Javanica*

Antagonism against eggs of *M. Javanica* by each *Trichoderma* strain was evaluated using EPI numbers (Egg-Parasitic Index) which was calculated by dividing the number of hatched larvae in the presence of *Trichoderma* by the number of hatched larvae in the axenically plated control (225 egg). EPI numbers varied among species and strains between 0.13-0.73.

EPI of *T. asperellum* strains were lowest from 0.13 to 0.18, *T. koningii* was 0.23 to 29, *T. viride* was 0.36 to 0.39 and *T. harzianum* strains was the highest by 0.23 to 0.73 EPI.

T. asperellum strains parasitized the most eggs during the time-course of the examination with hatching reduction rate by 82.6 to 88%. These data have shown that *T. asperellum* strains have the innate ability to parasitize nematode eggs (Table 2).

T. asperellum was able to adhere and immobilize of 68.7 and 59.4% of eggs and J2 of *M. incognita* in bioassays 1 and 2, respectively (Table 2). Sharon *et al*., [4] showed that eggs adhered with *Trichoderma* conidia became non-viable, thus decreasing the eclosion rate. Furthermore, Sharon *et al*., [3] carrying out *in vitro* bioassays, verified that extracts of *T. asperellum* released in soil were capable to immobilize J2 of *M. javanica* and reduce egg viability. These results suggest that there are both mechanisms of fungal parasitism and lethal activity of secondary metabolites on nematodes.

Table 2. Efficacy of *Trichoderma* spp. filtrates on egg hatching of *M. incognita*.

S. No.	Treatment	Egg hatched (of 225 eggs)	EPI	% of Hatching reduction
	Control	167		
1	***T. harzianum* (T1)**	86	0.51	48.5
2	***T. harzianum* (T2)**	117	0.70	29.9
3	***T. asperellum* (T3)**	20	0.12	88.0
4	***T. harzianum* (T4)**	73	0.44	56.3
5	***T. asperellum* (T5)**	27	0.16	83.8
6	***T. asperellum* (T6)**	21	0.13	87.4
7	***T. harzianum* (T7)**	53	0.32	68.3
8	***T. harzianum* (T8)**	50	0.30	70.1
9	***T. asperellum* (T9)**	29	0.17	82.6
10	***T. harzianum* (T10)**	47	0.28	71.9
11	***T. harzianum* (T11)**	103	0.62	38.3
12	***T. harzianum* (T12)**	53	0.32	68.3
13	***T. koningii* (T13)**	47	0.28	71.9
14	***T. koningii* (T14)**	37	0.22	77.8
15	***T. harzianum* (T15)**	37	0.22	77.8
16	***T. koningii* (T16)**	37	0.22	77.8
17	***T. harzianum* (T17)**	43	0.26	74.3
18	***T. koningii* (T18)**	40	0.24	76.0
19	***T. viride* (T19)**	63	0.38	62.3
20	***T. viride* (T20)**	57	0.34	65.9

3.2. Efficacy on 2nd stage larvae of *M. javanica*

All the tested strains of *Trichoderma* had significant efficacy on *M. javanica* compared to control treatment. *T. asperellum* proved to be the most effective among all examined *Trichoderma* strains on 2nd juvenile larvae (Table 3). They triggered 79.64, 76.07, 70.36 and 61.78% mortality after 48 hours. Furthermore, *T. harzianum* strains were also highly effective and had fast mortal effect, followed *T. koningii* while *T. viride* was the lowest effective one.

Among the *Trichoderma* species examined to their ability to kill plant-parasitic nematodes, mostly *T. asperellum* and *T. harzianum* has been tested [3]. Furthermore, many publications report interactions between plant-parasitic nematodes and soil fungi; however, most of the studies were performed on *Meloidogyne* species [26]. Among other *Trichoderma* species of this study, *T. asperellum* strains have caused significant reduction of *Meloidogyne* spp.

T. asperellum is a ubiquitous free-living mycoparasite that can be applied directly to the soil or on seedlings, associated or not to organic compounds, aiming at the control of soil-borne fungal diseases in protected crops. Moreover, the increase in crop yield provided by *T. asperellum* and its nematicidal effect add advantages to its use. Based on the results found in this study, we conclude that *T. asperellum* can be used as a bionematicide in the integrated pest management of *Meloidogyne* species especially *M. javanica* in protected horticultural crops, but not as a stand-alone control measure.

3.3. Molecular diversity for *Trichoderma asperellum* strains using RAPD Analyses

Total of 6 random primers were used in the present study to identify 4 strains of *T. asperellum*. These primers generated a number of amplified DNA fragments ranging from 5 to 13 amplicons and the size of amplified fragments ranging from <200 to >1500bp with different primers, (Fig. 1). Moreover, primer OPA-05 amplified the highest number of amplicons (13) among *Trichoderma* strains, while the lowest number was 5 amplicons with the primer OPA-06. The number of polymorphic amplicons ranged from 2 amplicons from primer OPA-06 to 13 amplicons from primer OPA-05. As shown in Table 4. The applicability of the method for determining genome similarities among *Trichoderma* strains was investigated by performing cluster analysis on the RAPD data. The UPGMA dendrogram generated from the similarity values is shown in Fig. 2. This dendrogram grouped the four *Trichoderma* strains into 2 main clusters, the first cluster contained one strain (T5). On the other hand, the second cluster was divided into 2 main subclusters; the first one contained strain T9, while the 2nd subcluster contained the other strains in one group contained strain T3 and T6. The close relationships between *Trichoderma* strains T3 and T6 suggesting close similarity of each of the 2 genotypes.

Table 3. Comparative effects of *Trichoderma* filtrates on mortality of *Meloidogyne javanica*.

Treatment	After 24 hrs	After 48 hrs	Mean
Control	0.00^{f}	0.00^{f}	0.00^{f}
***T. harzianum* (T1)**	34.64^{bcdef}	39.29^{abcdef}	36.97^{abcde}
***T. harzianum* (T2)**	4.64^{ef}	38.22^{abcdef}	21.43^{edf}
***T. asperellum* (T3)**	41.79^{abcdef}	79.64^{a}	60.72^{a}
***T. harzianum* (T4)**	1.07^{f}	33.21^{bcdef}	17.14^{ef}
***T. asperellum* (T5)**	38.22^{abcdef}	76.07^{ab}	57.14^{ab}
***T. asperellum* (T6)**	39.29^{abcdef}	61.78^{abcd}	50.535^{abc}
***T. harzianum* (T7)**	28.57^{cdef}	35.72^{bcdef}	32.14^{bcde}
***T. harzianum* (T8)**	31.07^{cdef}	36.79^{abcdef}	33.93^{abcde}
***T. asperellum* (T9)**	35.72^{bcdef}	70.36^{abc}	53.04^{abc}
***T. harzianum* (T10)**	33.93^{bcdef}	39.29^{abcdef}	36.67^{abcde}
***T. harzianum* (T11)**	20.36^{def}	58.21^{abcd}	39.29^{abcde}
***T. harzianum* (T12)**	22.50^{def}	39.29^{abcdef}	30.90^{bcde}
***T. koningii* (T13)**	20.71^{def}	48.93^{abcd}	34.82^{abcde}
***T. koningii* (T14)**	33.21^{bcdef}	53.57^{abcd}	43.39^{abcde}
***T. harzianum* (T15)**	34.65^{bcdef}	56.07^{abcd}	45.36^{abcd}
***T. koningii* (T16)**	29.64^{cdef}	45.36^{abcde}	37.50^{abcde}
***T. harzianum* (T17)**	33.21^{bcdef}	41.79^{abcdef}	37.50^{abcde}
***T. koningii* (T18)**	20.36^{def}	36.79^{abcdef}	28.57^{cde}
***T. viride* (T19)**	25.00^{def}	48.93^{abcd}	36.97^{abcde}
***T. viride* (T20)**	26.07^{def}	34.64^{bcdef}	30.3^{6bcde}
Mean	26.412	46.378	

Trichoderma sp. was treated by 1ml fungal filtrate/ 280 larvae

Data are means of five replicates. Means, followed by the same letter are not significantly different ($P \leq 0.05$).

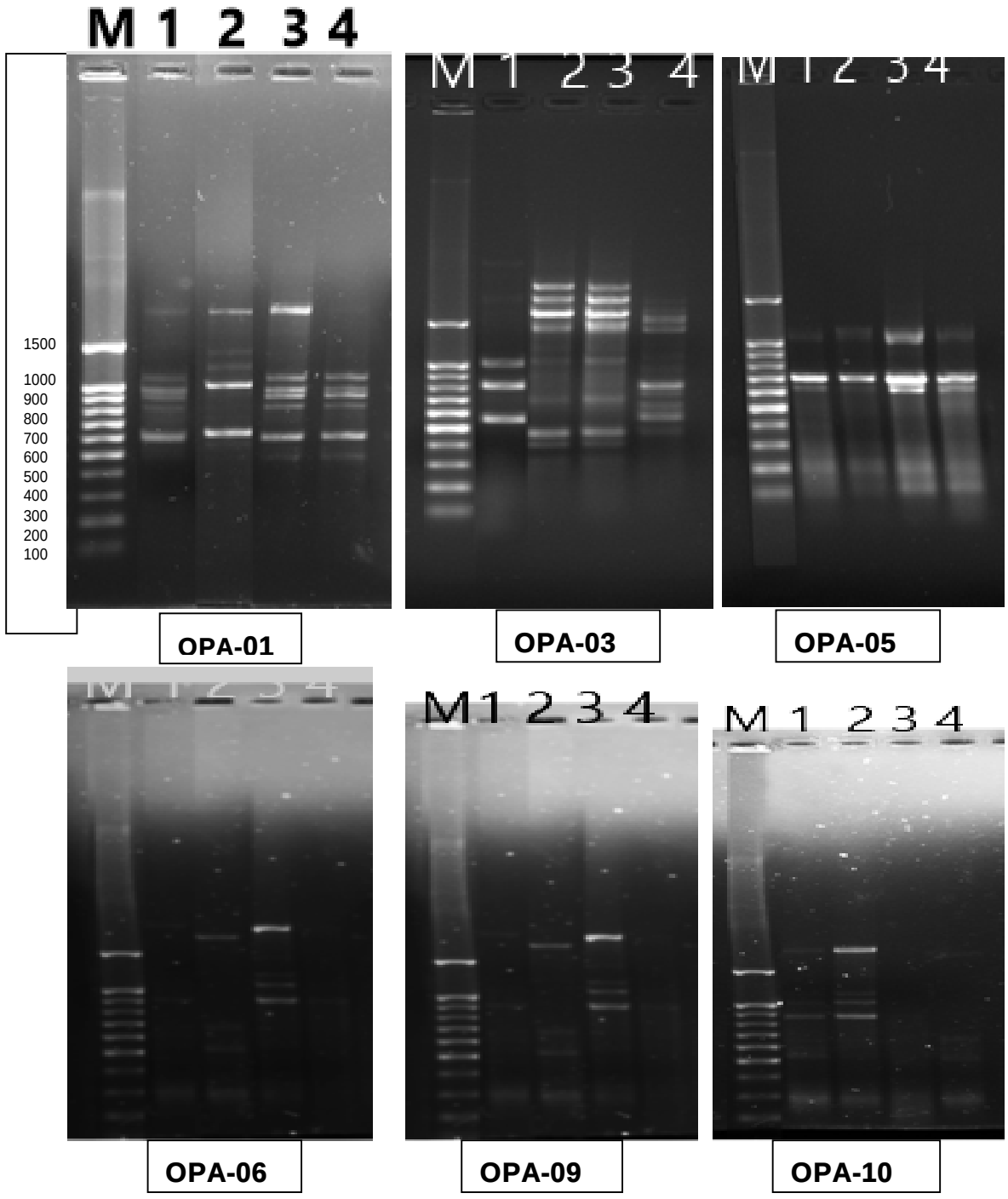

Fig. 1. RAPD–PCR patterns of four *Trichoderma asperellum* strains using six primers. Lane 1-4 represents strains T_3, T_5, T_6 and T_9, respectively. Lane M represents the molecular size marker (0.1 kb leader).

Table 4. Amplified bands obtained with RAPD markers between the four *Trichoderma asperellum* strains.

Primers	Total bands	Polymorphic	Monomorphic	Polymorphism %
OPA-01	11	11	0	100
OPA-03	12	11	1	91.7
OPA-05	13	13	0	100
OPA-06	5	2	3	40
OPA-09	9	9	0	100
OPA-10	7	6	1	85.7

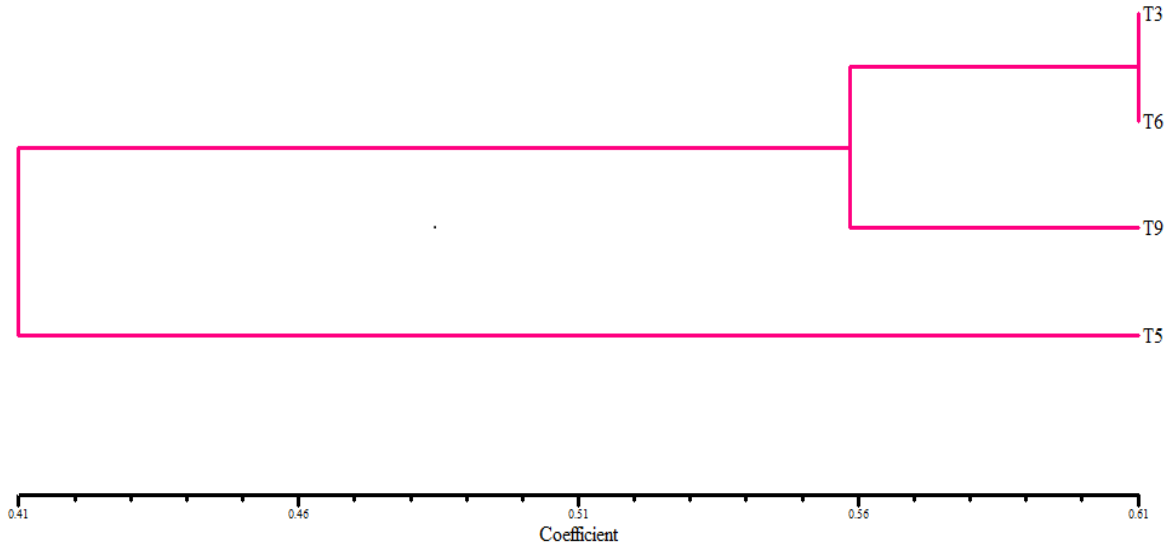

Fig. 2. Dendrogram between four *Trichoderma asperellum* strains using RAPD–PCR and using six Primers.

In RAPD technique, arbitrary short oligonucleotide primers targeting unknown sequences are used to generate amplified products that often show polymorphism within species [24,27]. Gopal *et al.*, [28] were used a random amplified polymorphic DNA (RAPD) marker to estimate the genetic variation among 17 strains of *Trichoderma*. Pandya *et al.*, [29] were found that by using RAPD analysis all the six native strains of *Trichoderma* species moderately (more or less) similar at molecular level. Sagar *et al.*, [30] was reported that the results of RAPD-PCR indicating their genetic diversity has opened new possibility of using the most efficient and more strains of *Trichoderma* in the preparation of effective biopesticide.

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