Research Article

Genetic Relatedness among the Indigenous Isolates of *Trichoderma harzianum*, using RAPD and their Nematocidal Capabilities against *Meloidogyne javanica*

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Abstract | *Trichoderma harzianum* is a well-known fungus and is being used as bio control agent for the management of important plant pathogens including root knot nematodes (RKN). In the present studies, 15 isolates of *Trichoderma harzianum* were collected from different the root knot nematodes-infested tomato field of Malakand and Swat, Pakistan. These isolates were tested for their nematocidal effects against *Meloidogyne javanica*. Anti-J2 antagonistic test divided the isolates into 5 antagonistic groups or AGs. AG1 was observed as the most antagonistic group, causing an average of 66.6% J2 mortality. Genetic variability among these isolates, was studied at molecular level by randomly amplified polymorphic DNA procedure. A selected set of three random primers was used for this purpose, which resulted in 113 bands on gel for the 15 isolates of *T. harzianum*. The analysis of the bi-variate data, using Unweighted Paired Groups Method Averages, divided the indigenous isolates of *Trichoderma,* into four well-defined clusters. These results clearly revealed that there were considerable genetic differences among the Trichoderma isolates. While, there was no correlation, found between RAPD clusters and aggressive groups (AGs). Isolates belonging to different AGs were found scattered among the four RAPD clusters. It was concluded that the indigenous isolates of Trichoderma harzianum were found effective against root knot nematodes and there was a significant difference among these isolates, at the molecular level.

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Introduction

Root knot nematodes (RKN) are important endoparasites of plants and are included in the list of destructive pests of agricultural crops. They are worldwide in distribution, have very wide host ranges and may cause 50-80% losses in vegetable crops (Walker, 1983). Control of these nematodes is very difficult. Chemical control is being considered the strategy for the management of plant diseases all over the world. However, chemicals besides being very expensive are a great risk to environment safety. Because of the public concerns about chemicals, many nematicides have been banned or their application is

restricted (Wesemael et al., 2011). Consequently, alternative approaches such as sanitation, solarization of soil, crop rotation, organic farming, use of resistant varieties and bio control strategies are encouraged (Collange et al., 2011).

Bio-control agents especially fungi from genera Trichoderma and Purpureocillium are very promosing for the control of RKN (Wilson and Jackson, 2013; Affokpon et al., 2011). *Trichoderma harzianum* can be used effectively against many plant pathogens. This fungus has been studied against various pathogens, including the phytonematodes (Saifullah and Thomas, 1996; Dos Santos et al., 1992; Rao et al., 1997; Meyer et al., 2000; Sharon et al., 2001; Goswami et al., 2006; Naserinasabi et al., 2011; Izuogu et al., 2014; Murslain et al., 2014). The possible mechanisms, involved for bio-control activity of *T.harzianum* including; antibiosis, parasitism, competition and enzymatic hydrolysis (Elad, 1995; Saifullah and Thomas 1996). *Trichoderma* spp. are found in all types of agriculture fields (Siddiqui and Shaukat, 2004 a, b) and are not reported to be harmful to human life, wild life and other beneficial soil microbes (Monte and Llobell, 2003). Several commercial preparations of *T. harzianum* such as KRL-AG2 (Plant Shield®), Binab-T®, Trichoderma-2000 and Trichodex®, are available in the market (Samuels, 1996). The ability of *Trichoderma* isolates to control RKN is expected to vary, may improve the bio-control efficiency of the fungus, by selecting the effective isolates, which is also adaptable to local geographical conditions. The differences in anti-nematode antagonistic ability of *Trichoderma harzianum* isolates could be correlated to their intra-species genetic variations.

To show genetic differences among the isolates of *T. harzianum*, several researchers, (Goodwin et al., 1991; Guthrie et al., 1992; Zimand et al., 1994; Arisan-Atac et al., 1995; Ranga Rani et al., 2017) have used randomly selected markers, such as the products of PCR-based Randomly Amplified Polymorphic DNA. Because of being simple, cost effective, speedy and effective to study diversity in pathogen populations, RAPD-PCR has been popular among the researchers (Jawhar et al., 2000; Hsiang and Wu, 2000). Factors that can make the reproducibility of RAPD fingerprints somewhat difficult, include; the reagents used in the PCR, the thermo cycler (Tyler et al., 1997) and the sharpness of the bands (Skroch and Nienhuis, 1995). However, under the well-established protocols, the results may be made reproducible further (Mello et al., 2008). Using Trichoderma as bio-control agent to control Fusarium wilt, Gupta et al. (2010) successfully differentiated the seven Trichoderma isolates they used through RAPD-PCR. They reported that the isolates were 61.84% similar and their RAPD results were reproducible.

Fujimori and Okuda (1993) studied the taxonomic relatedness of 74 Trichoderma strains, by examining their RAPD profiles. They found that the RAPD results were more consistent with the ecological, physiological and morphological parameters of the strains understudy. Schlick et al. (1994) used the RAPD to characterize various strains of *T. harzianum* and their gamma-induced mutants, and concluded, that this method was found useful for the identification of the strains. Employing RAPD markers to study natural genetic variation, present among different strains of Trichoderma, Gomez et al. (1997) were able to classify 14 isolates of the fungus into various groups, on the basis of their antagonistic capacity. In an attempt to relate the ability of the Trichoderma isolates against *Rhizoctonia solani* against their RAPD groups, Goes et al. (2002) found no correlation between isolates polymorphism and their origin, hardness and substrata. In another study, 25 selected isolates of Trichoderma were evaluated for the control of *Fusarium oxysporum* f.sp. *dianthi*, which cause carnation wilt, as well as their RAPD profiles were studied (Shanmugam et al., 2008). It was concluded that the genetic diversity in these isolates was not correlated at the antagonistic levels.

Root knot nematodes among the important plant pathogen in Pakistan and *T. harzianum* is found in most agricultural fields of Pakistan (Siddiqui and Shaukat, 2004 a; b). Therefore, we attempted to find isolates of *T. harzianum,* effective against root knot nematodes and to correlate the antagonistic ability of these isolates to their genetic differences. In this paper, we report the anti-nematode antagonistic ability of the selected *T. harzianum* isolates, which were collected from two different geographical areas of Pakistan and their possible genetic diversity, using PCR-RAPDs.

Materials and Methods

Isolation and identification of Trichoderma harzianum

Random samples were collected from 15 different localities of Khyber Pakhtunkhwa, Pakistan that included Jabban (Th-1), Heroshah (Th-2), Sakhakot

(Th-3), Dargai (Th-4), Dobandi (Th-5), Wartair (Th-6), Thana (Th-7), Zarakhela (Th-8), Shamozai (Th-9), Kabal (Th-10), Sharifabad (Th-11), Barikot (Th-12), Matta (Th-13), Charbagh (Th-14) and Mingora (Th-15). Isolation of *T. harzianum* from soil samples was made by suspension method as reported by Papavizas and Lundsden (1982). Bulk sample from each field was thoroughly mixed, homogenized and air dried. Soil suspension was then prepared by adding 1g of air-dried soil to 199 ml sterile tap water (1:200 dilutions) and hand stirred for 1-2 minutes. One ml aliquots were removed from soil suspension and spread over *Trichoderma* selective media (TSM). One liter of TSM included; $MgSO_4.7H_2O$ (0.2g), K2HPO4 (0.9g), KCl (0.15g), NH_4NO_3 (1.0g), Glucose (3.0g), Rose Bengal (0.15g), Agar (15g), Chloramphenicol (0.25g), Captan-500 (0.2g), Ridomil 273-50 (1.6g), as reported by Askew and Laing (1993). The cultures were then incubated at 25^{0}C for seven days. For the species identification of the bio-control fungus, pure cultures of the isolates, were sent to the Museum of Natural History Islamabad, Pakistan.

Antagonistic capacity of T. harzianum isolates

Small blocks of 2mm diameter, from 10-day old cultures of each *Trichoderma* isolate, were transferred to 250 ml Erlenmeyer flasks containing PDB (Difco) and incubated at 25^{0}C at 200 rpm in rotary shaker for 10 days. The fungal bio-mass was obtained by centrifugation for ten minutes at 12000 rpm. The liquid cultures were vacuum-filtered through Wattman No.1 (0.2 um) twice (Meyer et al., 2004). The culture filtrates were diluted to 75%, 50% and 25% of the original by adding sterilized water (Zareen et al., 1999). To see the effect of Trichoderma culture filtrates on nematode J2s, poured 2ml culture filtrates into each micro-well containing 50 J2s. Micro-wells, filled with 2ml sterile distilled water, served as control. These J2s were hatched from surface sterilized eggs placed in a sterile chamber. The treatments were arranged in CRD with 10 replications. After 48 hr, active and in-active J2s were counted. Water was added into treatments and motile J2s were counted again the next day. Juveniles, without any movements, when probed by fine needle, considered as dead (Siddiqui and Shaukat, 2004). Percent J2 mortality for each micro-well was calculated by dividing the dead J2s over the total J2s in that well, multiplied by 100. The trials were repeated twice and the values were combined for statistical analysis.

Extraction of template DNA

The isolates of *T. harzianum* were cultured on PDA (Difco). For DNA isolation, 0.05g fungal mycelium of each isolate, from the 7-days-old culture was added to 1.5ml eppendorf tube, put in liquid nitrogen and then homogenized in the tube with a glass rod. DNA was extracted from these preparations according to the method of Bullat et al. (1998) i.e. 500µl of DNA extraction buffer that contained Tris-HCl (0.5M), EDTA (0.5M) and NaCl (1.5M), at 7.8pH, was added to the samples. Phenol, Chloroform and Isoamyl alcohol were also added, at 25:24:1 ratio, respectively. The mixture was hand-shaken and the aqueous suspension was centrifuged for 5 minutes at 5000 rpm. The pellets were discarded and the supernatants were transferred to other tubes. The DNA precipitation process was carried out by adding 10% 3M Sodium Acetate with the same volume of concentrated isopropanol. The resultant precipitate was rinsed twice with ethanol (70%), dried at 37°C and suspended in TE buffer (1mM Tris-HCl and 0.1 mM EDTA at neutral pH 7.8). The DNA concentration was measured at 260 nm absorbance and the final concentration of DNA was adjusted at 50-100 ng/µl.

PCR amplification and RAPD analysis

The PCR was performed according to the protocols of Saitoh et al. (2006). The amplification conditions using thermo cycler (Gene Amp 2700) are presented as follows: Denaturing at 94°C for 2min, 30 cycles for each sample, of 94 °C for 30 second, followed by 57 °C for 30 second, 72 °C for one minute, and final extension for 7 minutes at 72 °C.

The RAPD DNA markers (Gene Link Tech, USA), were used in order to study the genetic diversity. The three primers used were; GLD-17, GLG-20, and GLD-08 with sequences TTTCCCACGG, TCTCCCTCAG, and GTGTGCCCCA, respectively. The amplification reactions were carried out in micro centrifuge tubes, in 25 µl reaction volume, which contain 17µl reaction mixture with 1µl template DNA, 1µl RAPD primers, 9µl ddH_2O and 5µl PCR mix. The PCR mix comprised of 460µl H_2O, 500µl buffer, 10µl dNTPs and 300µl $MgCl_2$). The PCR reagents were; 15µl ddH_2O, 7µl PCR mix, 1µl Taq, 1µl DNA, 1µl random primer, making it 25µl.

Analysis of the data

The PCR amplification products were resolved by loading 10µl of the reaction mixture in 1.2% Agarose

(Sigma) gel, followed by their electrophoresis in 1X Tris-borate-EDTA at 8.0 pH) buffer at 6 V. DNA ladder (1-kb), was run as molecular marker, for comparison. The images of ethidium bromide-stained gels, visualized with ultra violet (UV) light, were saved and transferred to computer. The polymorphic bands were observed on the gel that contains the amplified DNA of Trichoderma isolates. The presence of bands was recorded as 1, while the absence was recorded 0. The 1-0 bi-variate data matrix was thus, generated accordingly. By using reproducible bands, the data matrix (1-0 bi-variate) for every set of *T. harzianum* isolates, on the basis of three RAPDs, was used for the construction of a dendrogram. The absence or presence of the similar molecular size bands was considered to be 2 alleles on single locus and the difference in bands was recorded as a polymorphism. The data, obtained from the RAPD primers, were subjected to analysis to check genetic differences among the Trichoderma isolates, using the following equation;

$$F=2Nxy/Nx + Ny$$

Where F is the similarity co-efficient, Nxy represents the total bands, shared by x and y isolates, Nx indicates the bands present in isolate x, while Ny represents the presence of bands in y isolate (Nei and Li, 1979). The analysis of cluster was performed by the using Genetyx software, Un-weighted Pair-Group Method with Arithmetical Average or simply UPGMA, in which A is substituted by 1 and T is substituted by 0. The resultant clusters are given in the form of a dendrogram.

Results and Discussion

Anti-J2 antagonocity of T. harzianum isolates

The means of the un-diluted culture filtrates of the 15 *T. harzianum* isolates as well as those of the different dilutions differed significantly ($p \leq 0.5$) from each other and from the control (Table 1). However, after the data analysis, the 15 isolates of *T. harzianum* were found to be divided into five antagonistic groups (AGs). Members of the AG-1 were Th-1, Th-2, Th-3, Th-9 and Th-15, which exhibited maximum level of antagonistic ability against J2s of *M. javanica* (killing an average of 66.16% J2s) followed by those of AG 2, AG 3, AG 4, and AG5 respectively.

RAPD analysis of T. harzianum isolates

A total of 13 random primers (Gene link Tech, USA), were screened for the amplification of polymorphic bands of the 15 isolates of *T. harzianum*. However, only 3 primers were chosen for detailed studies as they produced reproducible results. Figure 1 shows that 113 bands were produced with the three primers designated as D-17, G-20 and D-08. Genetic polymorphism was observed, at different levels, for all the genotypes in the loci, detected by the primer D-17. In all the 15 isolates, only 31 bands were recorded, with 2.06 bands per genotype as average. Similarly, all the 15 isolates of *T. harzianum* (tested in this study), showed genetic variability in the loci when primer G-20 was used. Using primer D-08, 49 bands were observed, giving an average of 3.27 bands per isolate.

Table1: *Effect of the culture filtrates of Trichoderma harzianum isolates on Meloidogyne javanica J2 and grouping of the bio-control agent as determined by % J2 mortality test.*

Isolate	Locality	Mean% J2 Mortality[1]	AGs[2]
Th-1	Jabban	66.73a	AG 1
Th- 9	Shamozai	66.57ab	AG 1
Th-2	Heroshah	66.47abc	AG 1
Th-15	Mingora	65.57a-d	AG 1
Th-3	Sakhakot	65.47a-d	AG 1
Th-4	Dargai	65.37b-e	AG 2
Th-6	Wartair	65.13c-f	AG 3
Th-5	Dobandi	65.10c-f	AG 3
Th-7	Thana	64.70def	AG 4
Th-12	Barikot	64.70def	AG 4
Th-8	Zarakhela	64.67def	AG 4
Th-13	Matta	64.27def	AG 4
Th-14	Charbagh	64.13ef	AG 5
Th-10	Kabal	64.03f	AG 5
Th-11	Sharifabad	64.00f	AG 5

LSD value for isolates: *1.301;* [1]*Means % J2 mortality;* [2]*Antagonistic groups AG1, AG2, AG3, AG4 and AG5.*

The dendogram, constructed from RAPD analysis of Trichoderma isolates, divided the 15 isolates of Trichoderma into four groups as shown Figure 2. Group-1 (AG-1) was comprised of the five isolates; Th-5, Th-8, Th-2 and Th-12 and out of which only Th-2 belongs to the highly antagonistic group, AG1. The second RAPD group consists of six isolates (Th-3, Th-9, Th-13, Th-6, Th-14, and Th-1). In this case, only Th-1 and Th-3 fall in AG1 whereas the rest of the isolates are scattered among different AGs (Table 1). The third cluster consists of Th-10 and Th-15

while the fourth one is made up of Th-4, Th-7 and Th-11, where all of which represent different antagonistic groups.

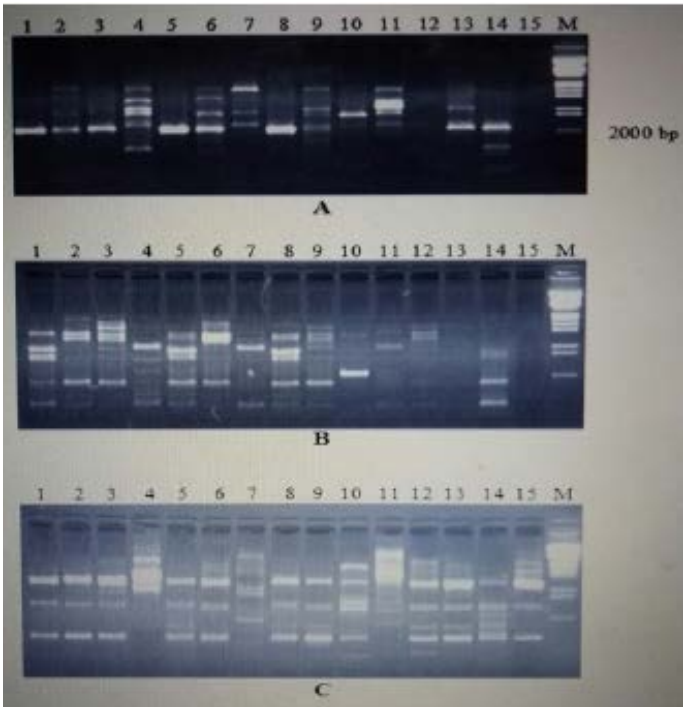

Figure 1: *PCR profile of T. harzianum isolates using RAPD primers: A. GLD-17, B.GL-G 20, C. GL-D08.*

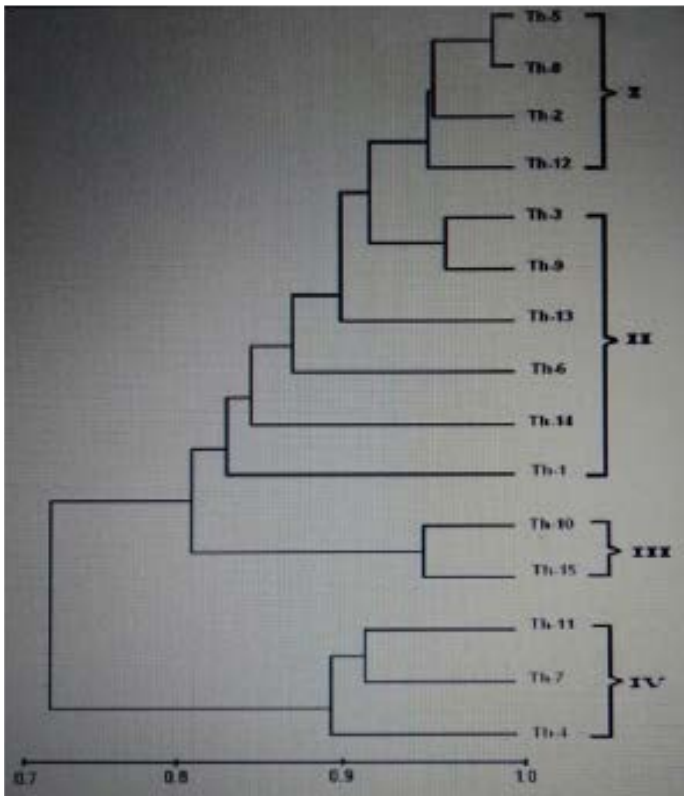

Figure 2: *Dendrogram (with three RAPD primers) showing genetic similarities among 15 isolates of T. harzianum using the unweighted pair group method with arithmetical average (UPGMA) of Genetyx software, (version 7.0). RAPD clusters are indicated in the right.*

The statistically significant differences in the abilities (to kill nematode J2s) of the members of different AGs suggests that there is intra-species genetic variation in *Trichoderma harzianum*. Similar results have been reported by Shanmugam et al. (2008), who found their *T. harzianum* isolates to be divided into 8 sub-clusters with a maximum of 35% dis-similarity among the isolates. Goes et al. (2002) also reported that Trichoderma isolates were considered aggressive against *Rhizoctonia solani*. They observed about 45% dis-similarity among the Trichoderma isolates. The intra-specific genetic variation is evident from our results as well (Figure 2). However, no close relationship was observed between our AGs and the geographic origin of the isolates. Members of any single antagonistic group were not found to be concentrated in a particular region. However, the most antagonistic or the least antagonistic Trichoderma isolates are seemed randomly distributed in the various tomato-growing regions that we used for soil sample collection. Similar findings were provided by several other researchers like Arabi and Jawhar (2007), Jawhar and Arabi (2009). This may be because of the genotype-isolate interactions, in which different virulent genes operate in a pathosystem as reported by Van der Plank (1984).

Likewise, the various RAPD groups of our dendrogram also did not correlate with the different antagonistic groups (AGs) of the bio-control agent or with the origin of the isolates. Members of the different antagonistic groups (AG1-AG5) shared the same RAPD clusters. Isolates belonging to different RAPD clusters were found to be scattered among the different regions used for soil sample collection. Other researchers like Goes et al. (2002), Shanmugam et al. (2008), also described that there was no correlation observed between the genetic polymorphism of the isolates and the aggressiveness they show. This lack of correlation in between the AGs of *T. harzianum* isolates and their phylogenetic structure could be because the isolates might be derived from similar source or the isolates may have recently evolved from the non-antagonistic isolates (Arabi and Jawhar, 2007). Although, the environment of all the tomato-growing regions (included in our studies) is the same, different tomato cultivars are grown in these areas. The fact that no correlation was found, between the AGs of *T. harzianum* isolates and their origin, suggests that there is no influence of the root exudates of different tomato cultivars on the rhizosphere *T. harzianum* population. Our finding

that some of the *T.harzianum* isolates were highly antagonistic against J2s of root knot nematode is significant because such isolates could be developed into commercial bio-control agents to control the local problem of root knot nematode disease.

Although the RAPD is considered fast, economical and easy method as compared to other techniques, but there is great concern with its reproducibility as reported by Kumari and Thukar (2014). Therefore, a great care should be taken to develop laboratory protocols, so that the results may be made reproducible.

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Author's Contribution

Baharullah Khattak: Designed and conducted research, measured observation and prepared draft.
Saifulla and Shaukat Hussain: Research supervisors, helped in planning the research and gave input during the course of this study.
Musharraf Ahmad and Asad Ali: Helped in review the manuscript.
Mohammad Junaid and Ijaz Ahmad Khan: Helped in manuscript editing and data analysis.
Taj Ali Khan and Mubbashir Hussain: Helped in preparation of methodology.

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