American Journal of Microbiological Research, 2015, Vol. 3, No. 1, 45-49
Available online at http://pubs.sciepub.com/ajmr/3/1/7

DOI:10.12691/ajmr-3-1-7

Development of SCAR marker for Specific Detection of *Trichoderma harzanium* and *Trichoderma viride*

H. J. Parmar[1], M. M. Hassan[3,4,*], N. P. Bodar[1], H .N. Lakhani[1], V. V. Umrania[2], B. A. Golakiya[1]

[1]Department of Biotechnology, College of Agriculture, Junagadh Agriculture University, Junagadh -362001, India
[2]Microbiology Dept, MVM Science and Home Science College, Saurashtra University, Rajkot -360001, India
[3]Scientific Research Center, Biotechnology and Genetic Engineering Unit, Taif University, KSA
[4]Genetics Department, Faculty of Agriculture, Minufiya University, Egypt
*Corresponding author: khyate_99@yahoo.com

Received January 05, 2015; Revised February 05, 2015; Accepted February 11, 2015

Abstract *Trichoderma* have been used as biological control agents against soil borne plant pathological fungi such as *S. rolfsii.* On this day molecular based techniques has been developed to detect *T. harzanium* using a fungus-specific marker derived from genomic DNA. An amplified RAPD product of 220 bp and 900 bp obtained in *T. harzanium* (NBAII Th 1) and *T. viride* (NBAII Tv 2) isolates, respectively. These two RAPD products were obtained using two random primers OPA-16 for *harzanium* (NBAII Th 1) isolate and OPC-05 for *T. viride* (NBAII Tv 2), thin RAPD products were sequenced. Based on sequences, one primers were designed, out of which a primer pair HAR220FP5 (CTTTTGGTTTGACACGGTTCT and HAR220RP5 (AAGCTTTGAAGTTGCGAGGA) amplified a sequence of 220 bp. and VIRI900FP7 (TACGCTCCAGGCTACCACTT) VIRI900RP7 (GAGATGAGCTCCTTGCTGCT) amplified a sequence of 900 bp. which was specific to *T. harzianum* and *T. viride*, respectively. The specificity of the marker when tested against six isolates of *Trichoderma* species showed a specific band of 220 bp. only in *T. harzanium* and a specific band of 900 bp. only in *T.viride* with the optimized PCR parameters. This sequence characterized amplified region (SCAR) marker was sensitive and could detect small quantities of *Trichoderma* DNA as low as 25 to 30 ng with high efficiency. This marker could also clearly distinguish *T. harzianum* and *T. viride* from other isolates of *Trichoderma*.

Keywords: *T. harzanium, T. viride, random amplified polymorphic DNA (RAPD), sequence characterized amplified region (SCAR) marker.*

Cite This Article: H. J. Parmar, M.M. Hassan, N. P. Bodar, H .N. Lakhani, V. V.Umrania, and B. A. Golakiya, "Development of SCAR marker for Specific Detection of *Trichoderma harzanium* and *Trichoderma viride*." *American Journal of Microbiological Research*, vol. 3, no. 1 (2015): 45-49. doi: 10.12691/ajmr-3-1-7.

1. Introduction

Antagonistic microorganisms, such as *Trichoderma*, reduce growth, survival or infections caused by pathogens by different mechanisms like competition, antibiosis, mycoparasitism, hyphal interactions and enzyme secretion. In addition, the release of biocontrol agents into the environment has created a demand for the development of methods to monitor their presence or absence in soil. Therefore, monitoring population dynamics in soil is of much importance. Previous methods employed to identify strains of *Trichoderma* spp. in soil samples have included the use of dilution plates on selective media. However, this method does not distinguish between indigenous strains and artificially introduced ones [1]. The *Trichoderma* isolates were differentiated by mycelia growth rate and colony appearance, as well as microscopic morphological features, including phialides and phialospores [2,3]. These can also be distinguished by randomly amplified polymorphic DNA (RAPD) - PCR, restriction fragment length polymorphisms in mitochondrial DNA and ribosomal DNA and sequence analysis of ribosomal DNA [4,5,6]. Now days, molecular techniques are gaining importance in characterization and diagnosis of microbial population. Molecular characterization and identification of biocontrol isolates of *Trichoderma* spp. has been reported by several researchers [6,7,8]. These techniques are not influenced by environment, independent of growth stage and reproducible when compared to conventional methods and helps in diagnosis of potential biocontrol agents in future. Here RAPD techniques has been utilized to generate unique PCR products in filamentous fungal species or strains of interest to be converted into species or strain specific sequence characterized amplified region (SCAR) markers [9,10,11,12]. SCAR markers differ from RAPD markers in that SCAR primers are designed based on known DNA sequences of the organism of study. This allows for the development of sensitive and diagnostic assays to amplify specific fungal DNA in laboratory cultures as well as field samples containing mixed DNA because primers anneal specifically to fungal sequences.

Thus, the present study was undertaken to assess the significant genetic variations by nucleic acid based marker

techniques using RAPD markers were converted to SCAR markers for development of a sensitive and reliable diagnostic assay for the selective detection of *T. harzanium* and *T. viride*.

2. Materials and Methods

2.1. Sources of *Trichoderma* spp.

Slants of six species of *Trichoderma* (*T. harzianum* NBAII Th 1; *T. hamatum* NBAII Tha-1; *T. koningi* MTCC 796; *T. pseudokoningi* MTCC 2048; *T. virens* NBAII Tvs 12; *T. viride* NBAII Tv 23) were obtained either from IARI, New Delhi or from MTCC, Chandigarh.

2.2. Isolation of Phytopathogenic Fungi

Sclerotium rolfsii was used as pathogen. Groundnut showing typical symptom of stem rot were collected from university fields. The fresh collected diseased plants of groundnut showing stem rot and on the roots were thoroughly washed under running tap water to remove surface soil, dust and other contaminants. Infected root tissues were cut out from the leading edge of lesion, and placed in 1% sodium hypochlorite for five minutes, washed briefly in sterile distilled water and dried on sterile filter paper. The dried pieces were cut into smaller pieces, plated onto PDA and incubated at 28°C. The cultures were purified by hyphal tip method [13].

2.3. DNA Extraction

Genomic DNA from microbes was isolated as method described by Doyle and Doyle [14], with some modification. *Trichoderma* strains and pathogen *S .rolfsii* were grown on PDB media. After 6 days of growth in incubation at 28°C and 120 rpm. mycelia were harvested by filtration (Whatman No. 1), washed successively with cold sterile water. Take 1 gm of fungi mycelia and crush with liquid nitrogen. Add preheated CTAB extraction buffer in a crushed mycelia and mix well. Transfer the mycelia in a centrifuge tube and keep at 65°C in a water bath for 1 h. Centrifuge the tubes at 10,000 rpm for 15 minutes. Take the supernatant and add equal amount of chloroform: isoamyl alcohol (24:1) and centrifuge at 10,000 rpm for 10 minutes. Again take the supernatant and add equal amount of chloroform: isoamyl alcohol (24:1) and mix gently then centrifuge it at 10,000 rpm for 10 minute. Take supernatant and add chilled isopropanol (double volume) and mix slowly to precipitate the DNA. Store the tubes at -4°C for 2-3 h. The DNA precipitate was collected by centrifugation at 14,000 rpm for 30 minutes and pellet were washed once with cold 70% ethanol and air dried for 30 min. The dry DNA pellet was dissolved in 50 µl of TE buffer.

2.4. PCR-RAPD

RAPD analysis was performed according to Shalini *et al.*, [15], using different twenty three Operon primers (Table 1). These 23 random primers were obtained from Bangalore Genei, India. The RAPD-PCR amplification reactions were performed in C1000^{TM} Thermo Cycler Bio-Rad, using the following PCR program: 1 cycle at 94°C, 5 min; 40 additional cycles consisting of 94°C for 45 s, 36°C for 60 s, 72°C for 2 min and final extension 72°C for 5 min. Amplified DNA products were analyzed by electrophoresis in 1.2% agarose gel run in TAE buffer. The gels were stained with ethidium bromide (5 µg ml^{-1}). 1 kb ladder (Bangalore Genie Pvt. Ltd, Bangalore) was used as a standard marker. DNA was visualized and photographed by Gel Doc system (UVitec, Cambridge, England).

RAPD profiles through polymerase chain reaction (PCR) twenty different random primers were screened for generating polymorphism among the isolates under the study. The experiment was repeated twice and results were reproducible. Band position for each isolate and primer combination were scored as either present (1) or absent (0) for phylogenetic analysis using NTSYS-pc (Numerical Taxonomy and Multivariate analysis) system version 2.2 by Exeter software.

The SIMQUALK programme was used to calculate Jaccard;s similarity coefficient and a graphical phenomenon (dendrogram) of the genetic relatedness among fungi was produced by means of the unweighted pair group method with arithmetic average (UPGMA) analysis [16]. Size of specific bands of DNA was determined using software Alpha imaginer 2200 manufactured by Alpha Ease FC, USA. The PCR mixture reagents were used as following.

Content	Quantity	Concentration
Taq buffer (10 x) with 15 mM $MgCl_2$	2.50µl	10 mM Tris 1.5 mM $MgCl_2$
dNTPs mix (2.5 mM each)	2.00 µl	0.2 mM
Taq DNA polymerase (3 U/µl)	0.5 µl	2.25 U
Primer (10 pmoles/µl)	2.00 µl	20 pmoles
Template DNA (25 ng/µl)	2.00 µl	50 ng
Sterile Millipore water	16 µl	
Total Reaction Mixture	25 µl	

2.5. Screening of RAPD Primers

In order to identify polymorphic (distinct) band for the development of SCAR markers, this experiment was repeated and 2 primers were found to give repeatable results. These two primers were further checked for consistency of RAPD banding patterns. PCR was repeated twice to ensure reproducibility and consistency of the banding patterns for each isolate. Two primers which produced consisted patterns were selected for development of the SCAR primer. Polymorphic band obtained from RAPD primers in different species was as following.

Primer	primer sequence	Species
OPA-16	5´-AGCCAGCGAA-3´	*T. harzianum*
OPC-05	5´-GATGACCGCC-3´	*T. viride*

2.6. Purification of PCR Fragments

The intense bands of different base pair were obtained from different strains of *Trichoderma* as well as plant pathogen were excised from the agarose gel with sharp sterile scapel blade by keeping gel on low intensity (70%) UV- trasilluminator. The agarose gel piece containing the fragment was collected in a sterile pre-weighed micro – centrifuge tube. The excised PCR fragments were subjected for Qiagen gel extraction kit to elute DNA from agarose gel. The eluted pure DNA is again re-amplified as shown. Prior to sequencing. The amplified DNA again added with master mix of sequencing kit, and corresponding primers were also added for sequencing.

2.7. Design of SCAR Primer and Sequencing of RAPD Amplicon

SCAR primers of *T. viride* and *T. harzianum* were synthesized against corresponding DNA fragments. Genomic DNA was amplified with these primers in a PCR. Primer sequence of SCAR primer was described. Prior to sequencing, the amplified DNA again added with master mix of sequencing kit, and corresponding primers were also added for sequencing. Reactions of were carried out in 25 µl volume containing 1.6 µl (30 ng) of genomic DNA, 8 µl Master mix, 1.5 µl of primer and deionized distilled water (up to a total volume of 25 µl).

3. Results and Discussion

In the present investigation, amplified products were observed when the genomic DNA of microbes (*Trichoderma* species and *S. rolfsii*) was subjected to RAPD analysis using 23 Random decamer primers. Out of 23 primers 2 primers were failed to give any amplified products of DNA. Possibly this may be due to absence of complementary sequence in the genomic DNA. Thus, 21 out of 23 fungal primers were selected for evaluating molecular differences existing in 6 species of *Trichoderma* which belongs for inhibiting the growth of fungal pathogen *S. rolfsii*. However, molecular characterization of pathogen *S. rolfsii* was also carried out to identify genetic variation and diversity, and compared it with various species of *Trichoderma*. The name and sequences of each primer are given in (Table 1).

3.1. RAPD Analysis

The amplified products generated using a common PCR protocol for all primers were separated on a 1.5% agarose gel matrix. The RAPD profiles visualized presence of high level of polymorphism between the species of *Trichoderma* and pathogen *S. rolfsii* (Figure 3). A total 305 bands were produced by 21 RAPD primers with an average frequency of 14 bands per primer. Total 305 polymorphic bands were generated out of which 266 were polymorphic and shared between at least two individuals, and 39 bands were polymorphic and unique while 3 were monomorphic. Two primer (OPC-13 and OPZ-10) out of the 21 RAPD primers produced monomorphic profiles. Primer OPF-09 generated the maximum 31 bands, whereas OPF-11 generated the lowest with 3 bands. The percent polymorphism furnished by each primer ranged between 50 and 100 (Table 3).

The calculated PIC values for RAPD markers were ranged from 0 to 0.911 (Table 3). The lowest PIC values obtained by OPF-11 and highest was with OPC-13. The present results showed only with 21 decamer random primers, hence screening of more number of primers is recommended to evaluate the present set of *Trichoderma* species effectively. Primer OPC-05 generated unique band of *T. koningii* (500 bp.) and *T. viride* (900bp.), OPA-16 generated unique band of *T. harzianum* (220 bp.) and *S. rolfsii* (500 bp.) and OPA-17 generated unique band of *T. koningii* (900 bp.) and *S. rolfsii* (500bp) which was purified and used to develop SCAR marker for particular species.

Literature showed efficiency of RAPD to identify various *Trichoderma* species and also fungal pathogen through molecular characterization. PCR fingerprinting with two different primers gave patterns that distinguished *T. viride* type-I species from those of type II [17].

Among 21 RAPD primers only two primers were selected for designing the SCAR primers. OPC-05 gave a unique fragment of size 900 bp in *T. viride* species and no amplification in other five species. OPA-16 gave a polymorphic fragment of size 220 bp in *T. harzianum* (Figure 2). The unique fragment of each species was not present in other species and directly sent as purified PCR product for sequencing. The sequences of these species were subjected for BLAST search at NCBI, but none of the sequence showed similarity with data base sequences of *Trichoderema*. SCAR primers were designed by using these sequences and sent for synthesis. The sequences of all the two SCAR markers are presented in (Table 2).

Dendrogram was constructed using the similarity matrix. The similarity coefficient ranged from 0.27 to 0.50 (Figure 2). The dendrogram obtained indicates that there was a major cluster consisting of 6 species of *Trichoderma* of total 7 micobes, whereas one fungal pathogen (*S. rolfsii*) was found to be different from the rest of the *Trichoderma*. The major cluster A and cluster B consisted of 6 species of *Trichoderma* and 1 pathogen *S. rolfsii* and shared 27% similarity. Major cluster A was further divided in to sub-cluster A_1 and A_2 and consisted of 5 and 1 species of *Trichoderma*, respectively, sharing 34.5% similarity. Sub cluster A_1 consist of 5 species of *Trichoderma*, *T. harzianum* and *T. viride* came in sub-sub group and having 43.5% similarity as well as *T. koningii* and *T. virens* having 41% similarity. Sub cluster A_2 consisted of 1 species *T. pseudokoningii* and shared 34.5% similarity with cluster A.

In view of the growth inhibition of fungal pathogen, *T. viride* and *T. harzianum*, which are highest inhibitory species also came in same group of dendrogram which were also in same cluster and shared 43.5% similarity (Figure 2). *T. pseudokoningii* strain remained single in sub sub-clustering and shared 34.5% similarity. Thus, dendrogram showed more or less grouping of the *Trichoderma* species by the level of antagonism.

This kind of tendancy was also observed in biocontrol agent *Pseudomonas fluroescens* which inhibits soil borne *Fusarium oxysporum f.sp.ciceri* [18]. (Kandoliya and Vakhariya, 2013).

3.2. Amplification using SCAR Primer

The designed putatively species-specific SCAR primer pairs for two species of *Trichoderma* (*T. harzianum* and *T. viride*) were used to amplify the genomic DNA of other 5 species A single and sharp 900 bp. band corresponding to the original RAPD fragment was obtained in *T. viride* with VIR-900 SCAR primer and no amplification was observed in the other six species (Figure 3). Similarly, single and bright 220 bp. band corresponding to the original RAPD fragment was obtained in *T. harzianum* with HAR-220 SCAR primer and amplification was not observed in the other six species.

In this study, efforts were made to develop SCAR markers for species identification in *Trichoderma*. The SCAR marker VIR-900 and HAR-220 are unique to *T.*

viride and *T. harzianum* can be used for identification of *T. viride* and *T. harzianum* from unknown culture collection.

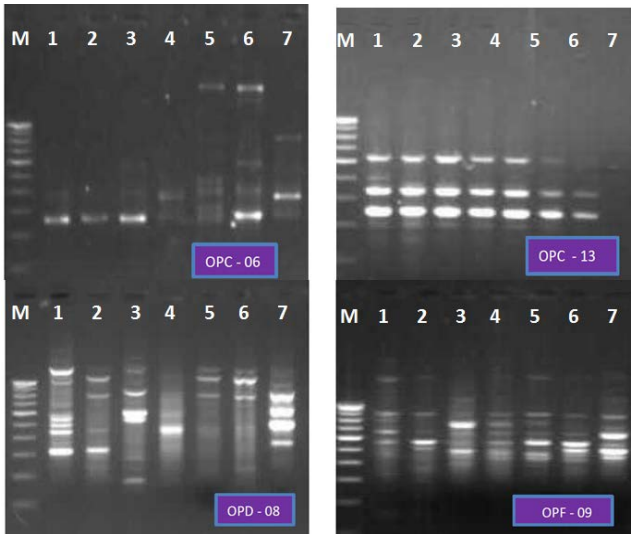

Figure 1. RAPD profile of six *Trichoderma* species and *S. rolfsii* obtained with OPA-06, OPA-08, OPA-09 and OPC-13 primers, 1= T. *harzianum*, 2=*T. hamatum*, 3= *T. koningii*, 4= *T. pseudokoningii*, 5= *T. virens*, 6= *T. viride* and 7= *S. rolfsii*

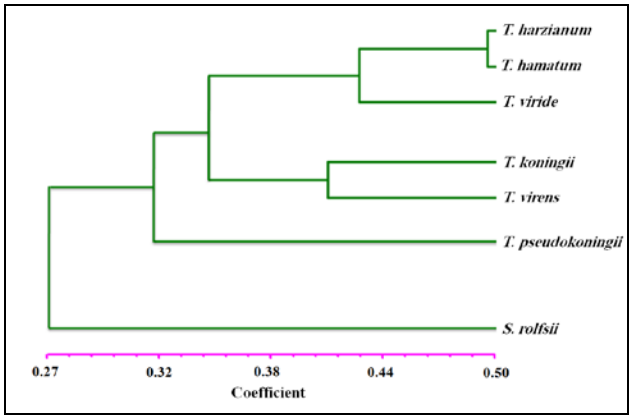

Figure 2. Dendogram depicting the genetic relationship among the six *Trichoderma* species based on the RAPD data

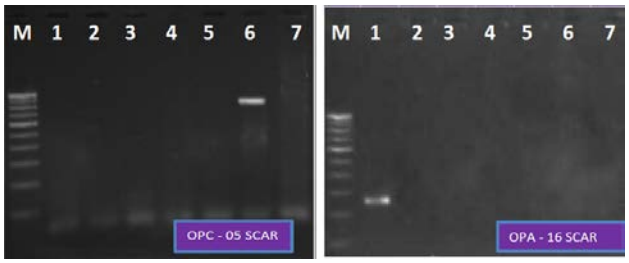

Figure 3. RAPD profile of six *Trichoderma* species and *S. rolfsii* obtained with OPA-16 and OPC-05 primers, 1= T. *harzianum*, 2=*T. hamatum*, 3= *T. koningii*, 4= *T. pseudokoningii*, 5= *T. virens*, 6= *T. viride* and 7= *S. rolfsii*

Table 1. List of primers name and sequence with GC content.

Sr. No.	RAPD Primer	Sequence (5`→3`)	Tm	GC (%)
1	OPA-15	5′-TTCCGAACC -3′	25	60%
2	OPA-16	5′-AGCCAGCGAA-3′	25	60%
3	OPA-17	5′-GACCGCTTGT -3′	25	60%
4	OPB-07	5′-GAAACGGGTG-3′	25	60%
5	OPB-08	5′-GTGACGTAGG-3′	23	60%
6	OPC-04	5′-CCGCATCTAC-3′	27	60%
7	OPC-05	5′-GATGACCGCC-3′	27	70%
8	OPC-06	5′-GAACGGACTC -3′	25	60%
9	OPC-11	5′-AAAGCTGCGG-3′	25	60%
10	OPC-13	5′-AAGCCTCGTC-3′	25	60%
11	OPD-05	5′-TGAGCGGACA-3′	25	60%
12	OPD-08	5′-GTGTGCCCCA-3′	25	70%
13	OPF-09	5′-CCAAGCTTCC-3′	25	60%
14	OPF-11	5′-TTGGTACCCC-3′	25	60%
15	OPG-12	5′-CAGCTCACGA-3′	25	60%
16	OPM-05	5′-GGGAACGTGT-3′	25	60%
17	OPN-04	5′-GACCGACCCA-3′	27	70%
18	OPN-10	5′-ACAACTGGGG-3′	25	60%
19	OPQ-12	5′-AGTAGGGCAC-3′	25	60%
20	OPQ-14	5′-GGACGCTTCA-3′	25	60%
21	OPZ-10	5′-CCGACAAACC-3′	25.0	60%

Table 2. Sequence of SCAR primers used for species identification

Primers	Primer sequences	Species
HAR220FP5	5′-CTTTTGGTTTGACACGGTTCT-3′	*T. harzianum*
HAR220RP5	5′-AAGCTTTGAAGTTGCGAGGA-3′	
VIRI900FP7	5′-TACGCTCCAGGCTACCACTT-3′	*T. viride*
VIRI900RP7	5′-GAGATGAGCTCCTTGCTGCT-3′	

Table 3. Jaccard´s similarity coefficient of six *Trichoderma* species based on RAPD data

Variety	*T. harz*	*T. ham.*	*T.kon.*	*T.psed*	*T.viren*	*T.viride*	*S.rolfsii*
T. harz.	1.000						
T. ham.	0.375	1.000					
T. kon.	0.500	0.571	1.000				
T. psed	0.333	0.571	0.500	1.000			
T. virens	0.500	0.333	0.500	0.125	1.000		
T. viride	0.600	0.363	0.454	0.454	0.300	1.000	
S. rolfsii	0.571	0.111	0.375	0.375	0.333	0.500	1.000

Zimand *et al.*, [19] used the RAPD procedure for the specific identification of T-39 in the *T. harzianum* group. They found that a set of nine primers was necessary to distinguish T-39 from other species of the group. Abbasi *et al.*, [9] stated that three RAPD markers were needed to distinguish *T. hamatum* 382 from other *T. hamatum* species. UP-PCR fingerprinting is another simple and reliable technique that can be used to detect genetic differences even at the strain level. It has proven to be a valuable tool for studying DNA polymorphisms in *Trichoderma* [6,20,21]. By the application of this approach we have identified three UP-PCR markers enabling the detection of a specific *T. harzianum* species which is an effective BCA against the rice sheath blight pathogen *R. solani* [22]. The identified markers discriminate strain AS12-2 from other species of *T.*

harzianum as well as 12 from other *Trichoderma* species. The results indicate the applicability of the UP-PCR method for distinguishing closely related species and for studies of diversity and genetic structure of populations.

References

[1] Knudsen IMB, Jensen B, Jensen DF, Hockenhull J (1996) Occurrence of *Gliocladium roseum* on barley roots in sand and field soil. Kluwer Academic Publishers, Netherlands.

[2] Seaby DA (1996) Differentiation of *Trichoderma* taxa associated with mushroom production. Plant Pathol 45: 905-912.

[3] Shahid M, Singh A, Srivastava M, Srivastava DK (2014) Molecular Characterization of *Trichoderma viride* isolated from rhizospheric soils of uttar pradesh based on rDNA markers and analysis of their PCR-ISSR profiles. J Mol Biomark Diagn 5: 169.

[4] Ospina-Giraldo MD, Royse DJ, Chen X, Romaine CP (1999) Molecular Phylogenetic Analyses of Biological Control Strains of *Trichoderma harzianum* and Other Biotypes of *Trichoderma* spp. Associated with Mushroom Green Mold. Phytopathology 89: 308-313.

[5] Hassan M M (2014). Influence of protoplast fusion between *Trichoderma spp.* enhances extracellular enzymes and antagonistic activity against some soil pathogens. Biotechnology and biotechnological equipments, 28: 1014-1023.

[6] Hassan M M, A Gaber and E I. El-Hallous (2014) Molecular and Morphological Characterization of *Trichoderma harzianum* from different Egyptian Soils, *Wulfenia Journal*, 21: 80-96.

[7] Hermosa, M. R., Grondona, I., Iturriaga, E. A., Dia-MinGuez, Castro, C., Monte, E. and Garcia-Acha, I. 2000. Molecular characterization and identification of biocontrol isolates of Trichoderma spp. Applied and Env. Microbiology. 66 (5): 1890-1898.

[8] Venkateswarulu, R., Reddi Kumar, M., Eswara Reddy, N. P. and Sudhakar, P. 2008. Molecular characterization of Trichoderma spp. used against Fusarium wilt with PCR based RAPD and ITS-PCR. J. Mycology and Plant Pathology. 38 (3): 559-563.

[9] Abbasi P. A., Miller S. A., Mealia T., Hoitink H. A. J., and Kim J. M. (1999). Precise detection and tracing of *Trichoderma hamatum* 382 in compost amended potting mixes by using molecular markers. *Appl. Environ. Microbiol*. 12: 5421-5426.

[10] Lecomte P, Peros JP, Blancard D, Bastien N, Delye C (2000). PCR assays that identify the grapevine dieback fungus Eutypa lata. Appl. Environ. Microbiol. 66: 4475-4480.

[11] Li KN, Rouse DI, Eyestone EJ, German TL (1999). The generation of specific DNA primers using random amplified polymorphic DNA and its application to Verticillium dahliae. Mycol. Res. 103: 1361-1368.

[12] Schilling AG, Moller EM, Geiger HH (1996). Polymerase chainreactionbased assays for species-specific detection of Fusarium culmorum, F. graminearum, and F. avenaceum. Phytopathol. 86: 515-522.

[13] Watanabe N (1984) Bulletin of Faculty of Agriculture, Maiji University, Japan 66: 45.

[14] Doyle J. J, Doyle J. L (1987) .A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem Bull* .19: 11-15.

[15] Shalini 1, Narayan, K. P. 1, Lata1 and Kotasthane, A. S. (2006). Genetic relatedness among *Trichoderma* species inhibiting a pathogenic fungi *Rhizoctonia solani. African Journal of Biotechnology* Vol. 5 (8), pp. 580-584.

[16] Sneath, P. H. A. and Sokal, R. R. (1973). Numerical Taxonomy: The Principles and Pratice of Numerical Classification. San Francisco: Freeman, 573 pp.

[17] Lieckfeldt, G.J. Samuels, H.I. Nirenberg, O. Petrini A. (1999). morphological and molecular perspective of *Trichoderma viride*: is it one or two species Appl. Environ. Microbiol. 65: 2418-2428.

[18] Kandoliya U.K. and D. N. Vakharia (2013) Antagonistic effect of *Pseudomonas fluorescens* against *fusrium oxysporum* f.sp. *Ciceri causing* wilt in chickpea. Legume Research., 36 (6) 569-575.

[19] Zimand, G., Valinsky, L., Elad, Y., Chet, I., Manualis, S. (1994). Use of RAPD procedure for the identification of *Trichoderma* species. *Mycol. Res*. 98, 531-534.

[20] Bulat, S. A., Lubeck, M., Mironenko, N. V., Jensen, D. F., Lubeck, P. S. (1998). UPPCR analysis and ITS1 ribotyping of *Trichoderma* and *Gliocladium* fungi. *Mycol.* 102: 933-943.

[21] Cumagun CJR, Hockenhull J, Lübeck M (2000) Characterization of *Trichoderma* isolates from Philippine rice fields by UP-PCR and rDNA-ITSI analysis: identification of UP-PCR markers. Journal of Phytopathology 148: 109-115.

[22] Naeimi, S. (2010). Development of a specific molecular marker for detection of the most effective *Trichoderma* strain against Than at ephoruscucumeris, the causal agent of rice sheath blight disease. Ph.D thesis, Faculty of Agriculture, University of Tehran, Karaj, Iran.