Journal of Sugar Beet
http://jsb.areeo.ac.ir/
Journal of Sugar Beet 2014, 30(1)

Pathogenicity and genetic variation of Iranian isolates of *Fusarium oxysporum* from sugar beet in Iran

S. Beladi Behbahani[(1)*], S. Rezaee[(2)] and S.B. Mahmoodi[(3)]

[(1)] *M.Sc. of Plant Pathology, Science and Research Branch, Islamic Azad University, Tehran, Iran.*
[(2)] *Assistant professor of Science and Research Branch, Islamic Azad University, Tehran, Iran.*
[(3)] *Associate professor of Sugar Beet Seed Institute (SBSI), Karaj, Iran.*

Beladi Behbahani S, Rezaee S, Mahmoodi SB Pathogenicity and genetic variation of Iranian isolates of *Fusarium oxysporum* from sugar beet in Iran. J. Sugar Beet. 2014; 30(1): 1-6. DOI:10.22092/jsb.2014.5853

Received June 14, 2012; Accepted April 15, 2014

ABSTRACT

Pathogenicity and genetic variability of 13 isolates of *Fusarium oxysporum* from sugar beet were evaluated. Samples were collected from six provinces of Iran during 1999- 2008. Pathogenic variability of the isolates was determined using a root-dip assay in spore suspension for 4-week-old plants. The isolates were classified into strong and weak pathogenic groups. DNA was extracted from *F. oxysporum* isolates and their molecular variability was investigated by 15 random amplified polymorphic DNA (RAPD) analysis of the PCR-amplified internal transcribed spacer (ITS) regions of ribosomal DNA. Digestion of the PCR products with the restriction enzymes EcoR1, Taq1 and HaeIII produced fragments of similar sizes. Cluster analysis using the unweighted pair-group method with arithmetic mean (UPGMA) clearly separated the isolates into five groups. A relationship was found between RAPD results and geographical origins of the isolates. However, no relationship was found between RAPD results and pathogenicity.

Keywords: *Fusarium oxysporum*, Sugar beet, RAPD- PCR, rDNA-RFLP

INTRODUCTION

Fusarium betae oxysporum Schlecht. emend. Snyd. & Hans. f. sp is a soil-borne pathogen reported in sugar beet planting regions in Iran. Interveinal chlorosis is occurring first on the older leaves, then progressing to the youngest ones. With disease progress, wilting of the individual leaves and plant death occur. At early stages, plants may wilt during sunny days but recover at night. Finally, leaves collapse on the soil surface and die but remain attached to the crown. No lesions or symptoms are found on roots. When the roots are sliced in cross section, they show a yellow-brown to gray discoloration in the water-conducting, vascular tissues. Older plants rarely die, but can experience a reduction in yield (Ruppel 1991). Fusarium yellows, caused by *Fusarium conglutinans* var. *betae*, was first reported from Colorado, US (Stewart 1931). Then, it changed to *F. oxysporum* f. sp *betae* (Ruppel 1991).

The pathogenicity of the fungus on sugar beet has been investigated through spore suspension of the roots (Hanson 2006). The genetic diversity of the pathogen has been studied by isozyme test, vegetative compatibility groups (VCG), and random amplified polymorphic DNA (RAPD). In Texas, isolates were differentiated by using isozyme analysis based on enzyme profiles discrepancies among them (Martyn et al. 1989). The genetic diversity among 160 isolates of *F. oxysporum* from Texas, classified them into seven vegetative compatibility groups (Harveson and Rush 1997). Using RAPD technique, the genetic diversity among isolates collected from five different regions in USA showed a high degree of polymorphism and isolates were differentiated based on geographical regions (Fisher and Gerik 1994). In another study, the pathogenic isolates of *F. oxysporum* f. sp. *be-*

*Corresponding author's email: shayesteh.beladi@gmail.com

tae (Fob) and *F. oxysporum* f. sp. *phaseolina* (Fop) from sugar beet and bean, respectively were evaluated using RAPD-PCR technique. Results showed a high degree of polymorphism in Fob and Fop isolates (Cramer 2003). In Iran, this pathogen was first reported from Isfahan by Behdad in 1969 (Behdad 1983). In a study in Khorasan province, *F. oxysporum* was introduced as a vascular wilt fungus and took the third rank among the all fungi causing root rot (Abasi Moghadam et al. 1997). In a study by Raufi et al. (2003), *F. oxysporum* had the second frequency among Fusarium species which were collected from different sugar beet production areas in Iran and different pathogenicity was also reported. Basically, in studies related to plant resistance against pathogens, sufficient knowledge should be gained regarding the genetic structure of the pathogens, and in the case of diversity among disease characteristics, identification of the most common isolates is necessary to use them in breeding and resistant germplasm selection (Mahmoodi *et al.* 2005). Prior to breeding programme implementation and in order to obtain cultivars with stable resistance, the population structure of the pathogen should be examined by reliable and accurate methods to identify resistant cultivar for each region. This study aimed to investigate the pathogenic and genetic diversity of *F. oxysporum* isolates from sugar beet in Iran.

MATERIALS AND METHODS

In this study, eight isolates of *F. oxysporum* were received from fungal isolates collection of Sugar Beet Seed Institute in Iran. Isolates were collected over a three year period (1999- 2001) from main sugar beet production areas. Five isolates were also separated from the samples collected from Qazvin, Hamedan, and Kermanshah regions.

Pathogenicity test

F. oxysporum isolates were examined for pathogenicity by dipping roots of 4-week-old susceptible sugar beet plants in a suspension of 10^6 spores per ml in a completely randomized design with 10 replications. Control plants were treated with sterile distilled water. Plants were visually assessed at 7 days after inoculation, using the 0-4 rating scale (Panella et al. 1995) as follow:

0 = no symptoms; 1 = chlorosis of leaves with necrosis on leaves edges; 2 = up to 50% chlorotic leaves and up to 50% of leaves become brown; 3 = all leaves become brown; and 4 = death of the seedling.

Genetic diversity

In order to determine the genetic diversity of the isolates, 15 random primers of ten nucleotide each (Table 1) were used. Each mycelium of the isolates were prepared in GYM medium containing 10 g glucose, 1 g $NH_4H_2PO_4$, 0.2 g KCl, 0.2 g $MgSO_4$, $7H_2O$, 5 g yeast extract, 1 ml $ZnSO_4$ (%1), and 1 ml $CuSO_4$ (%0.5). Three pieces of the agar (5 ml) containing fungal colony, grown for 5 days, were harvested and transferred to flasks containing 50 ml GYM medium. Each mycelium was harvested and DNA was extracted from each isolate according to CTAB method (Frederick *et. al*. 2002).

RAPD-PCR reaction

PCR was performed in a volume of 25 µl. The cycle parameters in the PCR program were as follows: 94°C for 5 min, 39 cycles of: 40°C for 40 seconds, 34°C for 94 seconds, 72°C for 80 seconds, and final extension at 72°C for 10 min (Toda et al. 1999).

The amplified DNA were electrophoresed on 1.2% agarose gel and TAE buffer (containing 0.04M Tris-acetate and 0.001M Na_2EDTA) at 2.5 V and visualized under UV light after staining with ethidium bromide. The presence and absence of the bands was recorded as one and zero, respectively in Excel software. Cluster analysis was performed with arithmetic mean (UPGMA) using MultiVariate Statistical Package software.

ITS-RFLP analysis

rRNA genes of fungal isolates were amplified with the primers ITS1 (19-mer) :5'- TCCG-TAGGTGAACCTGCGG-3' and ITS4 (20-

Table 1. Characteristics of the random primers used in the RAPD-PCR reaction.

No.	Primer	Sequence
1	UBC 203	CACGGCGAGT
2	UBC 208	ACGGCCGACC
3	UBC 211	GAAGCGCGAT
4	UBC 213	CAGCGAACTA
5	UBC 214	CATGTGCTTG
6	UBC 283	CGGCCACCGT
7	UBC 285	GGGCGCCTAG
8	UBC 286	CGGAGCCGGC
9	Takapoozist 1	CGTTGGATGC
10	Takapoozist 2	CCAGACAAGC
11	Takapoozist 7	CCGGCCTTAG
12	Takapoozist 8	CCTGGGCCTC
13	Takapoozist 9	CCTGGGCTGG
14	Takapoozist 10	CCGGCCCCAA
15	CinnaGene 9	CCTGGGCCT<A>

Table 2. Characteristics of *Fusarium oxysporum* isolates collected from sugar beet production areas in Iran and results of Kruskal-Wallis test[+]

No.	Isolate name	Year of sampling	Origin	Sum of ranks
1	104	2001	Lorestan	405.5
2	105	1999	Qazvin	825.0
3	109	2001	Hanedan	815.0
4	110	2001	Ardabil-Moghan	773.0
5	111	2001	Kermanshah	583.5
6	112	2001	Kerman	699.5
7	113	2001	Ardabil-Moghan	709.5
8	114	2001	Kerman	783.5
9	120	2007	Qazvin	218.0
10	129	2008	Hamedan-Nahavand	636.0
11	130	2008	Kermanshah-Bistoon	699.5
12	131	2008	Kermanshah-Bistoon	741.5
13	132	2008	Kermanshah-Bistoon	625.5

[+]Significant at 0.05 probability level

mer) : 5'-TCCTCCGCTTATTGATATGC-3' in 50 ng/ml (Salazar et al. 2000). The PCR conditions were as follows: 5 min preheating at 94 °C, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, and extension at 72 °C for 10 min. The amplified PCR products were electrophoresed on 2% agarose gel, stained with ethidium bromide and visualized under UV.

PCR digestion

Following amplification, 7 µl of PCR products were digested with 2 µl restriction enzymes *Hae*III, *Eco*RI and *Tag*I, 2 µl reaction buffer (10X), and 14 µl distilled water. The digestion was performed for 2 and half hours at 37 °C (for *Hae*III and *Eco*RI enzymes) and 65 °C (for *Tag*I) and samples were kept at -20 °C. The digested fragments were separated on 2% agarose gel, stained with ethidium bromide and visualized under UV.

RESULTS AND DISCUSSION

Seedlings were daily evaluated for disease symptoms (discoloration and necrosis). One week after planting, seedlings were harvested from spore-suspension and rated on the basis of 0-4 rating scale.

Cluster analysis (using average linkage between groups based on squared Euclidean distance) by SPSS software, separated isolates into two groups including weak and strong (Figure 2). The results of the ANOVA based on Kruskal-Wallis test, showed a significant difference among isolates, with the isolates 105 and 109 identified as strong pathogenic isolates and the isolates 104 and 102 as weak ones (Table 3).

RADP-PCR reaction

All 15 primers used in this study showed polymorphism among the isolates. The size of the bands was estimated between 120-3000 bp

Figure 1. Comparison of disease symptoms in pathogenicity test one week after inoculation.

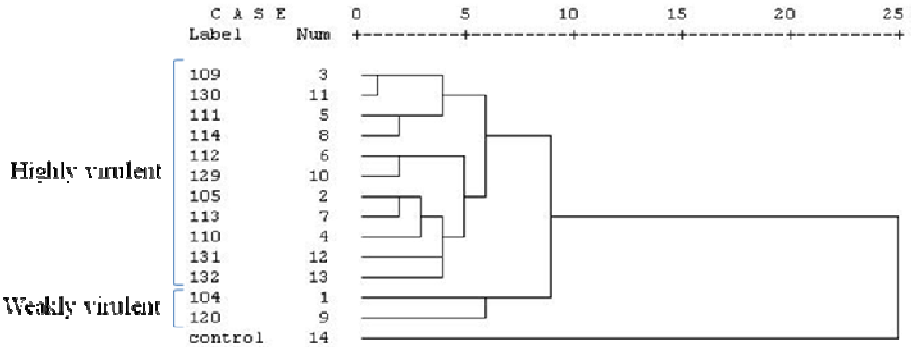

Figure 2. Cluster analysis of the isolates based on pathogenicity test

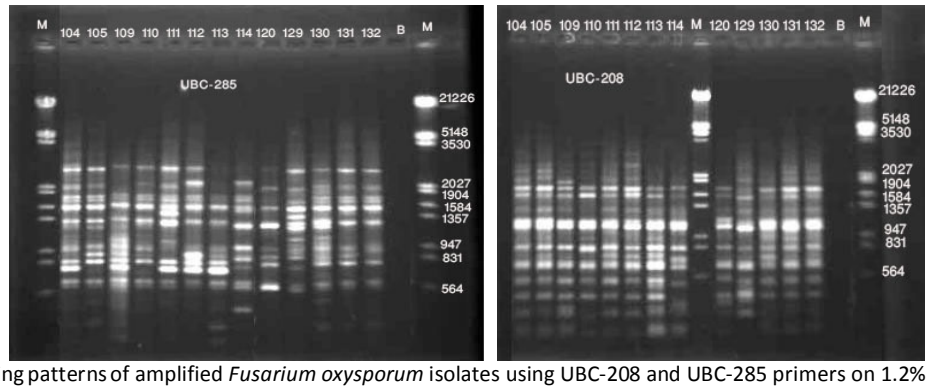

Figure 3. Banding patterns of amplified *Fusarium oxysporum* isolates using UBC-208 and UBC-285 primers on 1.2% agarose gel. M stands for the molecular weight marker.

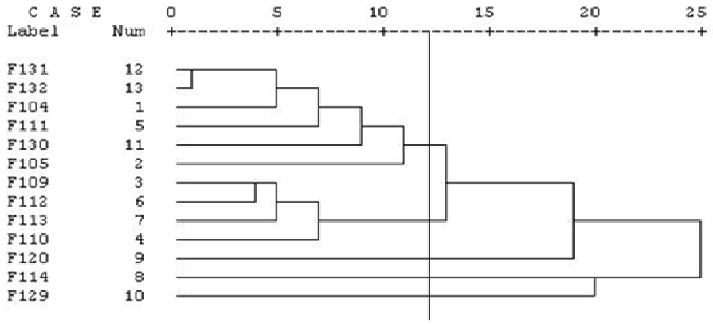

Figure 4. The cluster analysis of the Fusarium isolates based on RAPD-PCR results.

(Figure 3). The cluster analysis of the isolates classified them into five groups. Results showed that the differentiation of some isolates from others on the basis of RAPD-PCR was associated with their geographical regions. Four isolates from Kermanshah and two from Lorestan and Qazvin, respectively were clustered together in group I. The isolates 110 and 113 from Ardabil, and two isolates from Hamedan and Kerman, respectively were clustered in group II. The three isolates which were from Qazvin, Kerman, and Hamedan, respectively were clustered in the three remained groups. No significant correlation was found between pathogenicity and RAPD results (Figure 4).

ITS-RFLP analysis and comparison of restriction enzymes effect on PCR products

PCR amplification of genomic DNA extracted from *F. oxysporum* isolates with ITS1 and ITS4 primers resulted in the amplification of a product of approximately 550-570 bp. No amplified fragment was observed in negative controls. The pattern of ITS-RFLP bands upon digestion of the PCR products with the enzymes *Eco*RI, *Hae*III and *Taq*I indicated genetic diversity among the isolates which varied in length and number of fragments. Digestion of the PCR products with the restriction enzymes *Eco*RI, *Hae*III and *Taq*I produced 2, 3, and 4 fragments with 250 and 320-350 bp (for *Eco*RI), 90, 120 and 340 bp (for *Hae*III) and 70, 90, 150, and 230 bp (for *Taq*I) size, respectively (Figure 5).

A key factor in plant protection against diseases is obtaining sufficient knowledge about the genetic structure of pathogen population which may contribute in making more effective decisions (Martin and English 1997). On the other hand, the pathogen populations are under evolution to accommodate the environmental variations such as crop rotation, resistant cultivars, and fungicides. Therefore, in order to make control mechanisms more effective, instead of working on individual or specified genus, population should be studied (MacDonald 1997). *F. oxysporum* is a fungal plant pathogen which is widely distributed in soil and has drawn a lot of attention due to its capability in causing vascular wilt or root rot diseases that affect a wide range of plants. In this study, a total of 13 isolates from Iran were compared in terms of pathogenicity which were different in this respect. However, large variation was not found for isolates pathogenicity likely due to the low number of isolates. The isolates 104 and 120 showed low pathogenicity effect, which might be due to the

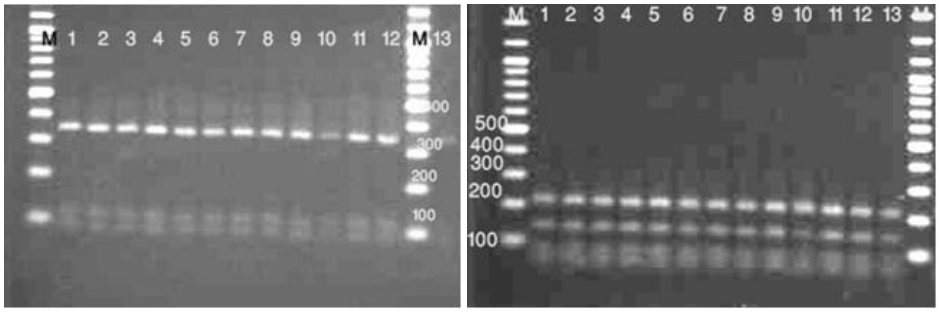

Figure 5. Restriction patterns of the PCR products of Fusarium isolates digested with *Eco*RI, *Hae*III (right) and *Tag*I (left). Numbers 1-13 are related to the isolates 104, 105, 109, 110, 111, 112, 113, 114, 120, 129, 130, 131, and 132. Lane M = molecular weight marker.

presence of mycovirus inside them (Noorifar et al., 2009). Several methods including VCGs (Puhalla 1985), isozyme test (Bosland and Elias et al. 1993), RFLPs (Williams 1987), DNA fingerprinting, differences in the fragment size of the RAPD or RFLPs digested products, and the sequence determination of the repeated DNAs were used to determine the genetic diversity of *F. oxysporum* isolates (Kistler 1997). Only a few studies were performed on the evaluation of *F.oxysporum* isolates characteristics in sugar beet compared to other forms. In a VCGs study, *F.oxysporum f. sp. betae* isolates showed high genetic diversity (Harveson and rush 1997). In this study, a remarkable genetic diversity was also found among isolates. In a study by Fisher and Gerik (1994), the RAPD analysis of *F. oxysporum f. sp betae* isolates collected from California and Oregon regions, showed different banding patterns and the isolates were classified based on geographical regions. In this study, it also appeared that for some isolates, classification was performed based on geographical regions. DNA amplification of the *F. oxysporum* isolate by using RAPD technique produced clear, reproducible, and polymorphic bands which facilitated isolates differentiation based on banding pattern. Fifteen primers which produced 229 polymorphic bands were selected for genetic diversity differentiation. Cluster analysis classified the isolates into five groups. In this study, the ITS region of nuclear ribosomal DNA, including ITS1, ITS2 and the intervening 5.8S rRNA gene, were amplified and evaluated on agarose gel. The amplified fragments showed similar size which is concordant with the results of Lee et al. (2000). Results of this study are in accordance with other studies which used *Eco*RI, *Hae*III and *Taq*I restriction enzymes (Lee et al. 2000; Arruda et al. 2005) in terms of length and fragment number.

CONCLUSION

In general, the results of this study showed that the genetic diversity in this fungus is high. Therefore, key factors influencing its creation and outbreak should be studied. Although, researchers found mutation, mating systems, and gene flow as the main factors influencing its genetic diversity but it seems that in addition to the aforesaid factors, the interplay between host and pathogen and also the lack of resistant host are also important. Among different Fusarium species, *F. oxysporum*, especially *F. oxysporum* f.sp. betae species showed the highest genetic diversity (Namiki et al. 1998; Woo et al. 1996). The results of this study are in accordance with previous studies which confirmed the presence of genetic diversity in this fungus and revealed the need for further investigations on this important specific form. No significant correlation was found between genetic diversity based on RAPD-PCR and isolates pathogenicity. However, the classification of some isolates was probably associated with their geographical origin. Sugar beet crop movement from one region to another and finally to sugar factory might be involved in the classification of the isolates with different geographical origin in one group. It seems that ITS-RFLP marker couldn't differentiate intera-specific variation which is corroborated by the results obtained from other studies. ITS-RFLP results showed similarity in banding pattern while IGS-RFLP results showed differences in banding pattern (Pavaanen et al. 1999). Although geographical regions could often differentiate species but most often the restriction sites of the restriction enzyme are key factor in differentiation of the isolates from different species. Although, 100% similarity among banding patterns do not indicate region similarity, which might be distinguished by other restriction enzymes, but it may itself be a reason for correct identification of the species.

REFERENCES

Abasi Moghadam E, Rastgar M, Jafarpoor B. Etiology of root

and crown rot of sugar beet in Khorasan. Proceedings of the 13th Iranian Plant Protection Congress, Karaj, Iran. 1997; P. 125. (In Persian, abstract in English)

Arruda GMT, Miller RNG, Ferreira MASV, Café-fiho AC. Morphological and molecular characterization of the sudden death syndrome pathogen of soybean in Brazil. Plant Pathology. 2005; 54:53-65.

Behdad E. Diseases of Agricultural Plants in Iran. Neshat Publisher, Isfahan. 1983. (In Persian)

Bosland PW, Williams PH. An evaluation of *Fusarium oxysporum* from crucifers based on pathogenicity, isozyme polymorphism, vegetative compatibility, and geographic origin. Can. J. Bot. 1987; 65:2067-2073.

Cramer RA. Characterization of *Fusarium oxysporum* isolates from common bean and sugar beet using pathogenicity assays and random-amplified polymorphic DNA markers. Journal of Phytopathology. 2003; 151: 352-360.

Elias KS, Zamir D, Lichtman-Pleban T, Katan T. Population structure of *Fusarium oxysporum* f. sp. *lycopersici*. Mol. Plant-Microb Intract. 1993; 6:565-572.

Ferderick MA, Roger Brent R, Kingston D, Seidamn J, Smith A, Strunl K. Preparation of plant DNA using CTAB. Short Protocol Molecular Biology. 2002; 2/4: 2/10.

Fisher GA, Gerik JS. Genetic diversity of *Fusarium oxysporum* isolates pathogenic to sugar beets. (Abstr) Phytopathology. 1994; 84:1098.

Hanson LE. Beet root-rot inducing isolates of *Fusarium oxysporum* from Colorado and Montana. Plant Disease. 2006; 90: 247.

Harveson RM, Rush CM. Genetic variation among *Fusarium oxysporum* isolates from sugar beet as determined by vegetative compatibility. Plant Disease. 1997; 81:85-88p.

Kistler HC. Genetic diversity in the plant pathogenic fungus *Fusarium oxysporum*. Phytopathology. 1997; 87:474-479.

Lee YM, Choi YK, Min BY. PCR-RFLP and sequence analysis of the rDNA ITS region in the *Fusarium* spp. The Journal of Microbiology. 2000; 38: 66-73.

Mahmoodi SB, Mesbah MR, Rahimian H, Alizadeh A, Noroozi P. Genetic diversity of sugar beet of *Rhizoctonia solani* revealed by RAPD-PCR and ITS-PCR and ITS-rDNA analysis. Iranian Journal of Plant Pathology. 2005; 41: 523-542. (In Persian, abstract in English)

Martin FN, English JT. Population genetics of soil born Fungal plant pathogens. Phytopathology. 1997; 87: 446-447.

Martyn RD, Rush CM, Biles CL, Bake EH. Etiology of a root rot disease of sugar beet in Texas. Plant Disease. 1989; 73: 879-883.

MacDonald B. The population genetics of Fungi: tools and techniques. Phytopathology. 1997; 87: 448-453.

Namiki F, Shiomi T, Nishi K, Kayamura T, Tsuge T. Pathogenic and genetic variation in the Japanese strains of *Fusarium oxysporum* f. sp. *melonis*. Phytopathology. 1998; 88:804-810.

Noorifar N, Etebarian HR, Mahmoodi SB, Hashemi M. Separation and identification of dsRNA related to some isolates of three species of genus *Fusarium* and study of their effects on isolates pathogenicity. Proceedings of the 6th National Biotechnology Congress of Iran, 13-15 Aug, Tehran, Iran; 2009. (In Persian)

Panella LW, Ruppel EG, Hecker RJ. Registration of four multigerm sugar beet germplasm resistant to *Rhizoctonia* root rot. Crop Science. 1995; 35: 291-292.

Pavaanen-Huhtala S, Hyvonen J, Bulat SA, Yli-Maltila T. RAPD-PCR, isozyme, rDNA RFLP and rDNA sequence analyses in identification of finfish *Fusarium oxysporum* isolates. Mycological Research. 1999;103:625-634.

Puhalla JE. Classification of strains of *Fusarium oxysporum* on the basis of vegetative compatibility. Can. J. Bot. 1985; 63:179-183.

Raufi M, Farokhinejad R, Mahmoodi SB. Identification and pathogenicity of *Fusarium* species associated with sugar beet root and crown rot in Iran. Journal of Sugar Beet. 2003; 19(2): 109-122. (In Persian, abstract in English)

Ruppel EG. Pathogenicity of *Fusarium* spp. from diseased sugar beets and variation among sugar beet isolates of *Fusarium oxysporum*. Plant Disease. 1991; 75:486-489.

Salazar O, Julian MC, Rubio V. Primers based on specific rDNA-ITS sequence for PCR detection of *Rhizoctonia solani*, *R. solani* :AG-2 subgroups and ecological types and binucleate *Rhizoctonia*. Mycological Research. 2000; 104: 281-285.

Toda T, Hyakumachi M, Suga H, Kageyama K, Tanaka A, Tani T. Differentiation of *Rhizoctonia* AG-D isolated from turf grass in to subgroups I and II based on rDNA and RAPD analyses. European Journal of Plant Pathology. 1999; 105: 835-846.

Woo SL, Zonia A, Del Sorbo G, Lorito M, Nanni B, Scala F, Noviello P. Characterization of *Fusarium oxysporum* f. sp. *phaseoli* by pathogenic races, VCGs, RFLP and RAPD. Phytopathology. 1996; 88:966-973.