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Root Diseases of Onion Caused by Some Root Colonizing Fungi in Northeast of Iran

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Abstract: Onion (*Allium cepa* L.) is threatened with infection caused by different pathogenic microorganisms. Soil borne fungi that infect roots and bulbs of this crop are one of the most important pathogens in Iran which cause severe damage annually. Pink root and Fusarium basal rot are important diseases of cultivated alliums. During 2007-2008 sampling was carried out from fields in northeast of Iran. After morphological and molecular studies and Pathogenicity test, the most frequently isolated fungi were *Fuasrium oxysporum*, *F. solani*, *F. proliferatum, Pyrenochaeta terrestris* and *Rhizopycnis vagum* respectively. In host range study all isolates of *Fusarium oxysporum* caused basal rot only in onion and proved to be *F. oxysporum* f.sp. *cepea*. The results showed that all the fusaria that isolated from roots, bulbs and seeds, caused root and basal rot on onion. Isolates of *P. terrestris* isolated from roots, could induced pink root and *R .vagum* caused necrosis of onion roots. Under greenhouse trail, no significant correlation between Fusarium species and their Pathogenicity power was observed. The results are thought to be the first report of *R .vagum* and *P. terrestris* from Khorasan province, Iran.

Key words: Basel rot · Fusarium · Iran · Onion · Pink root · Pyrenochaeta and Rhizopycnis

INTRODUCTION

Onions (*Allium cepa* L.) are one of the most important vegetables in Iran. Rotting of young and adult plants of onion is caused by different species of the genus Fusarium among which *F. oxysporum* f. sp. cepae is considered the most important onion parasite worldwide, causing rot of the basal plate of the onion bulb [1]. This fungus infects the roots or the basal plate of the bulbs. Further infection of bulb scales occurs later in the season and most severe losses are found at postharvest storage period. The fungus is spread worldwide and also infects other cultivated allium species, such as garlic [2].

Recently, *F. proliferatum* was found affecting onion [3, 4] and garlic [5]. *Fusarium proliferatum* isolated from onion and garlic in storage was reported in USA, showing bulb rot of onions caused by this fungus [3, 5]. *F. proliferatum* was also isolated from onion seeds in Europe [6] and Simey [7] is reported this fungus as an agent of bulb rot of garlic in Hungary during winter storage [7].

Another important disease of onion is pink root disease caused by the soilborne pathogen *Pyrenochaeta terrestris*. Pink root is a serious disease of onion bulbs and the infected roots turn pink initially and then become brittle and die. This fungus can survives for many years in soil. Although *P. terrestris* can be present in roots or dry outer scales of the bulbs, it does not invade the basal plate or stem of the bulb [8-10].

Rhizopycnis vagum D.F. Farr is a recently described coelomycetous fungus [11] known to contributes to 'vine decline' of cucurbits in several parts of the world [12, 13]. This fungus was first reported, as an unknown, Stagonospora-like fungus. *R. vagum* appears associated with other, noncucurbitaceous hosts. In Italy it was isolated from tomato (*Lycopersicon esculentum*) roots showing typical corky root symptoms, along with *P. lycopersici*, the causal of tomato corky root [14]. *Saccharum officinarum* (sugarcane) is another non-cucurbitaceous host of *R. vagum*. The fungus was isolated, although with low frequency, from roots of poorly-developed canes [15]. There was no repots of this fungus on onion.

The objective of this work was to survey onion fields in northeast of Iran for the identification of root-infecting fungi and to test their pathogenicity under greenhouse conditions. In addition, symptomology associated with root colonization by these fungi is also described.

MATERIAL AND METHODS

Disease Survey: To estimate the frequency occurrence of fungi associated with onion roots, samples were obtained over a 2-year period (2007 to 2008) from fields in

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8 counties in northeast of Iran. Samples were obtained from 37 fields where plants suffered from wilt, pink root, or root rot diseases.

The collected root samples were washed carefully under running tap water to get rid of adhering soil particles, then cutted to small pieces. The pieces of root and plate tissue were surface sterilized in 1% sodium hypochlorite for 1.5 min, followed by several rinsed in sterile distilled water and then dried in a laminar flow hood on sterile paper towel and placed into plates containing PDA (Potato Decstrose Agar).

After the plates were incubated at 20-25°C for 3 to 5 days, hyphal tips or single spores of various fungi were transferred to PDA. Fusarium spp. and picnidial fungi were identified based on morphological characteristics on carnation leaf agar and low nutrient medium (1 g MgSO 4, 3 g NaNO 3, 20 g agar in I-L H20) with pieces of dried wheat straw respectively. All dishes were incubated at 20-25°C under NUV + fluorescent illumination with a 12 h photoperiod. Fusarium species were identified according to Nelsone *et al.* [16] and Booth [3]. Frequency of isolation of each fungus from tissue types and field locations was recorded.

DNA Extraction and PCR Amplification: Isolates that morphologicaly were identified as *R. vagum* and *F. proliferatum,* tested positive using a pair of specific primers. To extract DNA from mycelium of fungi, the isolates were grown on PDA for 6-8 days and then transferred to 100^{mL} potato dextrose broth medium in 250^{mL} Erlenmeyer flasks and shaken at 150^{rpm} at 28 °C for 7 days. Aerial mycelium was then collected and dried in a laminar flow hood on sterile paper towel and then frozen in liquid nitrogen, ground to a fine powder using a mortar and pestle. Total genomic DNA was isolated from 0.1 gr of each sample using the GMO DNA extraction Kit of Bioneer following the manufacturer's instructions.

Specific primers Rv1-F (CCCCCGCTAGGACCCTTTATC) and Rv1-R (GGCTTCTGGATGCC CAT GTC), designed by Ghignone *et al.* [17], was used for *R. vagum*. 1µl of each primer (10 Pmol), 1µl of the DNA template and 16µl distilled water were added to 20µl reaction Accupower ™ PCR preMix tubes (BIONEER Com. - korea). Cycling parameters for the amplification reaction were: initial denaturation for5 min at 94 °C; 27 cycles of 40 s of denaturation at 94 °C, 50 s of annealing at 64 °C and 50 s of extension at 72 °C, final extension for 7 min at 72 °C. As for *F. proliferatum* the primer pair PRO1 (CTTTCCGCCAAGTTTCTTC) and PRO2 (TGTCAGTAACTCGACGTTGTTG), designed by Mule *et al.* [18], were used. 1µl of each primer (10 Pmol), 1µl of the DNA template and 16µl distilled water were added to 20µl reaction Accupower ™ PCR preMix tubes (BIONEER Com. - korea) and the following protocol was chosen: initial denaturation for5 min at 95 °C; 35 cycles of 50 s of denaturation at 94 °C, 50 s of annealing at 56 °C and 1min of extension at 72 °C, final extension for 7 min at 72 °C. Products (8 µl) were analyzed by electrophoresis in a 1.7%TBAE agarose gel, stained with 0.5µg/ml ethidium bromide and visualized by ultraviolet light.

Inoculums Production and Pathogenicity Test: All isolates used in pathogenicity tests were obtained from symptomatic onion bulb and roots in khorasan. Single-spore isolates of *Fusarium* spp. were obtained prior to use in pathogenicity tests, while hyphal tip isolates of *P. terrestris* and *R. vagum* were used. In order to compare the virulence of the Fusarium basal rot fungi, 20 isolates from each species were considered and stored at 4°C in sterilized soil and were increased on PDA at 24C. Mycelia plugs were transferred to potato-dextrose broth and grown at 26 C for *5* days on a rotary shaker.

The spore-containing pellet was resuspended in distilled water. Spore concentration was determined with a haemacytometer and the spore suspension was mixed with heat-sterilized sand (1×10^{5}spores ml^{-1}, 1×10^{4}spores g^{-1} dry sand).

P. terrestris and *R. vagum* were maintained on sterile soil at 4°C, transferred to potato-dextrose agar (PDA) and grown for 3 days at 24°C. After 11 days, 10 plugs (each 10 mm in diameter) were placed in 500-ml bottles with 200 ml of potato-dextrose broth and incubated at 20-25°C. The broth was shaken three times per week to break the mycelial mat. After 4 wk, the mycelium and broth were blended three times for 30 sec at low speed in a Waring Blendor, then 200 ml of inoculum and 1,400 ml of distilled water were mixed with 23.5 kg of heat-sterilized sand.

Pathogenicity was tested on onion cv. Texas. Three replications per isolate were tested, each consisting of 10 seeds sown in pots containing infested sand. Plants were maintained in a temperature and light-controlled greenhouse (12 h/12 h light/dark $25/21^{C}$). Symptoms on onion plants were observed 60 days after inoculation and disease rating was made based on the severity of rotting induced on the plants. Each seedling was rated for vigor

based on severity of disease symptoms using the visual numerical system according to Rengwalska and Simon [19]. Re-isolation of the pathogen from the lesions on the plants was conducted as described previously.

Host Range Study of *F. Oxysporum*: To determine host range and special form in *F. oxysporum,* healthy seeds or corms of 12 different species i.e., *Narcissus daffodil*, *Polianthes tuberosa*, *Gladiolus callianthus*, *Solanum tuberosum*, *Lycopersicum esculentum*, *Beta vulgaris*, *Raphanus sativus*, *Daucus carota, Cucurbita pepo, Allium cepea*, *Chenopodium album* and *Portulaca oleracea* were sown in sterilized earthen pots containing 2 kg infested sand as described above. After 60 days of sowing, the observations were taken on hypogeous parts of plants.

RESULTS

Disease survey: A total of 109 isolates was obtained from onion roots and bulbs (Table 1). Firstly they were identified based on their morphological characteristics (Fig. 1- 2). The morphological characteristics of three *Fusarium* spp. examined by the authors were consistent with those described by previous workers [1, 16]. Out of the 109 isolates, 52 isolates were identified as *F. oxysporum* Schlecht. Fr., 26 isolates as *F. solani* (Mart.) Sacc., 20 isolates as *F. proliferatum* (Matsushima) Nirenberg, 6 isolates as *R. vagum* and 6 isolates as *P. terestris* (Table 1). *Pyrenochaeta terestris* produced dark brown pycnidia (globose to subglobose, 180-250 µm) with brown setae 70-170 µm long. Setae were produced primarily around an astiole. Pycnidiospores (4.5-5.5 ·_1.5-2.0 µm) were hyaline, oblong ovoid and oozed from rupture or through the ostiole. The morphological characteristics of the isolates examined by the authors were consistent with those of *P. terrestris* described by Gorenz *et al.* [20].

The isolates of *Rhizopycnis vagum* developed spherical, ostiolate pycnidia on low nutrient medium with pieces of dried wheat straw. Conidia were hyaline to brown, cylindrical to fusiform, guttulate and 1-3 septate. Based on cultural characteristics and fungal morphology. These isolates were identified as *R. vagum* [11].

Isolates that morphologicaly were identified as *R. vagum* and *F. proliferatum,* tested positively using a pair of specific primers. PCR amplification resulted in all cases of *R. vagum* in the predicted single band of 369 bp (Fig. 3) and in the 587 bp for *F. proliferatum* (Fig. 4).

F. oxysporum, which was recovered from plants in 47.7% of the surveyed fields, was the fungus most commonly isolated from symptomatic onion bulbs and roots over a 2- year period. Other commonly isolated fungi were included *F. solani* (23.9%) and *F. proliferatum* (18.3%). *P. terestris* (4.6%) and *R. vagum* (5.7%), were infrequently isolated.

P. terrestris and *R. vagum* were isolated only once in the Razavi Khurasan province and frequently in Mashhad. The other fungi exhibited a more general distribution.

P. terestris was associated with reddish-brown lesions of the roots. Perithecia of *P. terestris* often were observed on symptomatic roots that were incubated in a moist chamber. *R. vagum* also was isolated occasionally from these pinkish, sunken lesions. Pycnidia of *R. vagum* were found occasionally on symptomatic roots that were

Table 1: Identification of fungi isolates from onion and Pathogenicity of isolates to the hosts by artificial inoculation

			Pathogenicity test		
Fungi identified	Isolated plant part	No. of isolates	No. of isolates tested	Mean disease severity score	Location
F. Oxysporum [w]	Root, bulb	52	20	3.6 a [y]	Esfaraien, Mane, Bojnord, Mashhad,
F. solani [w]	Root, bulb	26	20	2.8 ab [y]	Esfaraien, Mane, Bardaskan, Mashhad, Torbat H.
F. proliferatum [w]	Root, bulb	20	20	3 ab [y]	Esfaraien, Mane, Samalghan, Mashhad, Torbat H., Faruj
R.. vagum [w]	Root	6	6	2	Mashad
P. trestrris [x]	Root	5	5	3	Mashhad

[w] Disease classes: 1 = without symptoms, 2 --up to 10% rotted roots, 3 = 10-30% rotted roots with up to 10% rotted basal plates, 4 =completely rotted roots and 10-30% rotted basal plates and *5* = completely rotted roots and more than 30% rotted basal plates.

[x]: 'Disease classes: I = without symptoms, 2 = less than 10% pink roots, 3 = 10-50% pink roots with up to 10% rotted basal plates, 4 = more than 50% pink roots with 10-30% rotted basal plates and 5 = completely rotted roots and more than 30% rotted basal plates.

[y]: Weighted mean of disease classes for Fusaria species. Values in columns followed by different letters are significantly different (P= 0.05) according to Tukey's HSD test.

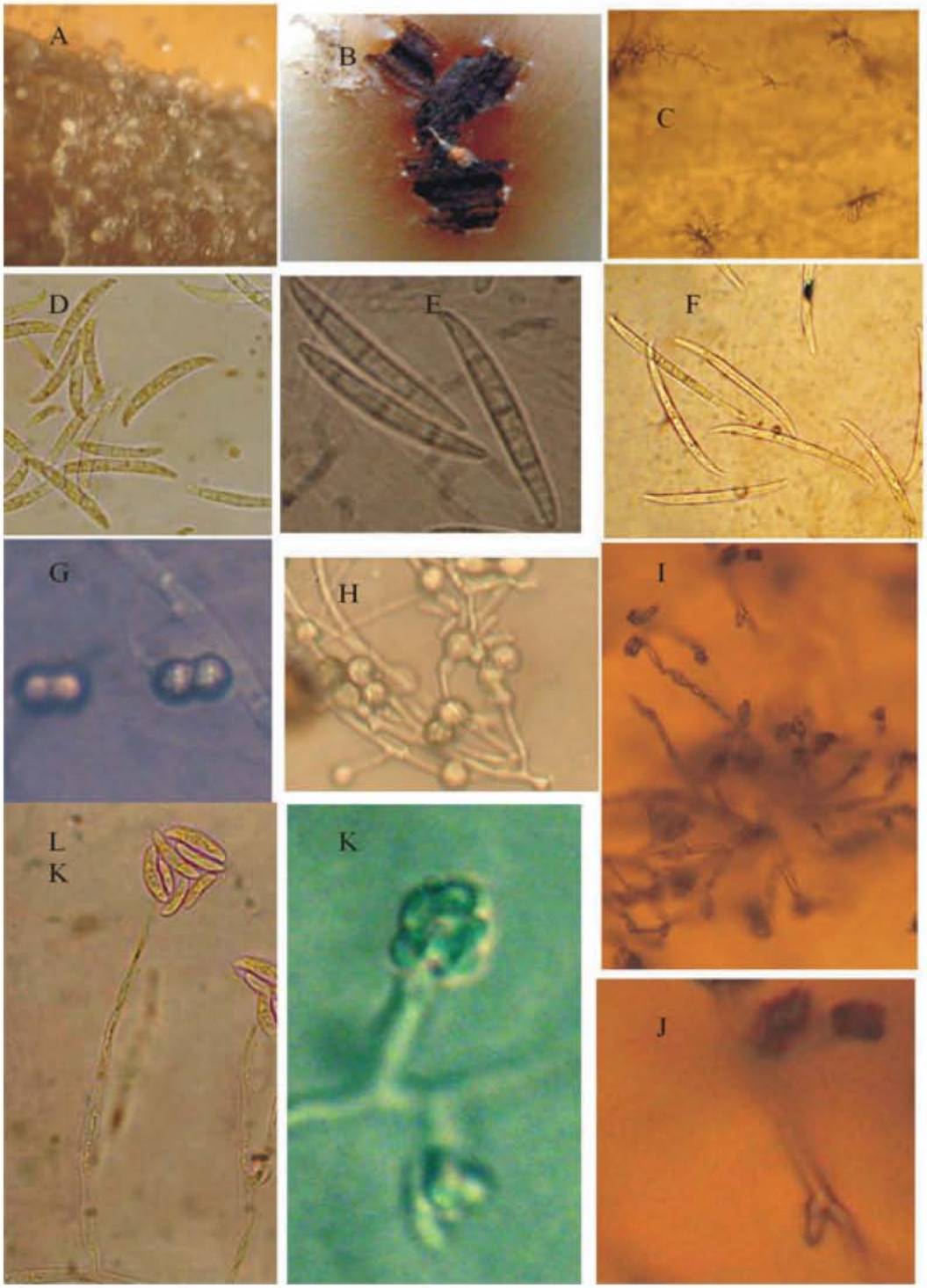

Fig. 1: Morphological characters of *Fuasrium* species. A: cream spordochium of *F. solani* on carnation leaf. B: orange spordochium of *F. oxysporum.* on carnation leaf and C: chain of microconidia of *F. proliferatum*. D: macroconidium of *F. solani*. E: macroconidium of *F. oxysporum*. F: macroconidium of *F. proliferatum* G: chlamydospore of *F. solani*. H: chlamydospore of *F. oxysporum*. J-K: chain of microconidia on poliphialid and monophialid in *F. proliferatum*. K: short phialid whit false head in *F. oxysporum*. L: long phialid whit false head in *F. solani*.

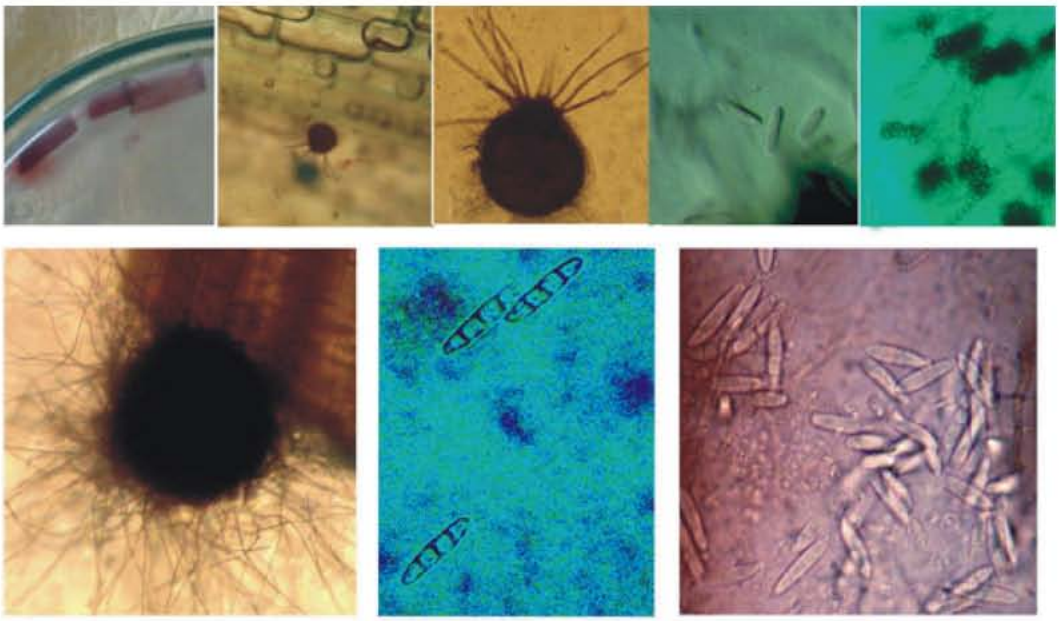

Fig. 2: Morphological characters of *Pyrenochaeta terrestris* (A-E)and Rhizopycnis vagum (F-H). A: pieces of dried wheat straw turn pink on low nutrient medium B-C: dark brown pycnidia, Setae were produced primarily around an astiole. D: unicellular Pycnidiospores. E: chlamydospore. F: dark brown pycnidia without Setae. G -H: 3-4 cellular Pycnidiospores

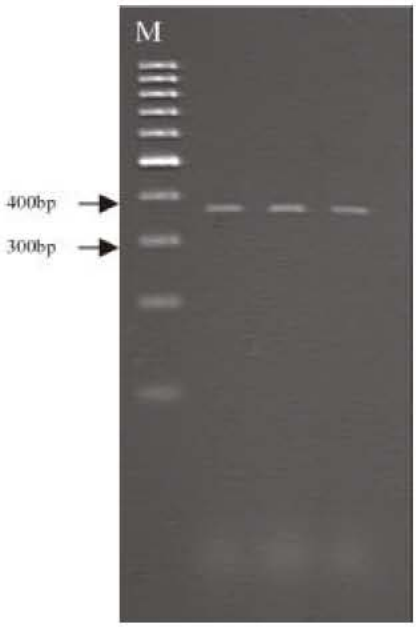

Fig. 3: Polymerase chain reaction (PCR) amplification products obtained from genomic DNA of *Rhizopycnis vagum*: RV11, Rv12 and Rv13 with the primer pairs (from left to right). M: molecular weight marker. Molecular weight in bp of some marker bands is specified on the right margin

incubated in a moist chamber. *Fusaria* species were associated with a wet, darkly discolored rot of the bulbs and roots, where with mycelia of this fungus, were embedded in the diseased tissue. Also orange sporodocia of *F. oxysporum* observed on infected bulbs in the filds.

Pathogenicity Tests: Symptoms on onion plants were apparent 60 days after planting in infested soil. The pink root disease symptoms were graded into five classes (Table1) on the basis of root pinking and basal plate rot as described by Rengwalska and Simon [19]. Variation in virulence among isolates of *P. terestris* was minimal. The pink root test results are given in Table 1 and Fig. 5. Fusarium basal rot symptoms were classified on a similar scale on the basis of root and basal plate rot. The calculated disease severity scores was differentiated both between the species and between the isolates within a species. The basal plate rot test results are given in Table 1 and Fig.6. About *R. vagum* all isolate caused low necrosis of roots (Table1, Fig. 7).

Host Range Study of *F. oxysporum*: In host range study of *F. oxysporum,* after 60 days of inoculation, hypogeous parts of plants were surveyed. All of the isolates caused basal rot only in onion and proved to be *F. oxysporum* f.sp. *cepea*.

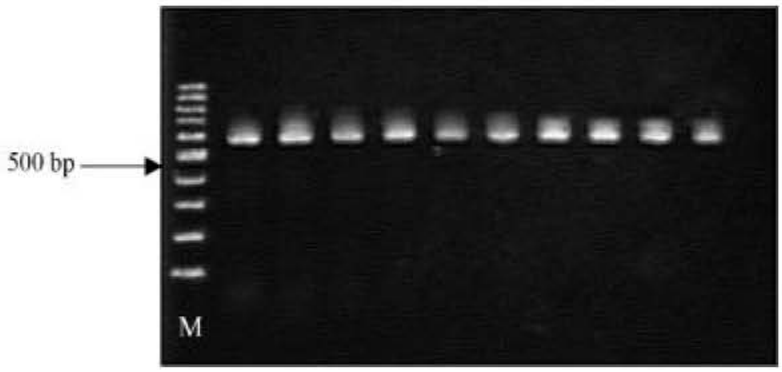

Fig. 4: Polymerase chain reaction (PCR) amplification products obtained from genomic DNA of *Fusarium proliferatum* isolates: E1, E2, E5, E7, E10, Eg5, Eg7, Td2, Td3 and R2 with the primer pairs (from left to right). M: molecular weight marker. Molecular weight in bp of some marker bands is specified on the right margin

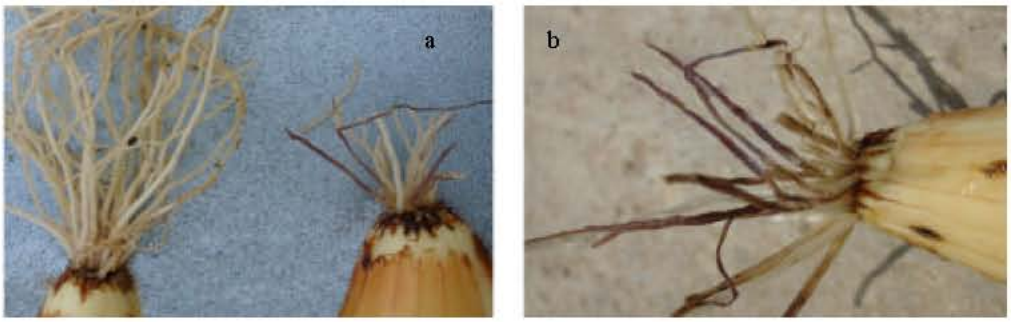

Fig. 5: Symptoms of pink root caused by *Pyrenochaeta terrestris* on onion grown in the greenhouse. A: comparison between control and infected plant. B: pinkish lesions on roots

Fig. 6: Symptoms of rot root caused by *Fusarium* species on onion grown in the greenhouse. A: comparison between control and. B: death of Ainfected pant. C-F: different degree of rot of a basal part and roots.

Fig. 7: Symptoms of *Rhizopycnis vagum* on onion grown in the greenhouse. A: comparison between control and infected plant. B - C: necrosis lesions on roots.

DISCUSSION

Fusarium spp. attack a variety of plants as a soil-borne pathogen [1, 16, 21]. The present study revealed that *F. oxysporum, F. solani* and *F. proliferatum* cause basal and root rot on onion plants. *F. oxysporum* was the most frequently isolated from the basal and root rot lesions. *Fusarium* spp. caused disease severity index (DSI) that ranged from 0 to 5; the highly pathogenic isolates caused severe basal rot. Isolates of *F. oxysporum* induced high mean DSI on plants. Results suggest that this fungus is the main pathogen of the disease. In contrast, isolates of *F. solani* and *F. proliferatum* gave lower DSI. Means. DIS of two fungi followed by the same letter (Table1) are not significantly different ($P = 0.05$) according to Tukey's HSD test [12, 19]. *Fusarium oxysporum* has a wide host range and several formae speciales of the fungus have been differentiated based on the pathogenicity of the isolates [1, 7]. *Fusarium oxysporum* Schlechtend. Fr. f. sp. *cepae* (H.N. Hans.) W.C. Snyder and H.N. Hans is the special form of this fungus that is the pathogenic on onion. Tsutsui, 1991 reported that *F. oxysporum*. f. sp. *cepae* also has the ability to infect squash (*Cucurbita pepo* L.) and pigweed (*Chenopodium album*) [22] but at this study non of the 12 tested species were host for this fungus.

F. proliferatum was another species isolated from visibly young and adult infected plants of onion in our survey. The performed data of the Pathogenicity tests showed that *Fusarium proliferatum* should be regarded as a potentially serious pathogen of onion in Iran. *Fusarium proliferatum* is a species that is easily misidentified as *F. verticillioides* due to closely related morphological traits [16, 23]. Mule *et al*. [18] concentrated on the calmodulin gene region in *F. proliferatum, F. subglutinans* and *F. verticillioides* and obtained a pair specific primer for each fungus. In this study all of the isolates that morphologically were identified as *F. proliferatum* were also confirmed with molecular method. The incidence of fusarium fungi associated with diseased plants don't varied within the plant parts and locations.

Hansen [24] first reported that pink root of onion was caused by *Phoma* sp. based on morphological characteristics but Gorenz *et al*. [20] found the pycnidia to be setose in all cases and transferred the species to the genus *Pyrenochaeta* in 1948. Also Nasr- Esfahani [25] first reported the pink root disease of onion in Esfahan province in Iran. In the present study, this is the first report of pink root disease from Khorasan province in northeast of Iran. However, *P. terestris* was isolated from diseased plants in only a few fields in our survey, symptoms of pink root were observed commonly in growing areas in the Mashhad and Esfaraien and lower in Bojnord and rarely in the Mane. Infected roots are easily invaded by other members of soil flora like bacteria and many species of *Fusarium* [9]. Hence, many saprophytic and pathogenic *Fusarium* species coexist with the casual agent on the diseased roots caused by *P. terrestris*. Since these fungi grow faster than *P. terrestris*, it makes it very difficult to isolate *P. terrestris* from the lesions of pinked roots.

Rhizopycnis vagum is a recently described phytopathogenic species associated with vine decline of cucurbits [11-13] and reported from other plants such as tomato [14]. *R. vagum* is also an endophyte in some plants [26]. This is the first report of *R. vagum* in Iran and onion is a new host. This fungus was co-isolated with *P. terrestris* from roots with typical pink root symptoms. But in Pathogenicity test there was no pink discoloration of roots, as observed in inoculations with *P. terrestris*. Although our glasshouse tests confirmed that *R. vagum* is pathogenic on onion, its role in field disease development is still unclear.

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