IJBPAS, November, 2016, 5(11): 2718-2729

ISSN: 2277–4998

International Journal of Biology, Pharmacy and Allied Sciences (IJBPAS)

'A Bridge Between Laboratory and Reader'

www.ijbpas.com

STUDY OF POPULATION STRUCTURE IN THE GIBBERELLA FUJIKUROI COMPLEX SPECIES, THE CAUSAL AGENT OF RICE FOOT ROT IN THE NORTHERN REGIONS OF IRAN, THROUGH APPLICATION OF SEXUAL CROSSING TESTS

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Received 9th July 2016; Revised 20th Aug. 2016; Accepted 15th Sept. 2016; Available online 1st Nov. 2016

ABSTRACT

In this study, the population structure of the complex species, *Gibberella funjkuroi*, the causal agent of rice foot rot, has been investigated. The samples were obtained from farms in various parts of the northern provinces. Of 41 isolates obtained from different cultivars, all except three had a microconidium chain. In order to study sexual fertility and determine the mating type and population of the isolates, each was crossed on the *in vitro* carrot agar feeding medium with six isolates of the female-fertilized standard representative/agent from one of three mating populations: A,C, and D. Two representative isolates from the two opposite mating types, *MATA-1* and *MATA-2* for mating population A, *MATC-1* and *MATC-2* for mating population C, and *MATD-1* and *MATD-2* for mating population D, were used. Therefore, ten isolates were grouped in mating population A (*Fusarium verticillioides*), two in mating population C (*F.fujiuroi*) and 29 in mating population D (*F.proliferatum*). All isolates of *F.verticillioides* belonged to mating type *MATA-1*. All isolates of *F.fujikuroi* belonged to mating type *MATC-1*, while both mating types *MATD-1* and *MATD-2* were identified for *F.proliferatum* isolates. Owing to the existence of two mating types in the

isolates of mating population D, the possibility of crossing revealed that only two of 36 crosses yielded prithecium possessing ascus and ascospore.

It is evident from this research that the mating populations A, C, and D of complex species of *G.fujikuroi* exist in the northern provinces. Mating population D is dominant.

Keywords: Fusarium, Mating type, Pathogenesis, Rice, Sexual fertility.

INTRODUCTION

The complex species, *Gibberella* Wollenwebr *fujikuroi* (Saw), the causal agent of rice foot rot, is regarded as one of the most significant seed- borne rice diseases. Using the various characteristics of non-sexual form as a criterion, researchers have introduced numerous different species under *Liseola* from the *Fusarium* genus. This has caused confusion in the *Fusariums* taxonomy. Thus, the fungus is also considered a complex species. One of the ways developed to overcome this difficulty was to employ sexual crossing tests for discerning species. Complex species of *G.fujikuroi* consist of nine mating populations, designated by the letters A to I **[1, 2, 3].**

In heterothallic ascomycete fungi, sexual compatibility is controlled by a two-state (dimictic) system in which a locus known as *MAT* with two alleles of *MAT-1* and *MAT-2* exists **[6, 13].** Sexual reproduction in most of the Fusariums, particularly in *G.fujikuroi*, is controlled by the above alleles **[11].** In Argentina, identified two mating populations, A (*F.verticilliodes*) and D (*F.proliferatum*), by crossing the isolates of the causal agent of corn stalk with the standard isolates possessing each of the two mating types, *MAT-1* and *MAT-2*, with various frequencies of each **[4].**

The objectives of this research were as follows:

Identification of the causal agents of rice foot rot in the complex species of *G.fujikuroi* and recognition of the mating populations and types of isolates of disease factor was performed using the standard (tester) representative isolates.

MATERIALS AND METHODS

Sampling

Samples of infected rice foot from transplanting as well as the stalks of diseased bushes filled with white mycellia and Conidia fungus were collected from various rice farms in the northern provinces. Considering the higher sensitivity of the Khazar cultivar to other cultivars, more samples were obtained from this cultivar. The samples were kept in a paper pocket in the refrigerator.

Isolation, purification, and keeping of isolates

Infected tissues were washed for 5 min by mild current of water faucet. Location of the plant's foot was cut into 1 1.5cm sizes and, following surface disinfection with 25% sodium hypochlorite solution for 1 min and washing with sterile distilled water, was transferred to PDA feeding medium and placed in the incubator in 27℃ for 48 h.

After the growth of the colony (mycillium) and sporogenesis, purification was conducted by making the spore single on 2% water-agar medium and by providing fungus suspension. In order to keep the isolates, 1.5ml micro tubes containing sterile sand were used (Padasht, 1993).

In order to eliminate probable available microorganisms in Autoclave, wet sands was sterilized three times in 1.5m/l micro tubes at 120ºC for 20 min. After pouring two drops of purified isolate suspension into sterile sand, the door of the micro tubes was sealed using parafilm. They were kept at 4℃ in the refrigerator until used.

Study of the isolates morphology and identification of the fungal species of disease factor

In this study, resources such as Nirenberg and O'Donnell (1998), O'Donnell et al.(1998) and, Leslie and Summerell (2006) were used to identify the fungal species of the disease factor. For this purpose, a SNA feeding medium was used. After culture of the fungus, small Petri basins were placed in 20℃ and absolute darkness, and, following the passage of 10 _ 14 days, they were studied according to the shape and size of the microconidium on the aerial mycelium; possession of a chain of microconidium on the aerial mycelium; length, existence or lack of clamidospore; existence of monophyalide; and existence of false heads.

Pathogenesis test

Khazar cultivar seeds were initially washed at the water faucet. After disinfection with 25% sodium hypochlorite for 5 min and washing with sterile distilled water, 15 seeds were planted into each vase.

When the transplants (seedlings) had grown sufficiently, five five-leaf transplants remained in each vase and the rest were omitted. Of each isolate, fungus suspension with a concentration of ten conidium per m/l was provided, and one m/l of fungus suspension was injected into each bush using an insulin syringe during the step of growing tall. In the control vase, 1m/l of sterile distilled water was injected into each bush.

Study of situation of the sexual fertility and determination of mating population of the isolates

For this purpose, a carrot-agar feeding medium (Rice chaff/hay obtained from the addition of 50 m/l of rice chaff extract to

500 m/l of carrot extract and promotion of the volume to 1000m/l) was employed for the first time (Correll et al., 1987)

Sexual fertility situation and determination of mating population and type of isolate was examined using the representative (tester) standard fertile-female isolates provided by South Africa. The mycelium ring or arch crossing method was used. In total, 41 Iranian isolates were crossed with three mating populations of A(*F.verticillioides*), C(*F.fujikuroi*), and D(*F.proliferatum*). Each isolate was cultured separately, with 2 to 3cm intervals, on the carrot agar medium inside the Petri dishes alongside one of two mating types, MAT-1 and MAT-2, belonging to each mating population.

Cultures were then placed in 26 to 27℃ and darkness for 5 _ 6 days until colonies had grown sufficiently and established contact with one another.

Next, the cultures, in 22 to 23℃, were placed under continuous florescent light. The formation of prithecium was evaluated once every week for almost two months. Two mating types belonging to each of the mating populations, A, C, and D, were crossed separately as well. These control cultures were placed under equal conditions with the other isolates.

RESULTS

Identification of disease caused agent fungus

Total of 41 isolates, three (GF-235, GF-217, and GF-233) lacked the microconidium chain on the SNA nutrient medium. On the basis of morphological studies of the isolates, three species, *F.proliferatum*, *F.verticillioides*, and *F. fujikuroi* were identified using keys.

Pathogenesis test

Of the 41 isolates, only GF-233 did not develop signs of foot rot. Disease signs for the rest of isolates were observed in the form of white-colored covering of the fungus colony on the stalks **[Figure 1].** The time of appearance of the signs was differed for various isolates so that the signs of the disease appeared on the stalks after 25, 31 or 45 days from the time of inoculation.

Identification of mating population and type of the isolates

By crossing each of the Iranian isolates of (*G.funjikuroi*) with two mating types related to each of three mating populations, A, C, and D (representative isolates), on the carrot agar medium, the kind of population and mating type of isolates were specified **[Figure 2].** Three isolates, GF-235, GF-211, and GF-233, which lacked the microconidium chain, produced perithecium and ascospore as a result of

crossing with the representative isolates related to mating population D.

Sexual infertility was observed in all isolates, and prithecium and ascospore was formed for all of them **[Figure 3].** Of 41 Iranian isolates, ten isolates belonged to mating population A(*F.verticillioides*), two isolates to mating population C(*F.fujikuroi*), and 29 isolates to mating population D (*F.proliferatum*) **[Figure 4].**

Figure 1: Signs of disease resulted when a suspension spore of *Gibberella fujikuroi*, the causal agent of rice foot rot, was injected into the Khazar cultivar in the green house.

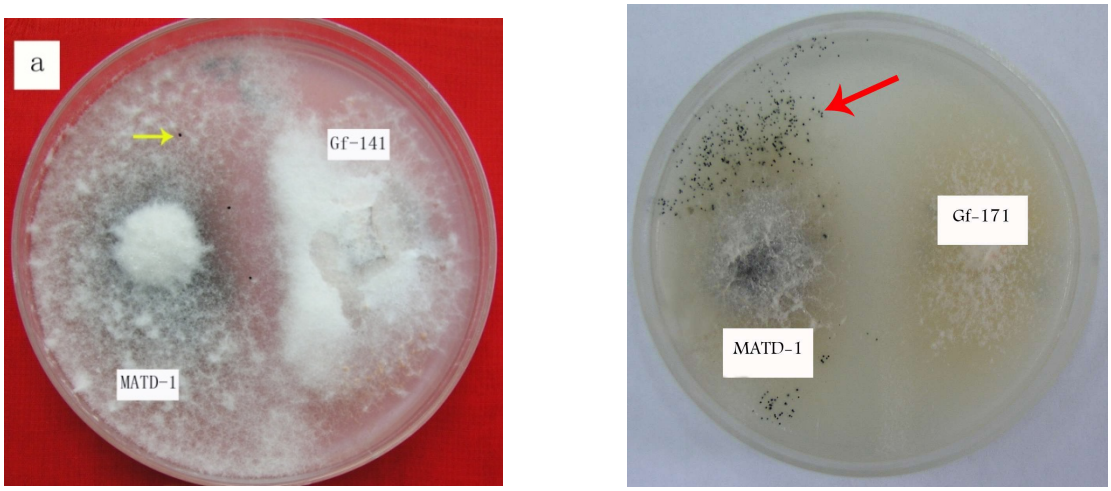

Figure 2: The formed pritheciums in crossing the isolates of GF-171 (Right) and GF-141 (left).Both with mating type *MAT-2* from the mating population of D in the complex species of *Gibberella fujikuroi* with isolate of standard representative/agent (tester) from mating type *MATD-1* on the carrot agar nutrient medium.

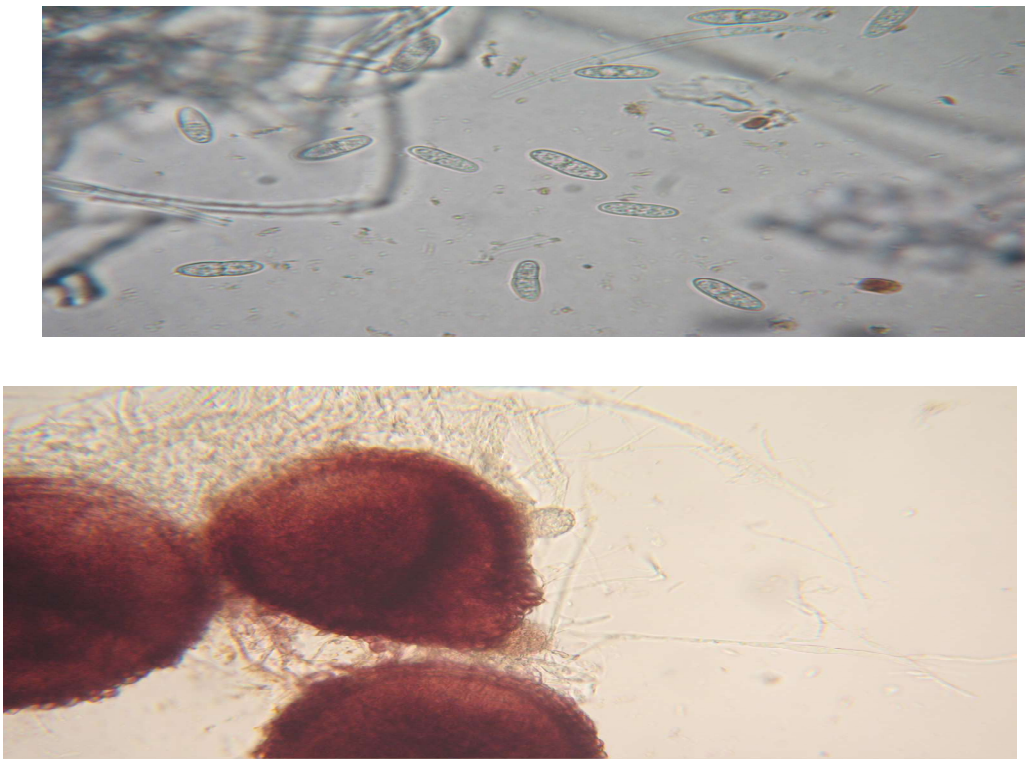

Figure 3: The pritheciums established from crossing isolate GF-237 with mating type *MATD-2* from mating population D, complex species *Gibberella fujikuroi* with the standard representative (tester) isolate (see below with 160- times magnification). Two-cell ascospores obtained from crossing of the isolate of GF-299 in this same population with standard representative isolate (see above with 400-times magnification).

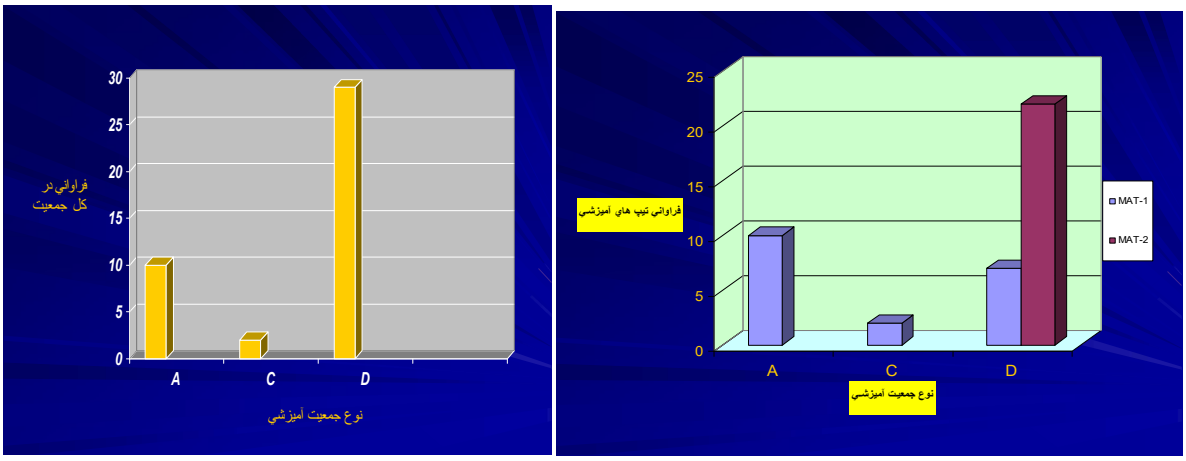

Figure 4: Frequency of each one of the mating populations of A, C, and D among the isolates of *Gibberella fujikuroi* complex species studied in this research

Positive crossing relates to that class of crossings in which prithecium forms and ascospore isolates from prithecium. All ten isolates of mating population (A) belonged to the mating type *MATA-1*. Two isolates of mating population (C) belonged to mating type *MATC-1*. Of 29 isolates of mating population (D), seven from mating type *MATD-1* and 22 from mating type *MATD-2* were identified **[Table 1].**

Both mating types required for successful fertility were observed only in the isolates of mating population (D) (Figure 5). Therefore, the possibility of crossing only in mating population (D) was noted, owing to the presence of both mating type

MATD-1 and *MATD-2*. Experimentally, only two of 36 crossings performed among Iranian isolates belonging to this population, GF-248 with GF-187 and GF-266 with GF-45, led to the formation of prithecium and ascospore. Fertility was not observed in any of the other crossings

Table 1: Specifications of the Iranian isolates of the *Gibberella fujikuroi* complex species acquired from rice on the basis of tests of fertility crossing and sexual compatibility with standard representative (tester) isolates from various mating populations. All isolates have been collected from the various regions of Iran northern provinces.

Name of isolate	Mating population	Mating type	Male/Female	Anamorph species of fungus
Gf-5	*MP-A	MATA-1	Male	*F.verticillioides*
Gf-13	MP-A	MATA-1	Male	*F.verticillioides*
Gf-37	MP-A	MATA-1	Male	*F.verticillioides*
Gf-49	MP-A	MATA-1	Male	*F.verticillioides*
Gf-89	MP-A	MATA-1	Male	*F.verticillioides*
Gf-91	MP-A	MATA-1	Male	*F.verticillioides*
Gf-93	MP-A	MATA-1	Male	*F.verticillioides*
Gf-111	MP-A	MATA-1	Male	*F.verticillioides*
Gf-115	MP-A	MATA-1	Male	*F.verticillioides*
Gf-173	MP-A	MATA-1	Male	*F.verticillioides*
Gf-105	MP-C	MATC-1	Male	*F.fujikuroi*
Gf-151	MP-C	MATC-1	Male	*F.fujikuroi*
Gf-201	MP-D	MATD-2	Male	*F.proliferatum*
Gf-155	MP-D	MATD-1	Male	*F.proliferatum*
Gf-243	MP-D	MATD-2	Male	*F.proliferatum*
Gf-187	MP-D	MATD-1	Male	*F.proliferatum*
Gf-215	MP-D	MATD-2	Male	*F.proliferatum*
Gf-19	MP-D	MATD-2	Male	*F.proliferatum*
Gf-237	MP-D	MATD-2	Male	*F.proliferatum*
Gf-266	MP-D	MATD-2	Male	*F.proliferatum*
Gf-35	MP-D	MATD-2	Male	*F.proliferatum*
Gf-41	MP-D	MATD-2	Male	*F.proliferatum*
Gf-43	MP-D	MATD-2	Male	*F.proliferatum*
Gf-69	MP-D	MATD-2	Male	*F.proliferatum*
Gf-81	MP-D	MATD-2	Male	*F.proliferatum*
Gf-141	MP-D	MATD-2	Male	*F.proliferatum*
Gf-211	MP-D	MATD-2	Male	*F.proliferatum*
Gf-229	MP-D	MATD-2	Male	*F.proliferatum*
Gf-233	MP-D	MATD-2	Male	*F.proliferatum*
Gf-248	MP-D	MATD-2	Male	*F.proliferatum*
Gf-123	MP-D	MATD-2	Male	*F.proliferatum*
Gf-117	MP-D	MATD-2	Male	*F.proliferatum*
Gf-45	MP-D	MATD-1	Male	*F.proliferatum*
Gf-239	MP-D	MATD-2	Male	*F.proliferatum*
Gf-131	MP-D	MATD-1	Male	*F.proliferatum*
Gf-99	MP-D	MATD-1	Male	*F.proliferatum*
Gf-203	MP-D	MATD-1	Male	*F.proliferatum*
Gf-139	MP-D	MATD-2	Male	*F.proliferatum*
Gf-183	MP-D	MATD-2	Male	*F.proliferatum*
Gf-171	MP-D	MATD-2	Male	*F.proliferatum*
Gf-235	MP-D	MATD-1	Male	*F.proliferatum*

***mating population**

DISCUSSION

The complex species, *G.fujikuroi*, is pathogenic on various herbal/plant hosts, creating dangerous diseases in some cases. As yet, on the basis of sexual compatibility tests in the laboratory, nine mating populations have been identified for this species, designated with letters from A to I. Each one of these populations is a biological species whose anamorphic morphological characteristics have been defined and whose teleomorphe has been identified and introduced **[3, 5, 6].**

Three species of *F.verticillioises*, *F. fujikuroi*, and *F.proliferatum* belonging to the mating populations A, C, and D from this complex species, respectively, have been isolated from rice in different parts of the world. These three species are morphologically similar and are all agents of rice foot rot**[3].** A study of the morphological attributes of isolates showed that the causal agents of rice foot rot in these provinces are highly similar to the species *F.verticillioides, F.fujikuroi and F.proliferatum*.

At 17 days post-infection, Padasht (1993) observed signs of foot rot in the rice seedlings. In the current research, the first signs of the disease were observed only after 25 days. In some cases, signs of disease were seen as late as 45 days post-infection. Medium temperature is an effective factor in this delay; the optimal temperature for germination of the fungus following injection is in the range of 25 _ 30℃ **[7].** However, because the pathogenesis test was performed in the green house in June and July, during summer, high temperature should be considered as a prohibitor of fungal growth. When the temperature was lowered up to approximately 30℃, signs of disease appeared. In the pathogenesis test in summer, no signs of growing should tall/elongation in the bushes were observed. Strains of the mating population C(*F.fujikuroi*) produce a large quantity of giberllic acid, leading to elongation (growing tall) of the infected bushes,while strains of the two mating populations, A and D, produced a large quantity of Fumonisin **[1,8,9].**

The observation that prithecium formed on the remaining stalks in the farm following the harvest of rice suggests fertility of the *G.fujikuroi* population in the farms**[9,10].** However, a low percentage of crossing on the carrot-agar nutrient medium of the Iranian isolates was accompanied by the production of prithecium and ascospore in the laboratory (*in vitro*) conditions. This might, due to differences in environmental conditions between the laboratory and nature.

The result of crossing Iranian isolates with the standard representative (tester) isolates showed that isolates belonging to the three mentioned mating populations of the disease factor in the northern provinces are active in terms of sexual fertility.

However, in the mating population A, mating type of *MATA-2* was not identified among the studied isolates. It appears that frequency of the mating type *MATA-2* in mating population A is low in the Iranian isolates. Of course, the number of isolates of this population applied in the current research was not high, and, probably, presence of the mating type *MATA-2* in population A will be evident by studying the higher isolates. Two isolates of the mating population (C) belonged to the mating type (*MATC-1*).Studying only these two isolates does not allow conclusions regarding sexual fertility and frequency of mating types in this population. Generally, this population is less important than populations (A) and (D) on the rice. In mating population (D), both mating types, *MATD-1* (eight isolates) and *MATD-2* (15 isolates) were identified. Considering the relatively high fertility shown by the isolates of this population in the laboratory as well as the existence of both mating types among isolates, it is anticipated that the occurrence of sexual reproduction and, subsequently, of sexual recombination in this population occur in nature. In a previous study on isolates obtained in Guilan province, 47 of 60 isolates, prithecium was isolated with the standard representative (tester) isolates, and ascospore was isolated from prithecium **[8].**

In this research, the existence of the mating type *MATC-2* was confirmed in three of ten isolates related to the mating population (C). The mating type *MATA-2* was not found in the 14 isolates related to the mating population (A).

With regard to the results obtained from sexual fertility studies of northern provinces isolates, *F.proliferatum* isolates are more fertile than two other species to which they were compared. In a number of the pritheciums formed in the sexual fertility tests and their fertility according to produced ascospores, this problem was also evident.

Considering that *F.prolifratum* is a dominant species in terms of frequency and power of sexual fertility in the northern provinces, it may be introduced as the most significant agent of rice foot rot in those regions.

In the majority of the total crossings performed for the various isolates in this research, prithecium was formed in the direction of the standard representative (tester) isolates, which demonstrates the fertility of the Iranian male isolates. In the

strains of *G.fujikuroi* studied by Leslie and Klein (1996), fertile female occurred infrequently, and populations were a combination of hermaphrodites and infertile female strains **[1].** However, strains of infertile male and fertile female were also identified **[11,12,13,14].** The number of pritheciums produced as a result of contact between the studied and representative (tester) isolate was different for various isolates, and related to the fertility power of the studied isolate because the fertility capability of the agent isolates, in terms of male and female sexual organs, is constant between crossings. In order to establish prithecium and produce ascospore, two isolates must be crossed with each other in a mating population; that is, two opposed mating types.

Padasht (1993) reported *F.fujikuroi* as a Bakane factor and causal agent of rice foot rot from Guilan farms **[7, 15, 16, 17].**

In the current research, on the basis of pathogenesis and sexual fertility tests, it was evident that, in addition to *F.fujikuroi* (mating population C), *F.verticillioides* (mating population A), and *F.proliferatum* (mating population C) are disease factors in northern provinces. *F.proliferatum* is most frequent, followed by *F.verticilliodes* and *F.fujikuroi*.

CONCLUSION

In one of the mating populations of the *G.fujikuroi* complex species, a high sexual fertility was observed. The existence of high fertility is confirmed by a study of higher isolates from two populations, (A) and (C), because most of the isolates belonging to these populations demonstrated successful sexual crossings in the laboratory. This finding suggests that sexual reproduction plays an active and important role in the life cycle of this group of pathogenic fungi, and gives the appearance of diversity. The importance of this problem should be noted in epidemiological studies and production and application of resistant cultivars.

In this study, three mating populations, A (*F.verticillioides*), C (*F.fujikuroi*), and D (*F.proliferatum*) were confirmed as the principal causal agents of the rice foot rot in northern provinces. Further, mating population (D) was confirmed as the dominant population.

ACKNOWLEDGMENTS

This research was conducted using the facilities of the Institute of Rice Research, Rasht, in the Phytopathology department. The writers/correspondent appreciate the heartfelt collaboration of the chairmanship of the institute, as well as the respectful person in charge of Phytopathology. The

representative (agent/tester) isolates were taken, by mediation of Dr. Gordon Shephard, from Prof. Walter Marasas in South Africa (PROMEC unit of Medical Research).

ETHICS

This study was confirmed in science and research branch of Islamic Azad University, Tehran, Iran with ethical number of 7667.

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