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## OCCURRENCE OF A<sub>2</sub> MATING TYPE AND MICROMORPHOLOGY OF OOSPORES OF *PHYTOPHTHORA INFESTANS* IN GOLESTAN PROVINCE OF IRAN

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### ABSTRACT

The aim of this study was to determine the mating types, origin and micromorphology of oospores of *Ph. infestans* isolated from the potato fields in Golestan province of Iran during 2014-16. In total, 197 isolates were assessed for mating type. The average frequencies of mating types A<sub>1</sub> and A<sub>2</sub> were 85.8 and 14.2% respectively. A<sub>2</sub> mating type was found only from two regions. A total of 9 self-fertile isolates (5.3%), which only belonged to the A<sub>1</sub> mating type were detected. The average value for oospore production index (OPI) within the interaction zone between the two isolates was estimated one (very low) in A<sub>1</sub>×A<sub>1</sub> and four (very high) in A<sub>1</sub>×A<sub>2</sub> crosses. Oospores of hybrid origin were observed in A<sub>1</sub> ×A<sub>2</sub> and self-fertile oospores only in A<sub>1</sub> ×A<sub>1</sub> paired cultures. Parthenogenetic oospores (without antheridia) were not observed in A<sub>1</sub> ×A<sub>1</sub> or A<sub>2</sub> ×A<sub>2</sub> paired cultures. The results of oospores micromorphology indicated that the antheridia in hybrid oospores were amphigynous and from half spheric to nearly spheric, almost hyaline, from 18.5-23.5 × 10.5-15.5 μm in size. Oogonia were nearly spheric from nacarat to brown in colour. The colour of the hybrid oospores varied from brown to dark-brown, spheric, varied from 25-35 μm in diameter with two thick walls with thickness of 2.5-5.5 μm. The self-fertile oospores were with a thin wall with thickness of 1.5 μm, spherical and varied from 40-60 μm in diameter. The monitoring of A<sub>1</sub> and A<sub>2</sub> mating type ratios of oospores are important to aid in the prediction of the extent of sexual recombination and thus the risk of long-lived oospores serving as primary inoculum sources in the main potato production regions.

**Keywords:** Iran, Mating type, Micromorphology, Oospore, Oomycetes, Potato, *Phytophthora infestans*.

### INTRODUCTION

Potato late blight, a disease caused by *Phytophthora infestans* (Mont.) de Bary is one of the most devastating plant diseases worldwide (Shaw, 1996) including Iran, especially in Golestan province. The disease was first reported from north of Iran in 1965 (Ershad, 2009). Golestan is one of the 31 provinces of Iran, located in the north-east of the country, south of the Caspian Sea (Long. 53°57' - 56°22' and Lat. 36°30' - 38°08'). It has a moderate and humid climate known as "the moderate Caspian climate". About 10 to 15 percent of the country's potato crop is produced in irrigated farms of this province (Aghajani, 2015).

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According to the Ministry of Agriculture Bureau in Golestan province, the potato late blight reached an epidemic level in 2006 in Golestan and resulted in severe damage to the crop (Sadraei, 2008). The same disease also reached an epidemic in Ardabil province of Iran (located in north-west of the country not very far from the Caspian Sea) in 1997 and caused an 80% crop loss (Zargarzadeh *et al.*, 2008)

*Ph. infestans* is a heterothallic oomycete with two mating types, A<sub>1</sub> and A<sub>2</sub> (Lehtinen *et al.*, 2009). Interaction between hyphae of opposite mating type can result in the formation of oospores. Initially, both A<sub>1</sub> and A<sub>2</sub> mating types were found only in central Mexico (Gallegly, 1958; Smoot *et al.*, 1958); while A<sub>1</sub> alone distributed worldwide (Drenth *et al.*, 1995). Later on A<sub>2</sub> which was earlier restricted to the Central Mexico only also reported from Switzerland (Hohl and Islen, 1984) and in many other countries in Europe, Africa, Asia and the America (Spielman *et al.*, 1991; Fry *et al.*, 1993; Drenth *et al.*, 1995).

Appearance of A<sub>2</sub> mating type led to reproducing sexually of heterothallic *Ph. infestans*, then producing oospores and recombining sexually by oospores formation. Oospores are able to overwinter in the remainders and soil, thereby it become new sources of infection and increase possibilities of more serious epiphytotic. Sexual reproduction results in a wider variety of genotypes (Zhang *et al.*, 2001) and more diverse in *Ph. infestans* populations (Drenth *et al.*, 1995) which might contain more virulent isolates or races which enabled the pathogen to avoid recognition by any specific resistance gene, or combinations of resistance genes in the potato population. Since fungicide resistance isolates produced through sexual

recombination, some fungicides will lose the value of application (Zhang *et al.*, 2001).

The aim of this study was to determine the mating types, origin and micromorphology of oospores of *Ph. infestans* in isolates obtained from the potato growing regions from different fields in Golestan province of Iran, during 2014-16.

#### MATERIALS AND METHODS

**Collection of samples:** During 2014-16, samples of blighted potato leaves were collected from most of the potato growing regions from different fields in Golestan province of Iran (Table 1). Samples were placed in labeled paper envelopes and transferred to the laboratory for isolation of the pathogen.

Table 1. Frequency of mating types for *Phytophthora infestans* isolates from blighted potato leaves from five regions from different potato fields in Golestan province of Iran.

Year	Region	Cultivar	Isolates	Mating types		A <sub>1</sub> Self-fertile isolates
				A <sub>1</sub>	A <sub>2</sub>	
2014	Valikabad	<i>Agria</i>	20	15 (75) <sup>a</sup>	5 (25)	2
	Feyzabad	<i>Marfona</i>	18	14 (77.8)	4 (22.2)	1
	Jelin	<i>Sante</i>	12	12 (100)	0 (0)	0
	Sorkhankalateh	<i>Arinda</i>	10	10 (100)	0 (0)	0
	Marzankalateh	<i>Draga</i>	10	10 (100)	0 (0)	0
Total			70	61 (87.1)	9 (12.9)	3 (4.9)
2015	Valikabad	<i>Agria</i>	18	12 (66.7)	6 (33.3)	2
	Feyzabad	<i>Marfona</i>	14	9 (64.3)	5 (35.7)	1
	Jelin	<i>Sante</i>	14	14 (100)	0 (0)	0
	Sorkhankalateh	<i>Arinda</i>	12	12 (100)	0 (0)	0
	Marzankalateh	<i>Draga</i>	12	12 (100)	0 (0)	0
Total			70	59 (84.3)	11 (15.7)	3 (5.1)
2016	Valikabad	<i>Agria</i>	16	12 (75)	4 (25)	2
	Feyzabad	<i>Marfona</i>	15	11 (73.3)	4 (26.7)	1
	Jelin	<i>Sante</i>	10	10 (100)	0 (0)	0
	Sorkhankalateh	<i>Arinda</i>	9	9 (100)	0 (0)	0
	Marzankalateh	<i>Draga</i>	7	7 (100)	0 (0)	0
Total			57	49 (86)	8 (14)	3 (6.1)
Total number			197	169 (85.8)	28 (14.2)	9 (4.6)

a: Percentage of mating type isolates.

**Isolation and maintenance of *Ph. Infestans*:** Isolation was made by placing blighted leaf fragments on potato tuber slices (about 5 mm thickness) of a susceptible cultivar (*Agria*) in sterilized humid chambers and incubating at 18°C. After 5-7 days, pathogen grew on the surface of the tuber slices. After visible intensive sporulation, mycelium with zoosporangia was transferred onto a small piece of a surface-sterilized tuber of cv. *Agria*. These pieces were put on rye B agar culture media (Caten and Jinks, 1968) with antibiotic

mixture (Ampicillin, Rifamycin, Vancomycin) and fungicide (Benomyl at a concentration of 500 ppm). Cultures were incubated in the dark at 18°C and transferred every 3-4 weeks. Stock cultures of all isolates were maintained on slants of rye A agar culture media at 15°C for further studies. In total, 197 pure isolates of *Ph. infestans* were obtained from the different potato fields in Golestan province of Iran.

**Determination of mating types:** The mating type of an isolate was determined by pairing of unknown isolates

with standard tester strains A<sub>1</sub> and A<sub>2</sub> (*Ph. infestans*) on rye B agar (Forbes, 1997) with tomato juice (Rafiee and Banihashaemi, 2015). A 5-mm agar discs of *Ph. infestans* were cut from the edge of 7-day-old culture of unknown mating type and paired 4 cm apart from a similar agar disc of either the A<sub>1</sub> or A<sub>2</sub> standard tester strain (dual culture) on the center of a rye B agar Petri dish (9 cm). Inoculated Petri dishes were incubated at 15-18°C in the dark for about 14 days or until oospores were formed (Tooley *et al.*, 1989). Four plates were prepared for each paired culture. After two weeks, each paired culture was mixed with 100 ml distilled water in a blender at high speed for 1 min. The oospore formation was examined by placing a single 10- $\mu$ l droplet by a micropipette on the microscopic slide for the presence or absence of oospores (Mazakova *et al.*, 2006).

Also, from the interface of two growing cultures mycelial plugs were randomly removed with the help of corkborer, squashed over microscopic slide and observed under the light microscope (Nikon: ALPHAPHOT-2 YS2). When oospores with antheridia formed in the A<sub>2</sub> pairing and did not form in the unknown isolate colony, the unknown isolate was rated as an A<sub>1</sub>. If oospores with antheridia formed in the A<sub>1</sub> pairing and not the unknown isolate colony, the unknown isolate was rated as an A<sub>2</sub> (Smirnov and Elansky, 1999).

**Determination of self-fertile isolates:** When oospores with antheridia formed in the A<sub>1</sub>×A<sub>1</sub> or A<sub>2</sub>×A<sub>2</sub> paired cultures, it was rated as a self-fertile isolate (Smirnov, 2001).

**Determination of parthenogenetic isolates:** When oospores without antheridia formed in the A<sub>1</sub>×A<sub>1</sub> or A<sub>2</sub>×A<sub>2</sub> paired cultures, it was rated as a parthenogenetic isolate (Smirnov, 2001).

**Determination of origin of oospores:** Each unknown isolate of *Ph. infestans* after determination of mating type (A<sub>1</sub> or A<sub>2</sub>) was paired and self-paired on Petri dishes containing rye B agar with tomato juice. After this, inoculated Petri dishes were incubated at 15-18°C in the dark for about 21 days. Oospores based on their origin were categorized to three groups: hybrid, self-fertile and parthenogenetic. If A<sub>1</sub>×A<sub>2</sub> paired cultures produced oospores with antheridia, it was rated as a hybrid oospore (Zhang *et al.*, 2001). If A<sub>1</sub>×A<sub>1</sub> or A<sub>2</sub>×A<sub>2</sub> paired cultures produced oospores with antheridia, it

was rated as a self-fertile oospore (Zhang *et al.*, 2001; Smirnov and Elansky, 1999). If A<sub>1</sub>×A<sub>1</sub> or A<sub>2</sub>×A<sub>2</sub> paired cultures produced oospores without antheridia, it was rated as a parthenogenetic oospore (Zhang *et al.*, 2001).

**Determination of oospore production index (OPI):** Oospore production index was estimated based on examination of 10 microscopic fields (0.2 mm<sup>2</sup> each) within the interaction zone of the two parental cultures (Smoot *et al.*, 1958). The zone in which oospores were formed was first located, and 10 microscopic fields were selected by random movement of the mechanical stage along this zone. The number of oospores in a cross-section from the agar surface to the bottom of the plate was quantified using an oospore production index (OPI). The OPI represents average oospore production in the mating region and is expressed in four classes: 0, 0 oospores mm<sup>-2</sup> (zero); 1, 1-50 oospores mm<sup>-2</sup> (very low); 2, 51-250 oospores mm<sup>-2</sup> (medium); 3, 251-500 oospores mm<sup>-2</sup> (high) and 4, >500 oospores mm<sup>-2</sup> (very high) (Flier *et al.*, 2001).

**Micromorphology of oospores:** The micromorphology of oospores was examined based on the presence of antheridia, presence and shape of oogonia, oospore diameter and wall thickness with a bright-field microscope at ×40 (Cohen *et al.*, 1997).

#### STATISTICAL ANALYSIS

All statistical analysis were performed using the SPSS v. 22 and Minitab v. 18 statistical packages.

#### RESULTS

**Mating types:** A total of 197 isolates of *Ph. infestans* isolated from blighted potato leaves collected from the most of the potato growing regions from different fields in Golestan province of Iran. Among them 169 isolates (85.8%) belongs to the A<sub>1</sub> mating type, 28 isolates belongs to the A<sub>2</sub> mating (14.2%) isolated from only two regions: Valikabad and Feyzabad. In 2014, the ratio of A<sub>1</sub> to A<sub>2</sub> mating types was 87:13, in 2015, the ratio was 84:16 and in 2016, the ratio was 86:14 for the benefit of A<sub>1</sub> mating type (almost 6:1) for every three years. The hierarchical cluster analysis of percentage of frequency of 197 isolates of *Ph. infestans* using Ward's method and squared Euclidean distance indicated that isolates grouped into two distinct clusters. Cluster I consisted of isolates from Valikabad and Feyzabad and had A<sub>1</sub> and A<sub>2</sub> mating types. Cluster II consisted of isolates from Jelin, Sorkhankalateh and Marzankalateh with the A<sub>1</sub> mating type (Figure 1).

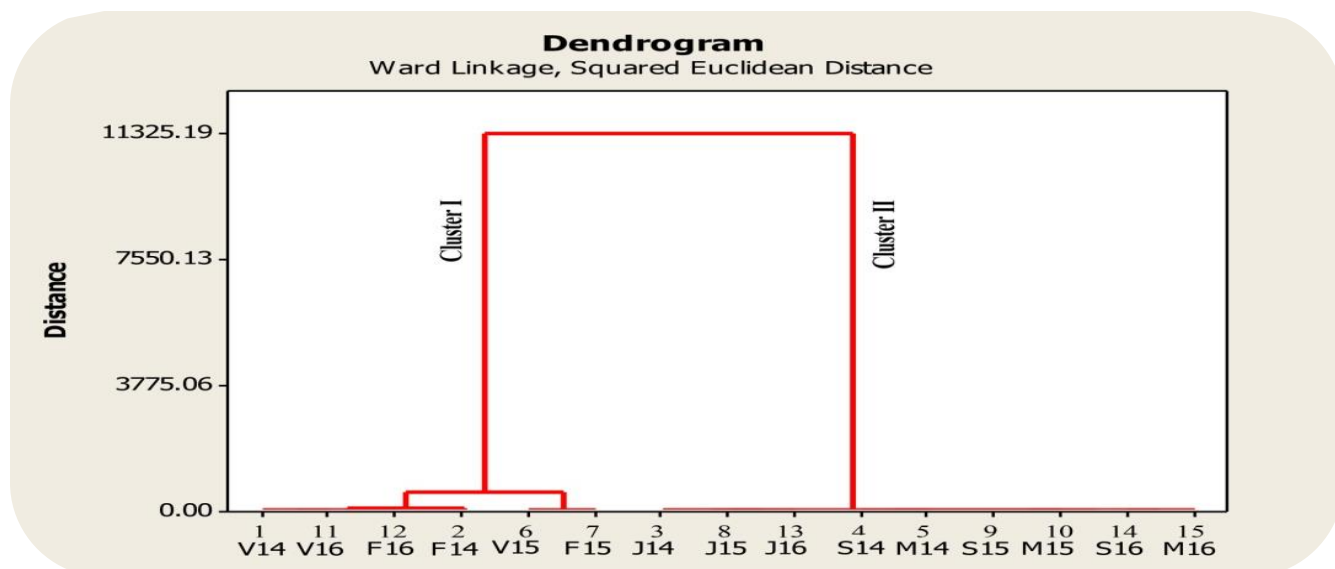


Figure 1. Clustering tree of percentage of frequency of 197 isolates of *Phytophthora infestans* during 2014-16 from the five potato growing regions in Golestan province of Iran using Ward's method and squared Euclidean distance.

Isolates:

\* V14: Valikabad 2014, V15: Valikabad 2015, V16: Valikabad 2016.

\* F14: Fazelabad 2014, F15: Fazelabad 2015, F16: Fazelabad 2016.

\* J14: Jelin 2014, J15: Jelin 2015, J16: jelin 2016.

\* S14: Sorkhankalateh 2014, S15: Sorkhankalateh 2015, S16: Sorkhankalateh 2016.

\* M14: Marzankalateh 2014, M15: Marzankalateh 2015, M16: Marzankalateh 2016.

**Self-fertile isolates:** During 2014-16, out of 169 isolates of *Ph. infestans* of  $A_1$  mating type, 9 were found self-fertile isolates (5.3%), including six isolates from Valikabad and three isolates from Feyzabad.

**Parthenogenetic isolates:** None of the 197 isolates were

found parthenogenetic.

**Origin of oospores:** In  $A_1 \times A_2$  paired cultures, oospores with antheridia (hybrid) were produced in great abundance. Self-fertile oospores (with antheridia) were observed only in  $A_1 \times A_1$  paired cultures. No oospores were produced in  $A_2 \times A_2$  paired cultures. Parthenogenetic oospores (without antheridia) were not observed in  $A_1 \times A_1$  or  $A_2 \times A_2$  paired cultures.

**Oospore production index (OPI):** The average value for oospore production index (OPI) at the interaction zone between the two isolates was estimated one (very low) in  $A_1$  self-fertile isolates from Valikabad and Feyzabad and four (very high) in  $A_1 \times A_2$  crosses from all five regions (Figure 2).

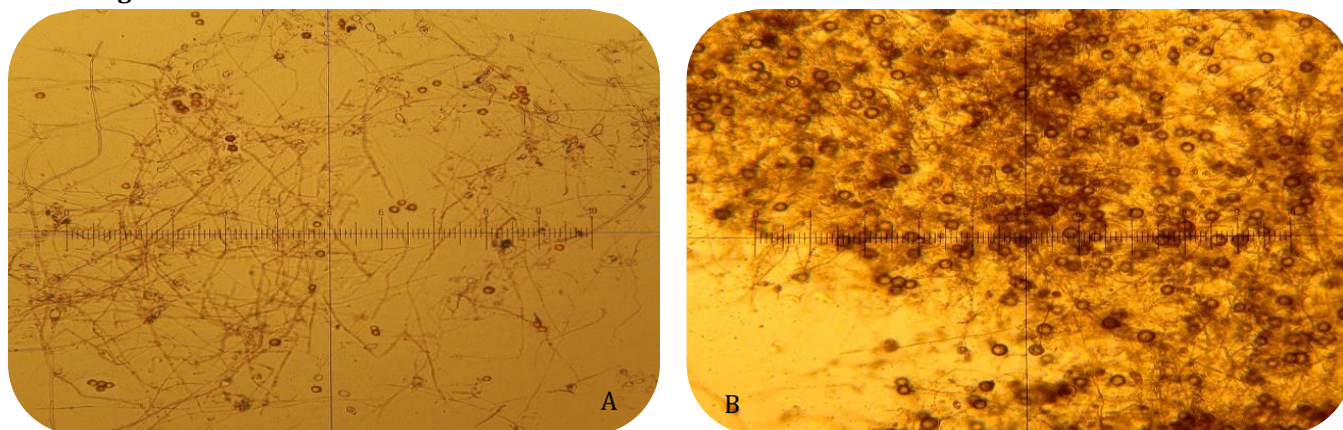


Figure 2. Oospores of *Phytophthora infestans* in microscopic field (0.2 mm<sup>2</sup>), A) 1, 1-50 oospores (very low) in  $A_1$  self-fertile isolates, B) 4, >500 oospores (very high) in  $A_1 \times A_2$  crosses. (0, 0 oospores mm<sup>-2</sup> (zero); 1, 1-50 oospores mm<sup>-2</sup> (very low); 2, 51-



250 oospores  $\text{mm}^{-2}$  (medium); 3, 251-500 oospores  $\text{mm}^{-2}$  (high) and 4, >500 oospores  $\text{mm}^{-2}$  (very high) (Flier *et al.*, 2001).

**Micromorphology of oospores:** The antheridia in hybrid oospores from  $A_1 \times A_2$  paired cultures were amphigynous and from half spheric to nearly spheric, almost hyaline, from  $18.5\text{-}23.5 \times 10.5\text{-}15.5 \mu\text{m}$  in size. Oogonia were nearly spheric from nacarat to brown in colour. The colour of the hybrid oospores varied from

brown to dark-brown, spheric, varied from  $25\text{-}35 \mu\text{m}$  in diameter with two thick walls with thickness of  $2.5\text{-}5.5 \mu\text{m}$ . The self-fertile oospores from  $A_1 \times A_1$  paired cultures were with a thin wall with thickness of  $1.5 \mu\text{m}$ , spherical and varied from  $40\text{-}60 \mu\text{m}$  in diameter (Figure 3).

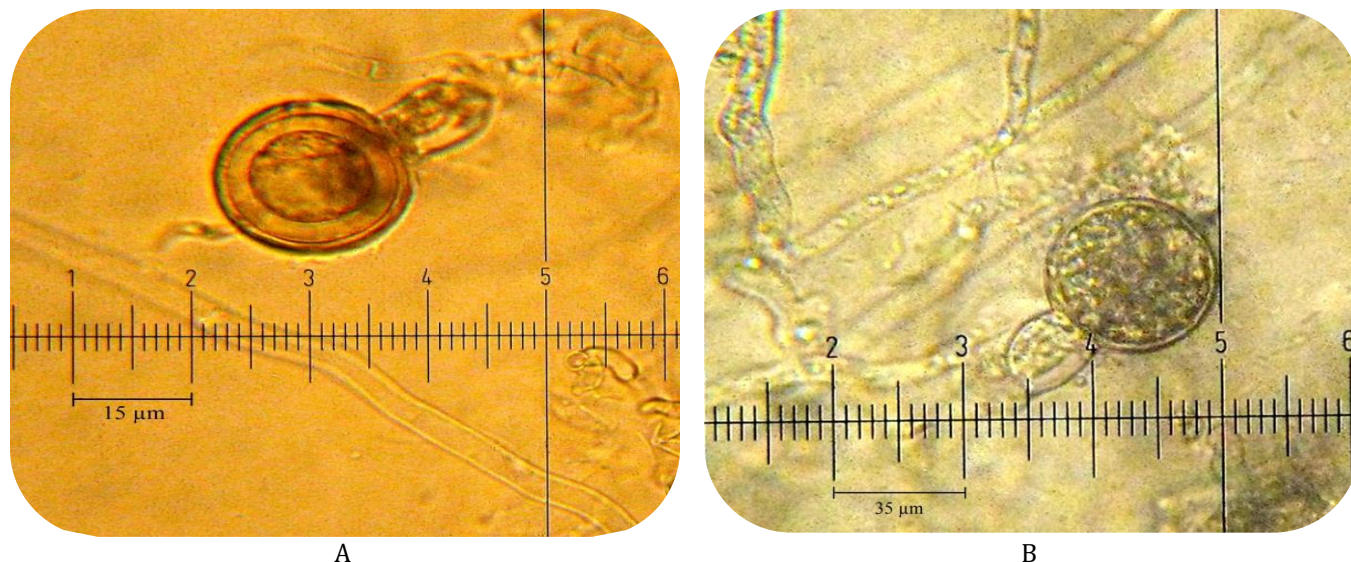


Figure 3. Microscopic view of oospores of *Phytophthora infestans*, A) hybrid oospore from  $A_1 \times A_2$  paired cultures, B) self-fertile oospore from  $A_1 \times A_1$  paired cultures.

## DISCUSSION

It is a very important problem whether  $A_2$  mating type occur in the main potato production regions, because the occurrence of  $A_2$  mating type means oospores formation and sexual reproduction of the pathogen in the life cycle. Sexual reproduction may generate genetic recombination and produce more virulent physiological races and may increase primary infection sources of the diseased, because oospores can overwinter in the remainders and soil (Zhang *et al.*, 2001). The monitoring of  $A_1$  and  $A_2$  mating type ratios is important to aid in the prediction of the extent of sexual recombination and thus the risk of long-lived oospores serving as primary inoculum sources. A study of the distribution of the  $A_1$  and  $A_2$  strains of *Ph. infestans* is fundamental to understanding the significance of mating type to both the generation and maintenance of genetic diversity and to disease aetiology (Cooke and Lees, 2004).

Afzali *et al.* (2008); for the first time observed the occurrence of the  $A_2$  mating types from the potato producing farms of Ardabil province of Iran. Zolfaghary and Gran (2015), have reported the first occurrence and

distribution of the  $A_2$  mating type of *Ph. infestans* from the potato fields in Golestan province in the north-east of Iran. These results after the first report on occurrence of the  $A_2$  mating type in Golestan province confirm the second detection of the  $A_2$  mating type of *Ph. infestans* in Golestan province of Iran and show the distribution of both mating types in several major potato producing regions. The results of this study indicated that the ratio of the  $A_1$  to  $A_2$  mating types of *Ph. infestans* from two regions: Valikabad and Feyzabad was almost 6:1 for the benefit of the  $A_1$  mating type for every three years. Afzali *et al.* (2008), have reported that the ratio of the  $A_1$  to  $A_2$  mating types of *Ph. infestans* from Ardabil province was 83.87 to 16.13%, almost 5:1 for the benefit of  $A_1$  mating type. Zolfaghary and Gran (2015); have reported that this ratio was 86.18 to 13.82%, almost 6:1 for the benefit of the  $A_1$  mating type in Golestan province. This ratio is not suitable for sexual reproduction in natural populations of *Ph. infestans* in potato fields. Therefore, the average value for oospore production index (OPI) for the  $A_1$  to  $A_2$  mating types of *Ph. infestans* from Valikabad and Feyzabad in potato blighted tissues (*in vivo*) can be

less than OPI in paired cultures (*in vitro*). The presence of the A<sub>2</sub> mating type during three years of research in Valikabad and Feyzabad perhaps was related to the conditions of Metalaxyl using in these regions. This systemic fungicide had been used more generally and earlier for several years in these regions. Metalaxyl was rarely used in Jelin, Sorkhankalateh and Marzankalateh. Thus, long time application of metalaxyl perhaps induced A<sub>1</sub> mating type turning into A<sub>2</sub> (Ann and Ko, 1988; Chang and Ko, 1990; Zhang *et al.*, 2001; Savenkova and Cherepennikova, 2002).

The presence of the A<sub>1</sub> self-fertile isolates only in Valikabad and Feyzabad perhaps was related to the effect of metalaxyl on the A<sub>1</sub> isolates (Elliot, 1983). On the other hand, potato cultivars which cultivated in Valikabad and Feyzabad (Agria and Marfona) for several years were susceptible to foliar late blight. Ranjbar *et al.* (2012), have reported that two potato cultivars namely, Agria and Marfona were susceptible to late blight on foliage. Zargarzadeh *et al.* (2008); were found that there were significant correlations between climatic factors, particularly temperature and rainfall, with the incidence of the late blight. It can be concluded that under the condition of about 30 mm rainfall and higher temperatures of about 19°C during the critical period of the growing season, we must expect the development of the potato late blight disease and must use preventive measure immediately. The weather data, including temperature, relative humidity and rainfall were obtained from the synoptic weather station of Golestan province in 2014-15, indicated that the weather conditions were favorable for the growth of pathogen of potato late blight. But, in 2016, reduction of rainfall and drought in Golestan province reduced the total number of isolates from 70 to 57. Our findings indicated that the self-fertile isolates only belonged to the A<sub>1</sub> mating type of *Ph. infestans*. Afzali *et al.* (2008); from Ardabil province have reported that self-fertile isolates of *Ph. infestans* only belonged to the A<sub>1</sub> mating type. Our investigation indicated that oospores of 197 isolates of *Ph. infestans*, obtained from the most of the potato growing regions from different fields in Golestan province of Iran (*in vitro*) had hybrid and self-fertile origins. Smirnov (2001); has categorized oospores of *Ph. infestans* into three kinds (hybrid, self-fertile and parthenogenetic) from point of the view of origin. The hybrid oospores are formed as cross-fertilization with a participation of *Ph. infestans* mycelia of different mating types. Self-fertile

oospores are formed as self-fertilization with participation of only one *Ph. infestans* mycelium. Parthenogenetic oospores can be formed as parthenogenesis- from unfertilized oogonia. Smirnov (2001); has suggested that the modern laboratory techniques (tracing of the hyphae, GUS gene transformation and genetic analysis of matings) are shown to be ineffective for determination of these types of oospores of *Ph. infestans* (hybrid, self-fertile and parthenogenetic) in the field populations. He suggested to analyze the oospore micromorphology (size, thickness of the wall, the form of oogonium and presence of antheridium) and mating types of the strains producing the investigated oospores. The analysis of these features should allow to distinguish all three kinds of *Ph. infestans* oospores *in vitro*. Smirnov (2001); has reported that *in vitro*, oospores of *Ph. infestans* had 15-60 µm in diameter. The hybrid oospores had spherical form, with antheridia, with two or more thick walls and often 25-35 µm in diameter. The self-fertile oospores had spherical form with a thin wall and often 40-60 µm in diameter. The parthenogenetic oospores had half spherical form with spheroid oogonia with a thin wall and often 15-20 µm in diameter. We did not find the parthenogenetic oospores *Ph. infestans* in isolates obtained from collected samples, but this will require further research.

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