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Disease severity and sporulation potential of *Pyricularia oryzae* in some native rice cultivars in Iran

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Abstract: Rice is one of the most important crops in Iran and the rice blast caused by *Pyricularia oryzae* causes significant losses in rice fields during outbreak season. At the beginning of the disease cycle, the primary inoculum imposes severe damage to plants as it leads to plant weakness. The damage will shortly become significant as the second wave of inoculum spreads. This study aimed to determine the occurrence and severity of blast disease on some native Iranian cultivars and estimate the number of conidia produced on second leaves. *Pyricularia oryzae* Cavara isolates were collected from rice leaves from the north of Iran. One out of 123 isolates was inoculated on seven Iranian native cultivars named Hashemi, Tarom Mahali, Sang Tarom, Binam, Champa, Alikazemi, and Domsiyah at the four-leaf stage. Lesions on leaves were scored and the percentage of the spotted area was calculated by software Image J1.52a. Various lesion types and a range of diseased areas varying from 30% recorded in Hashemi to 55% in Tarom Mahali were observed. Under optimum conditions, the sporulation potential was calculated. Isolate produced different amounts of spores on different cultivars; Hashemi showed the highest and Tarom Mahali showed the lowest sporulation with 63 and 11 spores per mm^2 of spotted area. The result indicated that the resistance threshold of the native cultivars could be different but not sufficient. Additionally, to estimate the damage on each cultivar on farms, we need to investigate the sporulation potential of the pathogen through time in each cultivar.

Keywords: Iranian cultivars, pathogenicity test, rice blast, spotted area.

INTRODUCTION

As a cereal grain, rice (*Oryza sativa* L.) is one of the most widely consumed staple foods globally (FAOSTAT 2021). After sugarcane and maize, rice represents the third most-produced agricultural commodity. The world dedicated 164.19 million hectares for rice cultivation (The Statistics Portal 2021), and the total production of this crop was 509.87 million tons in 2021 (FAOSTAT 2021). In Iran, rice has been grown on about 892.213 hectares, producing 4.5 million tons of this crop in 2020 (Ahmadi *et al*. 2020).

Pyricularia oryzae (Magnaportales, Ascomycota) is a notorious fungal pathogen causing disease on many gramineous plants. It is estimated that this destructive pathogen declines rice world's production by ~8% per year (Wilson and Talbot 2009). The outbreak of the rice blast is currently a threatening issue in all rice-growing regions globally. Rice blast disease was first reported by Sharif and Ershad (1966) from Iran in Guilan province. According to the reports, this disease

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has a high prevalence in different regions of northern Iran located in the southern areas of the Caspian Sea (Pordel and Javan-Nikkhah 2020).

The Lifecycle of rice blast begins by germinating the landed spore on the leaf surface, culminating in forming a specialized structure named appressorium (Wilson & Talbot 2009). This structure plays a pivotal role in the penetrating event by generating physical and chemical pressures. Following this stage, infective hyphae colonize the plant tissue, and diamond lesions, typically symptomatic of rice blast, are formed (Talbot 2003). The rice blast's causal agent attacks rice plants at all stages of development and may infect all parts of the rice plant. Blast symptoms on rice gradually enlarge, and these lesions decline the net photosynthetic rate of individual leaves to an extent far beyond the visible diseased leaf fraction (Wilson & Talbot 2009, Behdad 1979, Fukuta *et al*. 2014). The effect of genotype on rice blast severity has been proved in many reports (Valent *et al*. 1991, Fukata *et al*. 2014, Cruz *et al*. 2015). Additionally, there are several Iranian researchers whose work indicates a range of damage in different breeding or local cultivars (Javan-Nikkhah *et al*. 2003, Momeni *et al*., 2005).

Under favorable conditions of high humidity,the causal fungus sporulates profusely from formed lesions, allowing the disease to spread rapidly to adjacent rice plants by wind and dewdrop splash (Talbot 2003). The sporulation is a pivotal stage in the *P. oryzae* biology as this event leads to the generation of secondary inoculum and is an essential phase in disease spreading and imposing severe damage. The potential of sporulation in lesions increased rapidly and decreased slowly over time. The maximum number of conidia depends on the combinations of host cultivars, fungus isolates, and the growth stage of plants (Ebbole 2007). The inoculum on the first (n) or the second (n-1) leaf stages produces the maximum potential in the developing plants. Additionally, lesions on the second leaf below the flag leaf play a key role against panicles because the maximum peak in sporulation coincides with the initial heading stage (Kato 1974). Despite several reports describing blast lesions, there is very little information available concerning *P. oryzae* inoculum produced on rice leaves. However, a few reports showed a variance in sporulation potential in other crops (Momeni *et al*. 2002).

Applying a resistant variety is the best method to manage this destructive disease. However, this pathogen likely gains biological tools such as effector proteins during the co-evolution process to overcome resistance (Singh *et al.* 2014). So far, more than 48 new cultivars have been introduced in Iran using breeding tools (Alinia *et al*. 2015). The central purposes of this study were to 1)evaluate disease severity on different native rice cultivars against rice blast disease, and 2) estimate the number of conidia produced on rice second leave as an important source of secondary inoculum. To these aims, seven Iranian native cultivars have been selected based on the information obtained from the Rice Research Institute of Iran (RRII). It is worth mentioning that cultivars Hashemi, Tarom Mahali, and Alikazemi have the highest cultivated area among all the native ones. Domsiyah, Sang Tarom, and Champa have been used as the parent of some hybrid cultivars and Binam has been known for its susceptibility against *P. oryzae*.

MATERIAL AND METHOD

Plant materials

To evaluate the pathogenicity and sporulation of rice blast on different native cultivars, disease-free rice seeds were obtained from the RRII. The investigated native cultivars included Hashemi, Tarom Mahali, Sang Tarom, Binam, Champa, Alikazemi, and Domsiyah.

Fungal isolates

During July and October of 2019, a comprehensive sampling of two rice cultivars including consisting of Hashemi and Tarom Mahali (the most commonly cultivated native cultivar) in the North of Iran (Guilan and Mazandaran provinces) was conducted. Samples were placed in labeled paper bags and transferred to the laboratory for isolating disease-causing fungi. Isolates were purified on water agar by single spore method and maintained on sterilized paper for long-time preservation (Bussaban *et al*. 2005, Pordel *et al*. 2016).

Inoculation and evaluation of isolates

A total of 123 *P. oryzae* isolates were collected from leave samples. Of these, 61 were collected from Mazandaran and 62 isolates from Guilan provinces. To choose one isolate from the collection, isolates have been classified into 2 major groups based on the origin and then divided into four subgroups based on the cultivars they have been collected from. All isolates have been cultured in V8 media [Vegetable juice 100 ml, CaCO3 2 gr, Agar 15 gr, Streptomycin sulfate (after autoclaving, Duchefa Biochemi) 200 mg, distilled water 900 gr] and kept for 14 days at 25 °C (Wei 2015). One out of each four groups has been chosen for the next step, evaluated from their growth and sporulation rate.

Inoculation was basically in accordance with the method of Hayashi *et al.* (2009), the conidial suspension was prepared from 14-day-old culture. The suspension was adjusted to 10^5 conidia/ml containing 0.01% Tween 20, with the Fuchs-Rosenthal hemocytometer.

Pathogenicity was assessed by spraying prepared conidial suspension onto four-week-old rice seedlings of the Hashemi cultivar. Inoculated plants were maintained in a dew chamber at 30 degrees centigrade in darkness for 24 h, and then in the greenhouse with a 12/12 light and dark period and 75-95% relative humidity.

Symptoms were rated from three plants per isolate according to the rating index described by Valent *et al.* (1991) (Table 1).

Pathogenicity test

In order to, evaluate the disease severity of the representative isolate on seven different native cultivars, plants were grown for 28 days (to the stage of four leaves) in pots containing 1:1:1 of loam: sand: compost. These seedlings were inoculated with conidial suspension as explained previously and were kept in the greenhouse at a 12/12 dark/light period, 25-30 °C, and 75-90% RH (Hayashi *et al*. 2009).

Evaluation of pathogenicity in different cultivars inoculated with *P. oryzea* was measured in two methods. One method was described by Valent *et al*. (as explained before) in which the disease severity was recorded on days 6 and 10 post-inoculation. For the next method, pictures of the second leaves were taken and used for quantification of the diseased area. As mentioned previously, different leaves of the Poaceae family demonstrate variance in the disease symptoms and sporulation (Kato 1974), regards this fact to measure the percentage of spotted area the second leave is the reference. Leaf surface and lesion area were measured using the software Image J1.52a (Wayne, Rasband, National Institute of Health, USA) and the percentage of the spotted area was estimated. Then the data has been analyzed by using the SPSS software 18.0 (released 2009. PAWS Statics for windows, Chicago: SPSS Inc, USA)

Determination of sporulation in different cultivars (Epidemiological component)

The second leaves from all plants at 14 dpi were photographed and cut into four pieces. Leaf samples, to maintain high humidity and stimulate conidia production, were placed in a humid chamber, i.e. plastic boxes containing moistened filter paper. To quantify the spotted area pictures of the sampled area were used. The diseased area was measured using the software Image J1.52a (Wayne, Rasband, National Institute of Health, USA). After seven days under high-humidity conditions, each leaf piece was placed in a Falcon tube containing 4 ml of distilled water and the surfactant Silwet Gold® at 125 ppm. After mixing the sample and placing one aliquot of 50 µl from the spore suspension in a Fuchs-Rosenthal chamber, sporulation was measured. The spore concentration was calculated by the average of the three aliquots and divided by the diseased area. Individual treatment means were compared using Tukey’s honestly significant difference procedure (a = 0.05). (Cruz *et al*. 2015, Navaro 2020).

Table 1. Rating scales for the lesion type on rice leaves infected by *P. oryzae.* Modified from Valent et al. (1991)

Lesion type	Symptom
Type 0	no visible evidence of infection
Type 1	Uniform dark brown pinpoint lesions without visible centers. These lesions typically are barely visible but can reach 0.5 mm in diameter in some fungus-plant combinations
Type 2	Small lesions with distinct tan centers surrounded by a darker brown margin (approximately 1 mm in diameter)
Type 3	Small eyespot lesions approximately 2 mm in length with tan centers surrounded by dark brown margins
Type 4	Intermediate size eyespot lesions, approximately 3-4 mm in length
Type 5	Large eyespot lesions that attain the maximum size (approximately 5 mm in length)

RESULTS

Isolate selection

In an attempt to select one isolate to be used in the subsequent analysis, four fungal isolates representative of various sampling areas with the highest growth and sporulation rate were selected and included in an infection assay. Our infection assay demonstrated that there were no differences among the isolates in terms of disease severity evaluated by applying the Valent *et al*. (1999) scale on the applied cultivars. At 6- and 10-days post-inoculation (dpi) lesions attributed to type 2 were observed for all examined interactions. Based on this result, one of the employed isolates (IMT8) in the infection assay was selected for the subsequent assays, the isolate was collected at Rice Research Institute, Division of Amol, from Tarom Mahali cultivar.

Infection assay

The disease severity of the isolate IMT8 on seven native Iranian cultivars was investigated (Figures 1 and 2). To reach this goal, 28 days after cultivation, leaves of rice seedlings were inoculated via a mechanical sprayer as described in the material and method. The inoculated leaves were scored using the method described previously by Valent *et al*. (1991) at 6 and 10 dpi (Table 2).

Fig 1. Reactions of rice to *Pyricularia oryzae*, at the three-leaf stage, to inoculations with 10^5 conidia/ml containing Tween 20 suspension of IMT8 isolate at 6 days after inoculation and maintaining in the greenhouse with a 12/12 light and dark period and 75-95% relative humidity. A) Hashemi, B) Alikazemi, C) Domsiyah, D) Sang Tarom, E) Champa, F) Binam, G) Tarom Mahali.

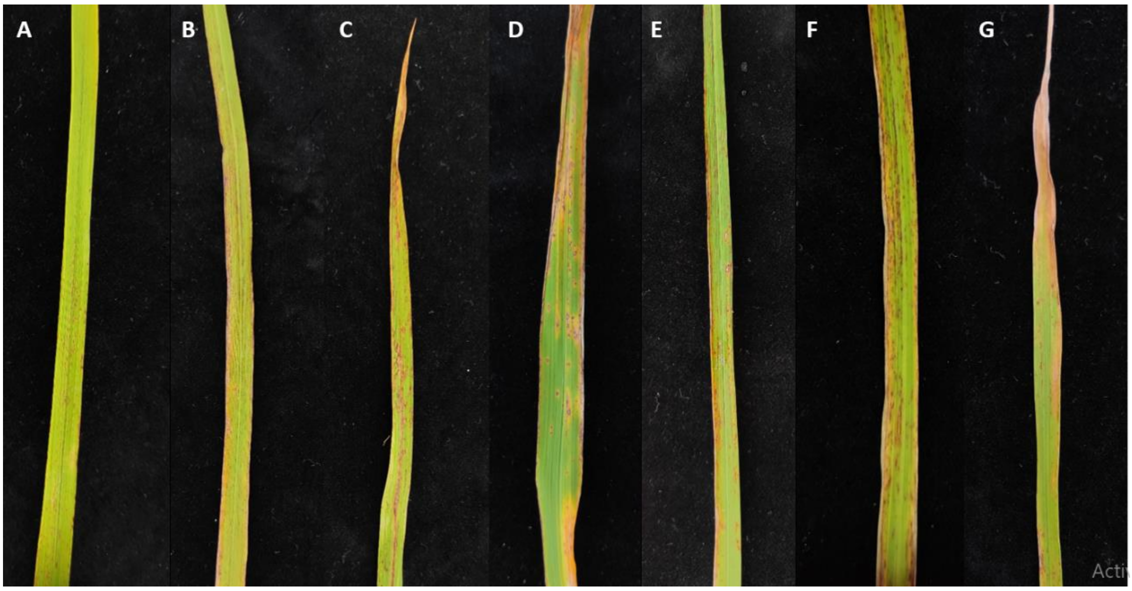

Fig 2. Reactions of rice *Pyricularia oryzae*, at the three-leaf stage, to inoculations with 10^5 conidia/ml containing Tween 20 suspension of IMT8 isolate at 10 days after inoculation and maintaining in the greenhouse with a 12/12 light and dark period and 75-95% relative humidity. A) Hashemi, B) Alikazemi, C) Domsiyah, D) Sang Tarom, E) Champa, F) Binam, G) Tarom Mahali.

Table 2. Scoring of the inoculated leaves of the seven examined native Iranian cultivars by *Pyricularia oryzae* IMT8 based on symptoms types (Table 1).

Lesion Scoring	**Cultivars**						
	Hashemi	**Alikazemi**	**Domsiyah**	**Sang Tarom**	**Champa**	**Binam**	**Tarom Mahali**
Day 6	2	2	3	3	3	3	5
Day 10	2	2	4	4	4	5	5

* Type 0, no visible evidence of infection; type 1, uniformly dark brown pinpoint lesions without visible centers. Less than 0.5 mm in diameter; type 2, small lesions with distinct tan centers surrounded by a darker brown margin and approximately 1 mm in diameter; type 3, small eyespot lesions 2 mm in length with tan centers and dark brown margins; type 4, 3-4 mm in length eyespot lesions; type 5, large eyespot lesions that attain the maximum size.
Lesion types 0 and 1 do not produce conidia however, in high humidity conditions types 2, 3, 4 and 5 produced conidia

Our infection assay revealed that all cultivars were susceptible to IMT8, however, the rate of symptom expansion was different in various cultivars. Among the seven cultivars, Tarom Mahali showed the highest lesion score on both recording days, as the score was rated at 5 on the sixth day. At 6 dpi, Binam, Champa, Sang Tarom, and Domsiyah were showing the same symptoms including small eyespot lesions with tan centers and dark brown margins, as was expected in 4 dpi the symptoms were expanding, but at a different rate. at 10 dpi, Binam displayed large eyespot lesions that were attached (score 5), however, this rate was lower in the others and their lesion diameter was grown 1 or 2 mm (score 4). Hashemi and Alikazemi represented the most resistant cultivars, showing the lowest lesion scoring, which was 1 mm lesions with distinct tan centers, and darker brown margin (score 2). It is worth mentioning that their type of lesion did not change at 4 dpi.

Additionally, we employed the method described by Cruz *et al*. (2015) to calculate the percentage of the diseased area due to infection by the IMT8 isolate (Figure 3). All cultivars have produced typical lesions but there were significant differences in the percentage of area affected by a pathogen, varying from 30% to 55% (p-value ≤ 0.05). This analysis demonstrated that cultivars were divided into four significantly different groups based on the percentage of the spotted leave surface. Hashemi with 30% had the less amount of spotted area followed by Alikazemi (38%), and Domsiyah (48%). There was no significant difference between Sang Tarom (51%), Champa (51%), Binam (52%); and Tarom Mahali showed the most area of disease with 55%. The outcome of this experiment and the previous one, not also demonstrates the fact that all cultivars are susceptible to this pathogen, but also that there is a range of variance in native cultivars towards infection by the IMT8 isolate.

Spore quantification

To evaluate and quantify the ability of the IMT8 isolate to sporulate on various native Iranian cultivars, second leaves of inoculated seedlings were harvested and then photographed at 14 dpi. The collected samples were incubated in plastic boxes for seven days, and subsequently, the number of spores in the diseased area was calculated. Data were analyzed and obtained findings were shown in Figure 4. This finding showed that the IMT8 isolate could generate spores on all studied cultivars, but the number of generated spores per mm^2 of the diseased area was significantly different among applied cultivars.

The highest sporulation of IMT8 isolate occurred on Hashemi cultivar with 63 spores per disease area. There was no significant difference between Alikazemi (60) and Champa (58) followed by Sang Tarom (40) and Binam (40). Domsiyah by presenting 29 spores in 1 mm^2 of disease area was in the next level and the lowest number of conidia were produced by Tarom Mahali calculated as 11 spores per disease area

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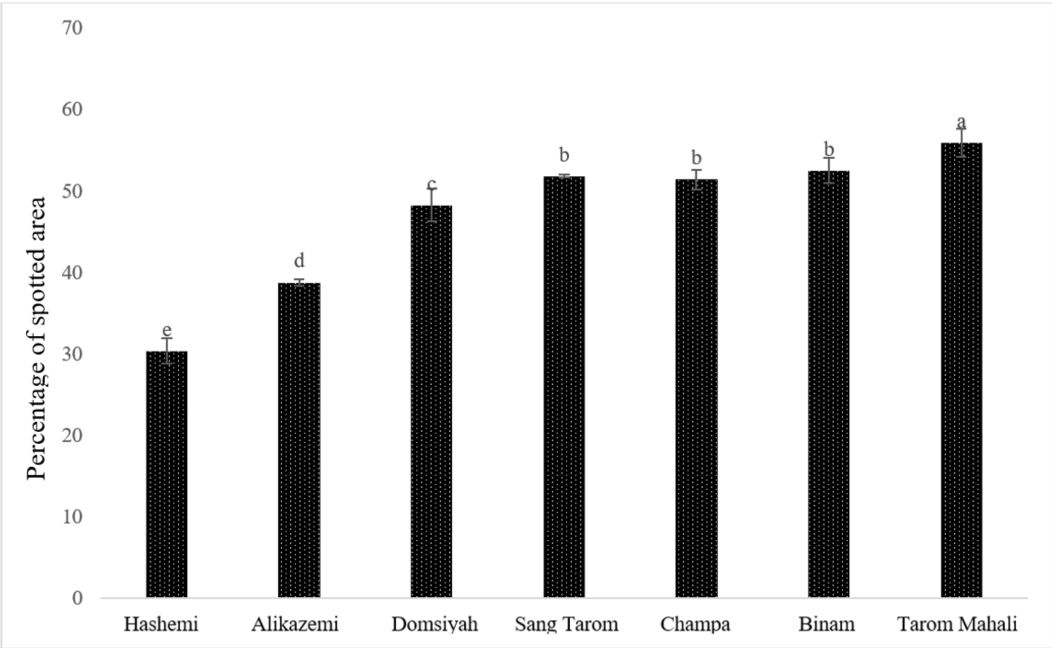

Fig 3. Percentage of the diseased area formed on the second leaf of seven native rice cultivars infected by *Pyricularia oryzae* IMT8. The bars indicate significant differences at p ≤ 0.05, calculated by the Tukey HSD test. Columns with the same letter are not significantly different.

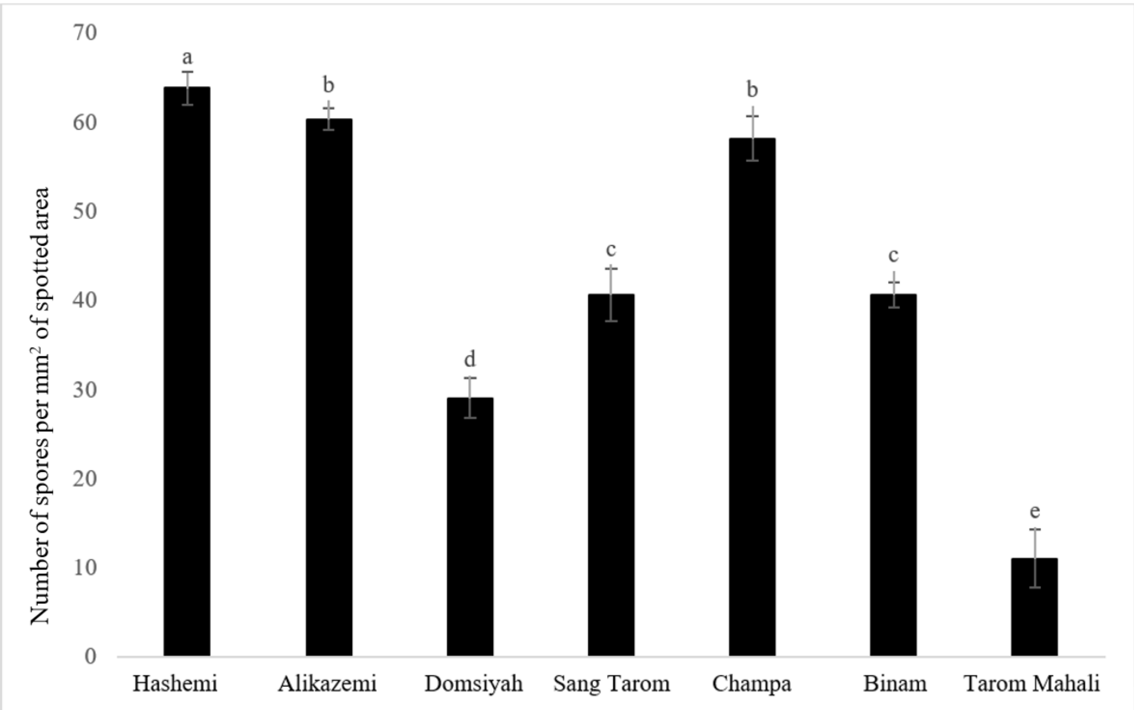

Fig 4: Numbers of conidia *Pyricularia oryzae* IMT8, averaged across treatments, produced on a diseased area of leaf samples in different rice cultivars, 14 day-post-inoculation. Bars indicate the standard error, calculated by the Tukey HSD test (significant differences at $p \leq 0.05$).

DISCUSSION

Different fungicides are currently applied to control blast disease, but they impose extra costs on rice production and chemical contamination of the environment and food chains are unavoidable (Jamal *et al*. 2012). Host resistance is the most environmentally sustainable and economically viable measure to control blast disease (Singh *et al*. 2014). Until now, successfully traditional molecular breeding approaches have developed blast-resistant varieties (Yuan *et al*., 1994, Guimarães *et al*. 1998, Mao *et al*. 1998, Dean *et al*. 2005). In a breeding approach, a delay in disease development and a slow disease growth rate are desirable features (Sigulas *et al*. 1988, Prabhu *et al*. 2002). Due to this fact studying the native cultivars' reaction against blast disease is crucial, to gaining novel knowledge to develop new blast resistance varieties.

Sporulation and lesion development are two main components of the rice leaf blast infection process under field conditions. Different parameters can make changes in sporulation and disease development such as genotype, plant age and environmental situations (Shahriar *et al*. 2020). Plant mortality and disease severity are often higher in young plants and seedlings, especially in crop plants. (Pordel *et al*. 2018, Pordel *et al*. 2021). As leaves get older and expand more, the resistance rapidly increases, resulting in a reduction of lesions and sporulation per leaf area (Roumen *et al*. 1992). It seems that studying the effect of genotype is essential to have a better picture of this pathosystem.

In our study, the percentage of diseased area agree with the results obtained from the lesion scoring and imply, there is a variance among native cultivars respectively from the most to the less susceptible one, Tarom Mahali, Binam, Champa, Sang Tarom, Domsiyah, Alikazemi, and Hashemi. Javan-Nikkhah *et al*. (2003) demonstrated that Binam, Alikazemi, and Domsiyah are more susceptible cultivars to blast than Hashemi. Amanzadeh *et al.* 2005 reported that there is a significant difference in Iranian cultivars. For instance, more lesions were produced on Champa and Tarom Mahali in response to infection by *P. oryzae* compared with that of the Sang Tarom.

These reports indicate that Iranian native cultivars are equipped with different resistance mechanisms and still, they are not providing sufficient resistance to inhibit completely the rice blast disease. Investigating the number of diseased areas (lesions) and their type is important factors, as lesions affect photosynthetic capacity, leading to a significant reduction of quantity and quality of yield crop. It is well documented that lesions on the central vein caused a marked reduction in the photosynthetic rate as they can disturb the transportation of water. Consequently, this event results in a remarkable reduction of the relative water content of the leaf (Burrell *et al*. 1974, Kaiser 1987, Bastiaans *et al*. 1993).

However, to have a bigger perspective about what happens on a farm with each cultivar, the sporulation and producing the second inoculum is important as it can cause the next waves of infection and even affect the neck blast occurrence.

The expression resistance mechanisms that are assumed to be different in native cultivars affected some epidemiological components, such as the ability of pathogens to sporulate. This process manipulates the apparent infection rate and the disease secondary cycle of most diseases (Parlevliet 1979, Abadi *et al*. 1989). Here, we observed a significant difference in the ability of the pathogen to sporulate on native cultivars that is presumably attributed to the genetic architecture of the examined cultivars. This finding is in disagreement with the infection assay where disease symptom type and the percentage of the diseased area are calculated. Although Hashemi and Alikazemi were cultivars with less amount of disease expansion at 6 and 10 dpi, the IMT8 isolate produced the highest amount of spore per mm^2 of the diseased area at 14 dpi on the mentioned cultivars. In other words, fungal sporulation in the less susceptible cultivars was higher than in the most susceptible ones. This sharp difference can be explained by mentioning the time every interaction of cultivars and IMT8 needs to reach the maximum of disease expansion and development, based on this hypothesis the pathogen on Tarom Mahali, Domsiyah and Binam might have its maximum sporulation potential before 14 dpi. On the other hand, although pathogen sporulation potential was high in interaction with Hashemi and Alikazemi cultivars and the number of spores per mm^2 of the diseased area was the highest among other interactions, it should be noted that the diseased area these two cultivars showed is significantly less than others, so it can be assumed that the final spore production on them should be less than those which have more lesion area. Momeni *et al.* (2002) tried to make a correlation between symptoms the occurred on the host and the ability of fungi to sporulate on the diseased area at 6 DPI. Interestingly, they recorded less sporulation in Domsiyah, which is the same result we gained. In another research on wheat blast done by Cruz *et al*. (2015), it has been revealed that there is a difference in sporulation on the wheat cultivar, suggesting that the examined cultivars are equipped with various tactics to hinder fungal sporulation.

In this research, we investigated the ability of the same isolate under similar environmental conditions to cause blast disease in some Iranian rice native cultivars having different genetic backgrounds. It is probable that the observed difference is attributed to the various mechanisms that existed in the native cultivar to reduce the fungal sporulation, which is a crucial factor to establish an epidemic in the fields. As there is not enough knowledge about the pathway Iranian native cultivars hinder the fungi growth and expansion and reduce the sporulation potential, more research is needed to gain adequate data for understanding the interaction between cultivars and pathogens and the relationship between disease expansion and sporulation.

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بررسی شدت بیماری و پتانسیل اسپورزایی *Pyricularia oryzae* روی تعدادی از ارقام برنج بومی ایرانی

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چکیده: برنج از نظر اقتصادی یکی از مهمترین محصولات زراعی ایران بوده است که متاسفانه سالانه متحمل خسارت زیادی از جانب بیمارگر قارچی *Pyricularia oryzae* عامل بیماری بلاست برنج می‌شود. در اوایل چرخه زندگی، مایه تلقیح اولیه باعث شروع بیماری شده و با کاهش توانایی فتوسنتز گیاه منجر به ضعف و نهایتا خسارت شدید به گیاه می‌شود. به سرعت میزان خسارت به سرعت در مزرعه با تولید و پراکنش مایه تلقیح ثانویه، چشمگیر می‌گردد. هدف این تحقیق، مطالعه وقوع و شدت بیماری بلاست در برخی ارقام بومی برنج و بررسی تعداد اسپور تولید شده روی برگ دوم ارقام مختلف بوده است. از برگ‌های جمع آوری شده از مناطق شمال ایران جدایه‌های *Pyricularia oryzae*، جداسازی شدند. یک جدایه ار ۱۲۳ جدایه روی هفت رقم بومی ایرانی شامل هاشمی، طارم محلی، سنگ طارم، بینام، چمپا، علی‌کاظمی و دمسیاه در مرحله چهار برگی مایه‌زنی شد. سپس نوع علائم ارزیابی و همچنین درصد آلودگی برگ توسط نرم افزار Image J1.52a محاسبه شد. دامنه اختلاف وسیعی در نوع لکه و درصد آلوده برگ بین ارقام، از ۳۰٪ در رقم هاشمی تا ۵۵٪ در رقم طارم محلی مشاهده شد. تحت شرایط بهینه، پتانسیل اسپوردهی جدایه محاسبه شد. جدایه بیمارگر پتانسیل اسپوردهی متفاوتی روی ارقام مختلف نشان داد. روی رقم هاشمی بیشترین و روی رقم طارم کمترین میزان اسپوردهی را با ۶۳ و ۱۱ اسپور در هر mm^2 منطقه لکه‌دار، نشان داد. ۶۳ و روی رقم طارم محلی ۱۱ اسپور در هر یک mm^2 لکه ثبت شد. نتایج بدست آمده نشان می‌دهند آستانه مقاومت در این ارقام متفاوت است اما کافی نمی‌باشد. به علاوه، می‌توان به این نتیجه رسید که تخمین خسارت هر رقم در مزرعه، به دیدگاه جامع‌تری احتیاج است که در آن نه تنها به نوع علائم و یا شدت بیماری توجه شود، بلکه پتانسیل اسپورزایی بیمارگر در طی زمان نیز در نظر گرفته شود.

کلمات کلیدی: ارقام ایرانی برنج، آزمون بیماریزایی، بلاست برنج، ناحیه لکه‌دار

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