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Research article

Elucidation of molecular variability among *Pyricularia grisea* isolates causing blast disease in forage pearl millet

Prerana Parihar[1], R. K. Pandya[1], Purnima Singh[1], Sushma Tiwari[1]*, M. K. Tripathi[1], Niraj Tripathi[2] and C Tara Satyavathi[3]

[1]Rajmata Vijayaraje Scindia Agricultural University, Gwalior-474002, India
[2]Jawaharlal Nehru Krishi Vishwa Vidyalaya, Jabalpur-482004, India
[3]ICAR-All India Coordinated Research Project on Pearl Millet, Jodhpur-342304, India
*Corresponding author e-mail: sushma2540@gmail.com

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Abstract

Pearl millet (*Pennisetum glaucum*) is an important nutri-cereal for humans and a forage crop for livestock. It is the only cereal crop that is proficient in adopting punitive climate conditions and peripheral soil. The blast disease caused by *Pyricularia grisea* is a major problem in pearl millet, causing significant losses in high-yield hybrids/varieties grown for fodder. Blast disease is a major biotic threat in the cultivation of pearl millet in northern Madhya Pradesh, especially Gwalior, Morena, Bhind and Sheopur. In this investigation, we studied eight isolates of *P. grisea* that cause pearl millet blast disease. We used RAPD and ISSR molecular markers to assess their morphology, pathogenicity and genetics. Mycelium growth was investigated maximum in isolate PGMP1, however, two isolates *viz*., PGMP1 and PGMP8 were found to be highly virulent. The pearl millet blast fungus population in northern Madhya Pradesh is genetically diverse. RAPD and ISSR markers reliably explained the relationships among the different isolates. Our results on the characterization of blast pathogen might be advantageous in devising location-specific disease management strategies in pearl millet against blast disease.

Keywords: Genetic diversity, Molecular markers, Pathogen, Population, Variability

Introduction

Pearl millet (*Pennisetum glaucum*), locally known as 'Bajra' (Choudhary *et al*., 2021a) is the most widely grown, drought-tolerant and multipurpose crop providing food, fodder and fuel worldwide (Yadav *et al.*, 2020; 2021; Kumawat *et al*., 2020; Kumar *et al*., 2023). It is a well-deserved crop that can deliver nutrition (Makwana *et al*., 2021; Choudhary *et al*., 2021b) without the need for much water and grown under harsh climatic conditions where other major crops like maize and sorghum cannot produce economic yield. India is the largest producer of this crop in Asia, replacing 7.52 million ha with a production of 10.28 million tons and productivity of 1386 kg ha^{-1} during 2019-20. Major pearl millet-growing states in India are Haryana, Gujarat, Rajasthan, Maharashtra, Uttar Pradesh, Karnataka, Andhra Pradesh, Madhya Pradesh and Telangana. In Madhya Pradesh, during 2019-20 the crop was planted on 0.28 million ha with an annual production of 0.63 million tons and productivity at 2219 kg ha^{-1} (IIMR, 2020).

The diseases are the main biotic constraints in obtaining the optimum yield potential of popular pearl millet hybrids and varieties (Reddy *et al.*, 2021). There are a number of diseases, *viz*. green ear, blast, smut, rust and ergot, which attack pearl millet, causing severe yield losses (Verma *et al*., 2021). In recent years, blast incited by *Pyricularia grisea* (Cooke) Sacc. has become one of the most prevalent and crucial diseases of pearl millet (Sharma *et al*., 2013; Parihar *et al*., 2022). At present, increased incidence has been recorded in most of the Indian states like Rajasthan, Haryana, Gujarat, Maharashtra, Madhya Pradesh, Uttar Pradesh, Karnataka and Andhra Pradesh. In Madhya Pradesh, more than 75% area of the crop is jointly shared by the Gwalior, Bhind, Morena and Sheopur districts where this disease has become one of the most important biotic restraints in attaining optimum yield in an array of popular hybrids especially grown for both grain and fodder purposes. The disease diminishes the fodder quality substantially (Bhardwaj *et al.*, 2022).

Blast pathogen is known for its high morphological, virulence and genetic variability. According to recent reports, leaf blasts occurred in many pearl millet cultivars in India. It may be owing to the highly unpredictable character of the pathogen or because of the emergence of new races. Thus, the use of resistant cultivars is limited to a certain place and time. Also, the repeated use of the same fungicide can lead to the development of resistance to that fungicide (Bhardwaj *et al.*, 2022). Understanding the genetic, pathogenic, and morphological variations of plant pathogens is crucial for implementing effective disease management strategies (McDonald and Linde, 2002).

Assessment of genetic variability is important to know the existing characteristics of an individual (Rana *et al.,* 2022; Barai *et al.,* 2022). Genetic diversity amongst blast pathogen populations has been studied using ISSR and RAPD markers (Chadha and Gopalakrishna, 2007; Sirithunya *et al.*, 2008). Combining RAPD and ISSR markers improves the accuracy of genetic assessments of pathogen populations (Schlotterer, 2004). This will help in the successful implementation of disease management strategies. Reports are lacking on pathogenic and molecular variability analysis of *P. grisea* collected from major pearl millet growing areas. So the present investigation was planned to recognize the *P. grisea* variability from disease locations of Madhya Pradesh, *viz*. Gwalior, Bhind, Morena and Sheopur by employing morphological, pathological and molecular marker analysis.

Materials and Methods

The current investigation was conducted at Department of Plant Pathology for laboratory and polyhouse-related work, and Department of Plant Molecular Biology and Biotechnology for Molecular work in College of Agriculture, Rajmata Vijayaraje Scindia Krishi Vishwa Vidyalaya, Gwalior, India.

Collection, isolation and purification of P. grisea isolates: Blast-infected leaf samples were collected from major pearl millet growing locations of Madhya Pradesh during the *Kharif* season of the years 2019 and 2020 (Table 1). Blast-infected leaf tissues were cut into small bits. These bits were washed twice in sterilized double distilled water and surface sterilized with 0.1 per cent sodium hypochlorite for two minutes then rinsed three times with sterilized double distilled water and dried on sterilized filter paper before being placed on petri dishes containing oatmeal agar (OMA) medium. Inoculated petri dishes were incubated for 7 days at 28±1°C. A dilute spore suspension was prepared in sterilized distilled water and plated on 4% water agar in petri dishes. After 10-12 h of incubation at 28±1°C, single germinating conidia were marked with the help of a dummy objective lens under a microscope and transferred to fresh petri plates amended with OMA medium. The petri plates were gestated at 28±1°C for 7 days and the identity of the fungal cultures developed from the single spore was established based on spore morphology. Cultures were maintained on the OMA medium for further investigation.

Morphological characteristics: The cultural characters of all monoconidial isolates of *P. grisea* were recorded by growing them on oat meal agar, rice meal agar, pearl millet leaf extract + 2% sucrose agar (PMLEA), pearl millet seed extract + 2% sucrose agar medium (PMSEA) and potato dextrose agar medium (PDA) for 14 days at 26°C. Cultural characteristics of *P. grisea* isolates were studied for colour of isolates, growth pattern, texture of colony, sectoring and wrinkles formation, radial growth (mm) and sporulation index. The colour and radial growth of *P. grisea* isolates on different media were recorded when the pathogen attained the maximum growth on petri dishes after 14 days of incubation. The sporulation capacity of each isolate on media was assessed by microscopic examinations. After 10 days of incubation at 28±1°C, each petri plate of *P. grisea* isolate was flooded with 5.0 ml of sterile distilled water and gently scraped with a sterile inoculation loop to produce spore suspension and the conidia were counted per microscopic field.

Pathogenic variability of P. grisea isolates: Dhanshakti cultivar of pearl millet was employed for testing the pathogenicity of each isolate (Table 1). Seedlings of the susceptible cultivar were grown in 15 cm diameter plastic pots filled with sterilized soil-sand-FYM mix in a 2:1:1 ratio and placed in a greenhouse at 30 °C with two replications. Excess seedlings were uprooted at one-leaf stage to keep 10 plants per pot. Mass multiplication of spores for inoculation was achieved by growing each isolate (3 discs/plate) on an OMA medium at 28±1ºC for 8 to 10 days. The plates were flooded with 10 ml of distilled water and the fungal growth containing mycelium and conidia was gently removed by scrapping with a sterile plastic inoculation loop. Harvested spores were filtered through a double-layer muslin cloth and

Table 1. Details of *P. grisea* isolates collected from major pearl millet growing districts of Madhya Pradesh

S.No.	District	Location	Isolates	Average severity (%)
1.	Gwalior	Gwalior	PGMP1	52.33
2.	Gwalior	Dabra	PGMP2	25.00
3.	Sheopur	Birpur	PGMP3	24.00
4.	Morena	Porsa	PGMP4	23.67
5.	Sheopur	Karahal	PGMP5	36.00
6.	Morena	Joura	PGMP6	29.00
7.	Bhind	Bhind	PGMP7	41.67
8.	Bhind	Gormi	PGMP8	50.69
SEM				1.42
CD (P<0.05)				4.19

serial dilution technique was used to prepare a suspension of about 10^5 conidia per ml as recommended by Jia *et al*. (2003) just before inoculation. Twelve-day-old pot-grown seedlings were artificially inoculated by spraying the inoculum on the foliage using a hand-operated atomizer. Seedlings sprayed with water were used as control. All the inoculated seedlings were covered with polythene bags and incubated at 25°C for 48 h to prevent cross-contamination. After 48 hours bags were removed and inoculated seedlings were exposed to more than 90% relative humidity (RH) under misting for six days in a greenhouse.

Genomic DNA isolation: For DNA extraction, isolates were grown in 100 ml of potato dextrose broth for 5 days at 28±1ºC in a rotary shaker at 100 rpm. Five hundred mg of mycelial mat was filtered, dried and ground to a fine powder in liquid nitrogen. The DNA extraction process was performed according to the method given by Jia *et al.* (2014). Dissolved DNA was stored at -20ºC for further downstream reactions. DNA was quantified and diluted in each sample for PCR amplification up to 25-40 ng/µL in nuclease-free water.

PCR and genetic diversity analysis of P. grisea using RAPD and ISSR molecular markers: A total of 15 RAPD and 3 ISSR primers were screened initially. Polymerase Chain Reaction was performed in a final volume of 10 µl consisting of 1 µl of 6X green taq buffer with $MgCl_2$, 0.2 µl dNTP's, 0.1 µl of *Taq* DNA polymerase (Dream*Taq*, Thermo Scientific, USA), 2.0 µl DNA, 1.0 µl of primer and sterile double distilled water. The PCR parameters for both types of markers were: initial denaturation of 5 min at 94 °C; tracked by 32 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 36 °C, and extension for 2 min at 72 °C and final extension for 5 min at 72 °C. The ISSR primers were selected based on earlier literature on pearl millet (Costa *et al.,* 2016; Longya *et al.,* 2020). The annealing temperature was decided according to previously published literature (Ratanacherdchai *et al.*, 2007). The amplified PCR products of both types of markers were resolved on 1.5% agarose gel and a 100 bp DNA ladder was employed for determination of amplicon size.

Data analysis: The banding patterns attained from RAPD and ISSR primers were scored based on the presence and absence of each band, coded as 1 and 0 correspondingly. The scores were used to create a data matrix to analyze genetic relationships using the NTSYS-pc programme version 2.2 (Exeter Software, New York, USA) described by Rohlf (2000). A dendrogram was constructed based on Jaccard's similarity coefficient (Jaccard, 1908) using the marker data from *P. grisea* isolates with an unweighted pair-group method on arithmetic average (UPGMA) cluster analysis to group the isolates based on their similarities. The relationships among the isolates were examined and presented as a dendrogram by using UPGMA.

Results and Discussion

Morphological analysis

Morphological characterization and pathogenicity: Pearl millet blast is the most serious disease in all pearl millet growing regions of the world (Rao *et al*., 2021). The fungus can overcome resistance within a short time after the release of a resistant cultivar and for that reason, breeding for

resistance has to be a consistent task (Srivastava *et al.,* 2014). The evaluation of genetic variation in plant pathogen populations is a crucial prerequisite for knowledge of co-evolution in the plant path system (Sankar *et al.* 2021). Morphological characteristics of eight isolates of *P. grisea* were carried out on different culture media. Colony colour was found to be grayish-black, black or white in different isolates (Table 2). The variations in colony colour and texture of *P. grisea* isolates were reported earlier by Kalavati *et al.* (2016) who experimented with the fungal isolates of *P. grisea* produced circular, irregular mycelium with smooth and rough margins on OMA and PDA media. Srivastava *et al.* (2014) reported that the colony colour of *P. oryzae* varied from buff to black colour with smooth and rough colonies. Isolate PGMP1 (85.19 mm) showed the highest mycelial growth (Table 3), which was at par with isolate PGMP5 (83.78 mm), however, minimum growth was recorded with isolate PGMP2 (63.79 mm).

The sporulation capacity of each isolate was assessed as per microscopic examination. A sporulation index (0-4 scale) was adopted during the present study as mentioned by Yashaswini *et al.* (2017) and Parihar *et al.* (2022). Data obtained after observation of eight isolates ranged from poor to very good (Table 4). It was categorized into five groups based on the number of spores per microscopic field with no spores as Nil (0); 1-10 spores as poor (1); 11-20 as fair (2); 21-40 as good (3) and above 40 as excellent (4). Among the eight isolates, PGMP8 was found to be the most effective with a sporulation score of 3.2, followed by PGMP1 with a score of 2.8. The lowest sporulation was observed in PGMP5 with a score of 1.0. The findings of the present investigation were in accordance with the results of Yashaswini *et al.* (2016), who reported the sporulation index of *P. grisea* isolates from excellent (scale 4) to poor (scale 1).

Isolates of P. *grisea* collected from major pearl millet growing districts of Madhya Pradesh based on their severity, lesion type and reaction differences on Dhanshakti cultivar were investigated. All the isolates were found to be virulent. The severity of isolates PGMP1 (52.33%) and PGMP8 (50.69%) were found to be significantly superior over the rest of the isolates and at par with each other (Table 1). However, minimum severity was recorded in isolate PGMP 4 (23.67%) being at par performance with isolates PGMP2 (25%) and PGMP3 (24%). In the present study, *P. grisea* showed a continuous array of symptoms in reaction to the infection of various isolates of the fungus from larger, distinct, spindle-shaped spots with a central ashy zone and marginal zones 3 to 5 mm broad and up to several sizes. In length (highly susceptible), broadly spindle-shaped spots only slightly longer than broad, 3-5 mm in diameter (susceptible), circular spots about 2 to 3 mm in diameter with a central ashy zone and a purplish brown margin lesion (susceptible) on Dhanshakti cultivar. These findings were supported by the research conducted by Takan *et al.* (2011). They tested the compatibility of thirty-one isolates representing diverse sampling locations and host ranges and revealed that all isolates were compatible with the tested 8 finger millet varieties and showed variability in aggressiveness, lesion number and leaf area affected. The consequences of this research have practical feasibility in the selection of geographically and pathologically diverse isolates for use in the identification and application of blast resistance in pearl millet. Differences in pathogenicity between individual isolates were used for a long time to assess variation in natural pathogen populations (Yadav *et al*., 2019). Based on severity and lesion type, eight isolates collected during the present study were grouped into three categories (Table 5).

Molecular analysis

ISSR marker analysis: In the current investigation, initially three ISSR primers were screened. Out of three, only two ISSR primers consistently produced strong amplicon. Data clearly outlined that the total number of alleles identified was six with an average of 3 alleles per locus (Table 6). The genetic diversity of *P. grisea* differed from zero (ISSR-3) to 0.25 (ISSR-1) with a 0.125 mean value. Further, polymorphism information content (PIC) values varied from 0.468 to 0.511 for ISSR-2 and ISSR-1 markers, respectively with a mean of 0.489. Major allele frequency arrayed between 0.50 and 0.645 with a mean worth of 0.562 (Table 6).

The UPGMA tree (Fig 1) displayed that *P. grisea* isolates were grouped into two clusters, a major cluster and a minor cluster. The major cluster included five isolates that again subdivided into two sub-groups. The first group had only one isolate (PGMP 5), whereas the second sub-group included four isolates namely PGMP1, PGMP2, PGMP7 and PGMP8. While the minor cluster had three isolates which again divided into two sub-groups. The first sub-group had only one isolate *viz*., PGMP 3,

Table 2. Cultural characters of eight isolates of *P. grisea* on five solid media tested after 14 days of incubation

Isolates	Oat meal agar	Rice agar	Pearl millet leaf extract+2% sucrose	Pearl millet seed extract+2% sucrose	Potato dextrose agar
PGMP1	Grayish black colour, raised aerial mycelium with sector formation	Grayish colour floccose aerial mycelium	Black colour flat mycelium	Gray colour raised mycelium	Black colour flat mycelium
PGMP2	Grayish white colour, floccose aerial mycelium	Grayish white colour floccose aerial mycelium	Black colour flat mycelium with ring formation	Blackish gray colour flat mycelium	Grayish white colour, raised aerial mycelium with sector formation
PGMP3	Grayish white colour, raised aerial mycelium with sector formation	Blackish colour raised mycelium	Black colour flat mycelium	Gray colour raised mycelium with ring formation.	Grayish white colour raised mycelium with ring formation
PGMP4	Blackish gray colour, fluffy aerial mycelium	Cottony raised mycelium with sector formation	Black colour flat mycelium	Gray colour raised mycelium with ring formation	Gray colour raised mycelium
PGMP5	Whitish gray colour, floccose aerial mycelium	Whitish gray floccose mycelium with ring formation.	Black colour flat mycelium with ring formation	Gray colour raised mycelium	Gray colour raised mycelium
PGMP6	Grayish colour raised aerial mycelium	Blackish white floccose mycelium	Black colour flat mycelium with zone formation	Black colour flat mycelium	Black colour flat mycelium
PGMP7	Whitish gray colour floccose aerial mycelium	Blackish white raised aerial mycelium	Blackish gray colour flat mycelium	Gray colour floccose mycelium	Blackish gray colour flat mycelium
PGMP8	Whitish gray raised aerial mycelium	Black colour raised aerial mycelium with ring formation	Black colour flat mycelium	Gray colour raised mycelium with ring formation.	Black colour raised aerial mycelium

Table 3. Radial mycelial growth of *P. grisea* isolates on different solid culture media

		Colony diameter (mm)*					
S. No.	**Isolates**	**Oat meal agar**	**Rice agar**	**Host leaf extract+2% sucrose**	**Host seed extract+ 2% sucrose**	**Potato dextrose agar**	**Mean**
1	PGMP1	90.00	76.22	81.67	88.86	89.21	85.19
2	PGMP2	52.19	57.26	51.09	78.64	79.75	63.79
3	PGMP3	89.34	80.67	51.78	88.65	70.55	76.20
4	PGMP4	83.07	87.00	65.85	71.39	81.38	77.74
5	PGMP5	83.05	88.29	81.18	86.67	79.72	83.78
6	PGMP6	79.84	76.29	66.42	63.31	52.95	67.76
7	PGMP7	84.42	77.07	54.32	57.07	85.53	71.60
8	PGMP8	90.00	79.89	34.80	90.00	81.80	75.30
Mean		**81.49**	**77.83**	**60.59**	**78.08**	**77.61**	**75.12**
		Isolates		**Media**		**Isolates*Media**	
SEM		0.60		0.47		1.33	
CD (P<0.05)		1.67		1.32		3.73	

Observation from seven days old culture plates; *Data presented in table was average of three replications

Table 4. Sporulation index (0-4 scale) of isolates on different solid culture media after ten days of inoculation

	Sporulation index (0-4 scale)					
Isolates	**Oat meal agar**	**Rice agar**	**Pearl millet leaf extract+ 2% sucrose**	**Pearl seed extract+2% sucrose**	**Potato dextrose agar**	**Mean**
PGMP1	4	2	2	2	4	2.80
PGMP2	2	1	3	3	3	2.40
PGMP3	3	3	1	1	2	2.00
PGMP4	3	0	1	1	1	1.20
PGMP5	1	1	1	1	1	1.00
PGMP6	2	2	2	2	3	2.20
PGMP7	3	2	1	1	3	2.00
PGMP8	4	4	3	2	3	3.20
Mean	**2.75**	**1.88**	**1.75**	**1.63**	**2.50**	**-**

Observation from ten days old culture plates; Data presented in Table was average of three replications

Table 5. Categorization of isolates in respect of lesion and reaction type

Category	**Severity range (%)**	**Isolate**	**Lesion type**	**Reaction type**
I	>50%	PGMP1,	Larger, distinct, spindle shape spot with a central ashy zone and marginal zones 3 to 5 mm broad and up to several cm. in length.	HS
II	26-50%	PGMP5, PGMP6, PGMP7	Broadly spindle shaped spots only slightly longer than broad, 3-5 mm in diameter.	S
III	<25%	PGMP2, PGMP3, PGMP4	Circular spots about 2 to 3 mm in diameter with central ashy zone and a purplish brown margin	S

HS : Highly susceptible; S : Susceptible

however, second sub-group had two isolates, *i.e*. PGMP 4 and PGMP6. Isolates collected from nearer locations, *i.e.* PGMP1, PGMP2 and PGMP7, PGMP8 were grouped. Moreover, isolate PGMP5 collected from distant location was placed in a separate cluster. Hence, results of ISSR markers revealed that a high measure of genetic variability of the *P. grisea* depends upon their sampling origin. Costa *et al.* (2016) also observed that genetic diversity among *P. grisea* isolates included inter-simple sequence repeat markers. Recently, Longya *et al.* (2020) analyzed genetic variability among *P. grisea* isolates of Thailand using 14 ISSR markers, and high genetic variations were evidenced among the collected 59 isolates in their study.

RAPD marker analysis: In the current investigation, initially a total of 15 RAPD primers were screened. Out of 15, only four RAPD primers steadily produced strong amplification products. Data recorded clearly outlined that the total number of alleles identified was 11 with an average of 2.75 alleles per locus (Table 6). Genetic diversity of *P. grisea* differed from zero (RAPD 2 and RAPD 4) to 0.25 (RAPD1) with a mean value of 0.0938. PIC value ranged between 0.3047 and 0.5547. While major allele frequency arrayed between 0.50 and 0.75 with a mean worth of 0.63.

The UPGMA (Fig 2) cluster analysis depicted that collected *P. grisea* isolates were divided into two groups *i.e*., group A and group B. Group A contained only one isolate namely, PGMP 8, whilst second group B was again divided into two sub-groups *i.e*., sub-group IB and IIB. Isolate PGMP 4 separated alone in sub-group IA, while sub-group IIB comprised six isolates *viz*., PGMP1, PGMP2, PGMP3, PGMP5, PGMP6 and PGMP7. Results of this investigation revealed that the genetic diversity of *P. grisea* depicted by RAPD primers did not depend upon their geographical origin.

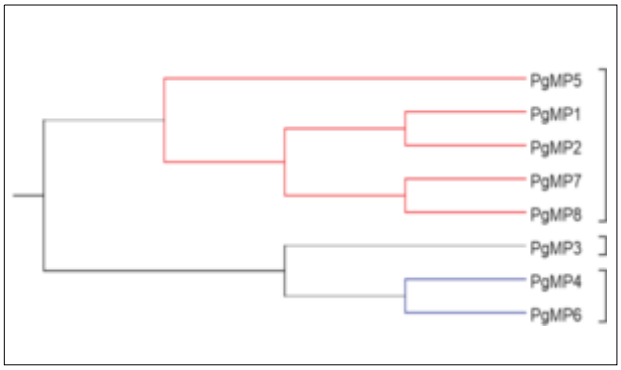

Fig 1. UPGMA clustering among *P. grisea* isolates based on ISSR markers

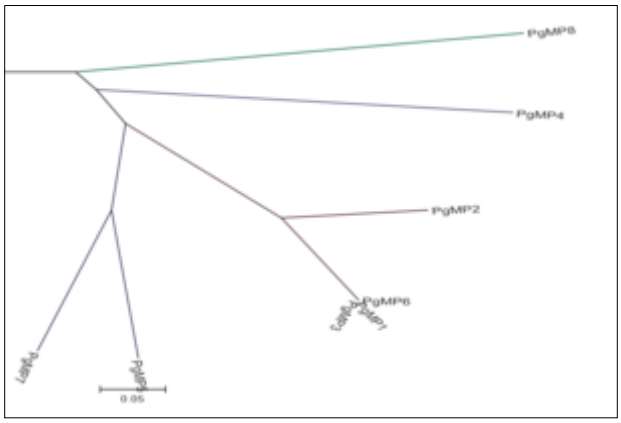

Fig 2. UPGMA clustering among *P. grisea* isolates based on RAPD markers

Table 6. ISSR and RAPD markers presenting major allele frequency, number of alleles, gene diversity and PIC value in *P.*

Markers	Major allele frequency	Allele number	Gene diversity	PIC
ISSR				
ISSR1	0.50	3	0.59	0.51
ISSR3	0.63	3	0.53	0.47
Mean	**0.56**	**3**	**0.56**	**0.49**
RAPD				
OPA-01	0.50	3	0.63	0.55
OPA-02	0.50	3	0.59	0.51
OPA-04	0.75	2	0.38	0.30
OPA-09	0.75	3	0.39	0.35
Mean	**0.63**	**2.75**	**0.49**	**0.43**

PIC: Polymorphism information content

DNA fingerprinting of 48 rice isolates of *P. grisea* collected from Himachal Pradesh through RAPD was attempted by Rathour *et al.* (2004) and high genetic diversity with 83% polymorphisms was detected. Sharma *et al.* (2008) also used RAPD markers for diversity analysis among *P. grisea* isolates collected from the Himalayan region of India. They reported high genetic variability among the isolates collected from Himachal Pradesh in comparison to the isolates of Uttaranchal. However, Singh and Kumar (2010) observed high genetic variability among finger millet isolates of *P. grisea* in three different geographical areas of Uttarakhand after evaluation with RAPD markers.

Conclusion

It was concluded that *P. grisea* collected from different regions of northern Madhya Pradesh *viz.,* Gwalior, Morena, Bhind and Sheopur consisted of a variable population based on morphology, virulence pattern and molecular analysis. The fungal strains varied with respect to morphology and severity. Colony colour varied from white to grey, with differential growth rates leading to unique patterns. This led to the production of different mycelium morphologies. Mycelial growth was maximum in isolate PGMP1 collected from the Gwalior location, while isolate PGMP8 collected from the Gormi location of Bhind district produced a greater number of conidia. Blast severity on the Dhanshakti cultivar was higher in isolates PGMP1 and PGMP8. Molecular phylogenetic grouping obtained by RAPD analysis did not correlate with morphological characteristics and virulence patterns. However, ISSR markers revealed the presence of a high level of genetic diversity, and *P. grisea* strains could be clustered based on geographic distribution. These results indicated the presence of a certain level of genetic diversity among *P. grisea* isolates. Pathogenicity tests exposed that these isolates expressed different levels of virulence which could be important for disease dynamics and managing diseases, like developing resistant crops.

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