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Diversity of *Macrophomina phaseolina* Based on Morphological and Genotypic Characteristics in Iran

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Fifty two *Macrophomina phaseolina* isolates were recovered from 24 host plant species through the 14 Iranian provinces. All isolates were confirmed to species using species-specific primers. The colony characteristics of each isolate were recorded, including chlorate phenotype, relative growth rate at 30°C and 37°C, average size of microsclerotia, and time to microsclerotia formation. The feathery colony phenotype was the most common (63.7%) on the chlorate selective medium and represented the chlorate sensitive phenotype of the Iranian *Macrophomina phaseolina* population. Mean-time, inter simple sequence repeats (ISSR) Markers were used to assess the genetic diversity of the fungus. Unweighted pair-group method using arithmetic means (UPGMA) clustering of data showed that isolates did not clearly differentiate to the specific group according to the host or geographical origins, however, usually the isolates from the same host or the same geographic origin tend to group nearly. Our results did not show a correlation between the genetic diversity based on the ISSR and phenotypic characteristics. Similar to the *M. phaseolina* populations in the other countries, the Iranian isolates were highly diverse based on the phenotypic and the genotypic characteristics investigated and needs more studies using neutral molecular tools to get a deeper insight into this complex species.

Keywords : charcoal rot, chlorate phenotype, ISSR

The causal agent of charcoal rot, *Macrophomina phaseolina* (Tassi) Goidanich, is a soil- and seed-borne pathogen of over 500 host plant species (Dhingra and Sinclair, 1978) and it causes significant damage in Iran to soybean (Raeyatpanah and Forootan, 1993; Raeyatpanah et al., 2002) and sunflower (Razavi and Pahlavani, 2004). The high levels of morphological variability of *M. phaseolina* across different hosts and geographical regions suggest that this species may be divided into subgroups (Aboshosha et

al., 2007; Beas-Fernandez et al., 2006; Hawatema and Hameed, 2006; Karunanithi et al., 1999; Mayek-Perez et al., 1997; Mihail and Taylor, 1995; Omar et al., 2007). However, grouping of isolates by *formae specialis*, subspecies, or physiological race was challenged due to variation in the morphology of isolates from a single host (Dhingra and Sinclair, 1972).

Molecular markers are powerful tools for assessing genetic variation and elucidating genetic relationships within and among species (Chakravarthi and Naravaneni, 2006). Different molecular methods have been used for differentiating *M. phaseolina* populations including Restriction Fragment Length Polymorphism (RFLP) of rDNA-ITS regions (Aghakhani and Dubey, 2009; Almeida et al., 2003; Purkayastha et al., 2006; Su et al., 2001), Random Amplified Polymorphic DNA (RAPD) (Aboshosha et al., 2007; Aghakhani and Dubey, 2009; Almeida et al., 2003; Almeida et al., 2008; Babu et al., 2010; Das et al., 2006; Jana et al., 2003; Omar et al., 2007; Purkayastha et al., Rajkumar and Kuruvinashetti, 2007; Su et al., 2001; 2006; Zade et al., 2009), Amplified Fragment Length Polymorphism (AFLP) (Brooker et al., 2008; Mayek-Perez et al., 2001; Reyes-Franco et al., 2006; Saleh et al., 2010; Vandemark et al., 2000), Universal Rice Primer PCR (URP-PCR) (Jana et al., 2005b), Inter simple sequence repeats (ISSR) (Jana et al., 2005a; Purkayastha et al., 2008), Repetitive Sequence-Based Polymerase Chain Reaction (Rep-PCR) (Purkayastha et al., 2008) and SSR (Baird et al., 2010). Inter simple sequence repeat (ISSR) markers are powerful tools which can be utilized to access the variation in the flanking regions of microsatellite loci that are dispersed throughout all genomes (Zietkiewicz et al., 1994). In this study we used some conventional techniques and ISSR markers to assess the morphologic and genetic variability among 52 isolates of *M. phaseolina* from different hosts and origins within Iran and identifying insights to host specialization.

Material and Methods

Fungal isolates. Isolates of *M. phaseolina* were recovered from 24 different host species showing typical symptoms

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Fig. 1. Iranian provinces where samples, showing charcoal rot, of different host species were collected for *Macrophomina phaseolina* isolation.

and signs of charcoal rot in 14 Iranian provinces (Fig. 1 and Table 2). To isolate *M. phaseolina* from infected tissue, five small (0.5 cm) epidermal sections were excised from symptomatic roots or stems. Tissue sections were surface disinfested with 75% ethanol for 30 s and 2.5% sodium hypochlorite for 30 then washed in sterile water for 1 min. and placed on potato dextrose agar (PDA, Merck, Darmstadt, Germany) containing chloramphenicol (0.1 mg/ml). Plates were incubated at 30 °C in the dark for 5 days. Pure cultures were obtained by the hyphal tip technique and maintained on sterile toothpicks at room temperature (Edmunds, 1964). Isolates were identified to species using species-specific primers (MpKFI/MpKRI) according to Babu et al. (2007). Controls included a no DNA negative control, DNA of isolates of *Trichoderma harzianum*, and *Cytospora schusleri*, *Sclerotinia sclerotiorum*. A known marker isolate of *M. phaseolina* was used as a positive control (Fig. 2).

Fig. 2. Identification of *M. phaseolina* isolates using species-specific primers (MpKFI/MpKRI), M: 1 kb molecular ladder; m1-m3: *M. phaseolina* isolates from Iran; T: *Trichoderma harzianum*, C: *Cytospora schusleri*, and S: *Sclerotinia sclerotiorum*, are negative controls; PC: positive control marker isolate of *M. phaseolina*; NC: negative control without DNA

Fig. 3. Colony phenotypes of *Macrophomina phaseolina* isolates grown on PDA amended with 120 mM potassium chlorate. A. dense colony phenotype B. feathery colony phenotype and C. restricted colony phenotype

Morphological analysis. The morphological characteristics of the recovered isolates were investigated, including colony phenotype on medium containing 120 mM potassium chlorate (Fig. 3) (Pearson et al., 1986), relative growth rate at 30 °C and 37 °C (Beas-Fernandez et al., 2006; Mayek-Perez et al., 1997), height of aerial mycelium, average length and width of 50 microsclerotia, and time to microsclerotia formation.

Genomic DNA extraction. Isolates were grown in the dark at 28 °C for 5 days in 250 ml glass bottles containing 50 ml potato dextrose broth (PDB). Mycelia were filtered through Whatman No. 1 filter paper and lyophilized by vacuum pump. Genomic DNA was extracted according to Lee and Taylor (1990) with some modifications (excluding phenol-chloroform step) as described by Safaie et al. (2005). DNA concentrations were determined by BioPhotometer at 260 nm and were stored at –20 °C for further use.

PCR amplification and gel electrophoresis. ISSR analysis

was carried out amplifying the genomic DNA using ISSR primers (Table 1). The PCR amplification conditions were: initial denaturation at 95 °C for 3 min followed by 35 cycles of denaturation at 94 °C for 2 min, primer annealing according to Table 1 for 1 min, primer extension at 72 °C for 2 min, and a final primer extension at 72 °C for 7 min. The amplification product was separated in a 1.5% agarose gel. The gel was stained with ethidium bromide (0.50 µg/ml) and visualized under UV to confirm DNA amplification. Amplifications and gel separation were repeated at least twice with each primer. A total of nine primers were screened initially. Six primers were selected for final analysis based on informative banding patterns, clarity, and repeatability.

Statistical analysis. Statistical analysis of morphological characteristics was performed in SAS.9.1.3.Service Pack 2. Means comparisons were performed by Duncan's Multiple Range Test. Relatedness among the 52 isolates was estimated by comparing the amplified DNA bands across the six

Table 1. List of ISSR primers, their annealing temperatures, and the resulted DNA polymorphisms applied to differentiate isolates of *M. phaseolina*

Primer	Primer sequence (5–3')	Annealing temperature (°C)	No. of amplified DNA fragments	No. of polymorphic fragments	Percent polymorphic fragments	Reference
ISSR2	5'-ACTGACTGACTGACTG-3'	48	26	22	84.6	Jana et al., 2005
ISSR5	5'-GACACGACACGACACGACAC-3'	50	25	21	84.0	Jana et al., 2005
ISSR10	5'-CACCACCACCACCAC-3'	52	20	18	90.0	Jana et al., 2005
PcMs	5'-GTCGTCGTCGTCGTCGTCGTC-3	57	20	18	90.0	Islam et al., 2003
ISSR09	5'-CCACCACCACCACCA-3'	47	17	14	82.4	Vasserur et al., 2005
P4	5'-ATGATGATGATGATGATG-3'	47	16	14	87.5	Stepansky et al., 1999
P5	5'-ACACACACACACACACYC-3'	–	–	–	–	Stepansky et al., 1999
P6	5'-GAGAGAGAGAGAGAGAYG-3'	–	–	–	–	Stepansky et al., 1999
LB-B	5'-GACAGACAGACAGACATT-3	–	–	–	–	Arbaoui et al., 2008
Total	–	–	124	108	87.1	–

Table 2. Host-, geographic-origins, and morphological characteristics of the studied *M. phaseolina* isolates of Iran

Isolate No.	Host	Location	Colony morphology	Chlorate sensitivity	Growth rate at 30 °C (mm/hr)	A	B	C
1	Sunflower	Khozestan	Feathery	Sensitive	1.385	M		
2	Sunflower	Fars	Restricted	Sensitive	1.156	L		
3	Sunflower	Markazi	Restricted	Sensitive	0.906	L	+	
4	Soybean	Golestan	Feathery	Sensitive	1.021	M	+	
5	Soybean	Golestan	Restricted	Sensitive	0.833	M	+	
6	Soybean	Golestan	Restricted	Sensitive	1.115	M		
7	Olive	Khozestan-bagh malek	Feathery	Sensitive	1.323	H		+
8	Olive	Khozestan-haftapeh	Feathery	Sensitive	1.448	M		+
9	Olive	Khozestan-ahvaz	Feathery	Sensitive	1.135	M		+
10	Cantaloupe	Khorasan	Restricted	Sensitive	1.198	M		
11	Cantaloupe	Fars	Dense	Resistant	1.083	L		+
12	Cantaloupe	Isfahan-kashan	Dense	Resistant	1.240	M		
13	Common Bean	Khorasan	Feathery	Sensitive	1.302	M		+
14	Common Bean	Isfahan	Feathery	Sensitive	1.115	L		+
15	Common Bean	Ghazvin	Restricted	Sensitive	1.010	H	+	
16	Tomato	Khorasan	Restricted	Sensitive	1.240	L		
17	Tomato	Qom	Feathery	Sensitive	1.219	H		
18	Tomato	Ghazvin	Feathery	Sensitive	1.125	M		+
19	Cucumber	Tehran-karaj	Dense	Resistant	1.542	L		
20	Cucumber	Hamedan	Restricted	Sensitive	1.010	L		
21	Cucumber	Qom	Feathery	Sensitive	1.292	H		
22	Melon	Khorasan	Restricted	Sensitive	1.458	H		
23	Melon	Semnan	Dense	Resistant	1.104	L		
24	Melon	Qom	Feathery	Sensitive	1.260	M	+	
25	Sesame	Isfahan-kashan	Feathery	Sensitive	1.406	H		
26	Sesame	Khozestan	Feathery	Sensitive	1.500	M		+
27	Sesame	Kerman	Feathery	Sensitive	0.844	L	+	
28	Kiwi	Golestan	Feathery	Sensitive	1.010	M	+	
29	Kiwi	Golestan	Feathery	Sensitive	0.990	M	+	
30	Kiwi	Golestan	Feathery	Sensitive	0.927	M	+	+
31	Hemp	Isfahan-kashan	Feathery	Sensitive	1.385	M		+
32	Hemp	Isfahan	Restricted	Sensitive	1.125	M		
33	Hemp	Isfahan	Feathery	Sensitive	1.188	L		
34	Pumpkin	Markazi	Feathery	Sensitive	1.344	M		
35	Pumpkin	Ghazvin	Feathery	Sensitive	1.208	M		
36	Pumpkin	Ghazvin	Feathery	Sensitive	1.177	M		+
37	Mungbean	Kerman	Feathery	Sensitive	1.146	M		
38	Mungbean	Kerman	Feathery	Sensitive	1.250	L		
39	Mungbean	Kerman	Feathery	Sensitive	1.208	M		
40	Okra	Khozestan	Feathery	Sensitive	1.406	M		
41	Okra	Yazd	Feathery	Sensitive	1.073	L		
42	Sugar beet	Khorasan-fariman	Dense	Resistant	1.240	L		
43	Canola	Qom	Feathery	Sensitive	0.948	H		
44	Datura	Markazi	Feathery	Sensitive	1.021	L		
45	Eggplant	Hormozgan	Feathery	Sensitive	1.260	L		
46	Sonchus	Tehran-karaj	Restricted	Sensitive	1.219	H	+	+
47	Maize	Isfahan-kashan	Feathery	Sensitive	1.115	H		
48	Maize	Golestan	Feathery	Sensitive	1.333	M		+
49	Watermelon	Isfahan-kashan	Feathery	Sensitive	1.021	H		+
50	Marigold	Golestan	Restricted	Sensitive	1.198	H		+
51	Sorghum	Golestan	Feathery	Sensitive	1.271	M		
52	Turnip	Isfahan-kashan	Feathery	Sensitive	1.115	L		+

A: Height of aerial mycelium: L=low (0 mm), M=moderate (1–2 mm), H=high (> 2 mm). B: Isolates with microsclerotia formed before 48 h of incubation at 37 °C, C: Isolates with microsclerotia formed after 60 hr of incubation at 37 °C

ISSR primers. Each band was considered either present (coded as 1) or absent (coded as 0). The binary data were analyzed using the NTSYS-pc (Numerical Taxonomy and Multivariate Analysis System) package (Rohlf, 2000) to determine the similarities among individuals. Similarities were estimated using Jaccard's (J) coefficient (Jaccard, 1908). Clustering analysis was performed using NTSYS-PC software programs by the unweighted pair-group method using arithmetic averages (UPGMA).

Results and Discussion

Fungal isolates. Fifty two *Macrophomina phaseolina* isolates were recovered from 24 plant species collected from the 14 Iranian provinces (Fig. 1 and Table 2). This was the first report of *M. phaseolina* from hemp, turnip, okra, mungbean, marigold and *Sonchus* in Iran. The species-specific primers designed by Babu et al. (2007) amplified one 350 bp band in all isolates of *Macrophomina*, confirming that the isolates obtained belonged to the species *M. phaseolina*. No bands were present in the negative controls, confirming that the primers were specific to *M. phaseolina*. In most cases, we selected three isolates from each host, but from different regions, to try to determine whether isolates of one host from three regions have host-specific fingerprint profiles, this is an insight into host specialization.

Morphological analysis. Most *M. phaseolina* isolates (67.3%) were characterized by feathery colony phenotype when grown on PDA medium containing 120 mM potassium chlorate while 9.6% of the isolates showed dense growth and 23.1% of the isolates showed restricted growth (Table 2). Isolates characterized with a dense growth phenotype were considered resistant to chlorate, while isolates characterized with feathery or restricted growth phenotype were considered resistant to chlorate. All resistant isolates, except one, were collected from cucurbitaceous host plants, the exception was collected from sugar beet. All isolates from olive, kiwi, pumpkin, mungbean, tomato, okra and maize had a feathery phenotype, while two isolates from sunflower and soybean had a restricted phenotype (Table 2). Isolates were characterized according to the height of aerial mycelium when grown in PDA culture. Low aerial mycelium measured 0 mm, medium aerial mycelium measured 1–2 mm, and high aerial mycelium measured greater than 2 mm. Among the isolates collected in Iran, 30.8% had low aerial mycelium, 48.1% were moderate, and 21.2% had high aerial mycelium (Table 2). Isolates No.10 and No.12 had the largest microsclerotia, and isolates No.41 and No.27 had the smallest microsclerotia. Isolates from common bean (No.13, No.14 and No.15), maize (No.47 and No.48), mungbean (No.38 and No.39), kiwi (No.29 and No.30), cucumber (No.19 and No.20), olive (No.8 and No.9), sun-

Fig. 4. Dendrogram constructed with UPGMA clustering method for 52 *Macrophomina phaseolina* recovered from different provinces in Iran. Dissimilarities were computed from chlorate phenotype, relative growth rate at 30°C and 37°C, height of aerial mycelium, average length and width of 50 microsclerotia, and time of microsclerotia formation.

flower (No.1 and No.3), soybean (No.4 and No.6) and hemp (No.31 and No.33) have the same size of sclerote and grouped according to host. Finally, isolates were clustered using all morphological characteristics (Fig. 4).

All the dense chlorate-resistant isolates, except one, were recovered from cucurbitaceous hosts, the exception was collected from sugar beet in Khorasan province. Chlorate is an analog of nitrate, and the reduction of chlorate via nitrate reductase can result in chlorate toxicity in plants, algae, bacteria and fungi. Chlorate-sensitive strains can reduce nitrate to nitrite, and the chlorate resistant strains cannot (Pearson et al., 1986). All isolates from olive, kiwi, pumpkin, mungbean, tomato, okra and maize were sensitive to chlorate, as recognized by the feathery phenotype on potassium chlorate medium. These results support reports that isolates of the same host species usually have the same colony phenotype (Cloud and Rupe, 1994; Pearson et al., 1987; Purkayastha et al., 2006; Su et al., 2001). As observed among *M. phaseolina* isolates from olive, kiwi, pumpkin, mungbean, tomato, okra and maize, the association of chlorate sensitivity and host may be due to the selection pressure posed by the host over time. Host-specific isolates may have had time to establish a genetic change based on the physiology of nitrogen utilization in the host. However, colony phenotype of *M. phaseolina* isolates of cucumber and melon grown on chlorate medium was not consistent

within each host. The host from which an isolate was collected may not be the only host to which the isolate was exposed (Pearson et al., 1986). Inorganic nitrate serves as an excellent nitrogen source for many fungi, including *M. phaseolina*, but it is generally not utilized unless the cells lack a favored nitrogen source, such as ammonia, glutamine or glutamate (Dhingra and Sinclair, 1978; Marzluf, 1997). Maximum invasion in *Macrophomina* took place during the grain development stages because of competition for carbohydrates with developing grains that physiologically weaken the plant (Dodd, 1980). Under such conditions it is highly probable that fungi might utilize nitrate as a source of nitrogen (Das et al., 2006).

ISSR analysis. DNA fragments that resulted from amplification with six ISSR primers ranged in size from 250 to 3,300 bp (Fig. 5). The number of bands ranged from 16 to 26, of which 84–90% were polymorphic (Table 1). Dendrogram of pooled six primers data is presented in Fig. 6. Based on the resulting dendrogram, isolates did not clearly differentiate to the specific group according to the host or geographical origins, however, usually the isolates from the same host or the same geographic origin tend to group nearly. For example, isolates from hosts olive (No.7 and No.8), sunflower (No.2 and No. 3), Kiwi (No.28, No.29 and No. 30), mungbean (No.38 and No.39), hemp (No.32

Fig. 5. ISSR banding pattern of 52 *Macrophomina phaseolina* isolates generated by primer PCMS (B). M: standard 1kb molecular ladder, NC: negative control without DNA.

Fig. 6. Dendrogram of 52 isolates of *Macrophomina phaseolina* derived from ISSR analysis with all six primers by UPGMA.

and No.33) and isolates from provinces Qom (No.17 and No.24), Golestan (No.4, No.28, No.29, No.30 and No.5), Ghazvin (No.18 and No.26), Kerman (No.38 and No.39), Isfahan (No.32 and No.33) appeared to be closely related (Fig. 6).

The ISSR technique is an appropriate molecular analysis approach to identify genetic differences in *M. phaseolina* populations (Jana et al., 2005a). The results of the present study clearly demonstrated that *M. phaseolina* from different parts of the country were highly variable and ISSR markers are suitable to reflect the genetic diversity among the populations. A number of molecular studies elsewhere have shown a high level of polymorphism in this fungus when isolates from different host or geographical origins were compared using different molecular tools (Almeida et al., 2003; Jones et al., 1998; Mayek-Perez et al., 2001; Su et al., 2001; Vandemark et al., 2000). Other recent studies have demonstrated the genetic diversity among *M. phaseolina* isolates (Aboshosha et al., 2007; Aghakhani and Dubey, 2009; Babu et al., 2010; Baird et al., 2010; Beas-Fernandez et al., 2006; Das et al., 2006; Jana et al., 2005a; Jana et al., 2005b; Omar et al., 2007; Purkayastha et al., 2008; Rajkumar and Kuruvnashetti, 2007; Reyes-Franco et al., 2006; Saleh et al., 2010). The high genetic diversity of this pleomorphic fungal species is reflected not only in isolates from distinct hosts and geographical origins but also within isolates collected from a single host or geographical origin. The

activity of transposons and a high mutation rate of micro-satellites could be crucial for genetic variability generated by parasexual reproduction of the fungi (Purkayastha et al., 2008). Based on the resulting dendrogram for ISSR markers, the isolates were not completely differentiated to specific groups according to host or geographical origins. However, usually isolates from the same host or origin tend to group together. Several studies have found no association between DNA genotype and host origin in populations (Almeida et al., 2003; Reyes-Franco et al., 2006; Vandemark et al., 2000). Recently Saleh et al. (2010) used AFLP markers and ITS region sequences to assess the genetic diversity and relationships of *M. phaseolina* isolates from maize, sorghum, tallgrass prairie and soybean. Using Bayesian cluster analysis based on the estimation of co-ancestry coefficients they demonstrated the incomplete specialization by host in charcoal rot fungus, in contrast to results reported by earlier studies (Jana et al., 2003; Jana et al., 2005a; Jana et al., 2005b; Mayek-Perez et al., 2001; Su et al., 2001). Recently, Baird et al. (2010) used twelve simple sequence repeat (SSRs) loci to evaluate genetic diversity of *M. phaseolina* collected from different geographical regions and host species throughout the United States. Their results showed some clustering by host and geography, but the dendrogram generally grouped isolates independent of host or geography. Three Primers in the present study, ISSR2, ISSR5, and ISSR10, were used by Jana et al. (2005a) to separate

isolates according to their hosts and geographical origins. Unlike the present study, these primers did not completely separate isolates according to their hosts. The controversial results could be due to the high number of isolates and hosts in our study compared to theirs. Isolates from hosts olive (No.7 and No.8), sunflower (No.2 and No. 3), Kiwi (No.28, No.29 and No. 30), mungbean (No.38 and No.39), hemp (No.32 and No.33) and isolates from provinces Qom (No.17 and No.24), Golestan (No.4, No.28, No.29, No.30 and No.5), Ghazvin (No.18 and No.26), Kerman (No.38 and No.39), Isfahan (No.32 and No.33) were closely related (Fig. 6). In part similar to our results, several other studies have found an association between DNA genotype and host or geographic origin in populations (Jana et al., 2003; Jana et al., 2005a; Jana et al., 2005b; Mayek-Perez et al., 2001; Purkayastha et al., 2006; Su et al., 2001). The present findings are partially in accordance with the earlier results that the *M. phaseolina* isolates of same host were genetically similar and differed distinctly from the isolates of other hosts (Purkayastha et al., 2006; Su et al., 2001). The existence of genetic diversity among the isolates from the same provinces might be due to the movement of *M. phaseolina* through infested seeds and soil (Aghakhani and Dubey, 2009). Some of the isolates within a province showed similarity suggesting that these isolates may be considered as a part of the same ancestral population (Almeida et al., 2003).

Conclusion

A considerable degree of diversity was revealed in the Iranian population of *M. phaseolina* in the present study. It is suggest that this enhanced its ability to adapt to diverse conditions and overcome host resistance deployed by growers (Trigiano et al., 2008). Basic morphological characteristics are the primary tools to detect diversity in all organisms; however, such morphological phenotypes are subjected to modification by environmental factors or by interaction with products of unknown genes in the genetic background. Molecular markers are ideal tools for assessing variation rapidly within and among species (Chakravarthi and Naravaneni, 2006). In the present study, 52 *M. phaseolina* isolates were recovered from 24 host plant species throughout 14 Iranian provinces surveyed. All isolates were confirmed to species based on species-specific primers according to Babu et al. (2007).

Our results revealed that like *M. phaseolina* populations in other countries; the Iranian populations are highly diverse based on phenotypic and genotypic data. High levels of variability are most likely due to the exposure of the pathogen to diverse climates, hosts, cropping patterns and movement of the pathogen within the country. In recent

years the country has experienced severe drought conditions possibly due to global warming. *M. phaseolina* capable of becoming a severe problem in many regions and on several host plants, it is tempting to call it a “global warming plant pathogen” as this may influence its current importance in countries such as Iran.

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