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Detection of double stranded RNA in phytopathogenic *Macrophomina phaseolina* causing charcoal rot in *Cyamopsis tetragonoloba*

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Abstract One hundred one isolates of *Macrophomina phaseolina* from various hosts and eco-geographical locations were employed for elucidating relationships among genetic diversity and virulence. Highly pathogenic, moderately pathogenic, and hypovirulent cluster bean specific isolates were identified. In order to correlate respective phenotypes of plant pathogenic fungus multiple and complex patterns of dsRNA elements were analyzed. Double-stranded ribonucleic acids (dsRNA) are ubiquitous in all major groups and most of them have vast potential as biological control agents for fungi. Rate of virulence and its further association could ascertain by host plant and their fungal genotypes. Variability of the fungal genotypes decides the link between the complexity of dsRNA with different variants and the change in virulence pattern. Double-stranded RNA was identified in approximately 21.7% of *M. phaseolina* isolates from charcoal rot infected cluster bean varieties. After recurrent laboratory transfer on culture media, the preponderance of the isolates harboring dsRNAs developed degenerate culture phenotypes and showed reduced virulence (hypovirulence) to cluster bean. *Macrophomina* has successfully showed diversified and reproducible banding profile in dsRNA containing/free isolates. This is the first report of hypovirulence and detection of dsRNA in *Macrophomina phaseolina* isolates of cluster bean origin.

Keywords Cluster bean · Detection · Double stranded RNA (dsRNA) · *Macrophomina phaseolina*

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Introduction

Cluster bean (*Cyamopsis tetragonoloba*) the cash crop contributing 80% shares of its total worldwide production has tremendous application in textile, paper, petroleum, mining, pharmaceuticals, explosives, and food industries. It is widely cultivated in countries such as India, Pakistan, USA, Italy, Morocco, Germany, Greece, and Spain, thus considered as a new crop for western agricultural practices [1]. Owing to its immense importance, there is a strong need for appropriate addressing and well documentation of the germplasm, by biocontrol of phytopathogenic *Macrophomina phaseolina*. The fungus is a non-specific pathogen of charcoal rot disease and hampers a broad spectrum of economically important crops such as common beans, maize, soybean, sorghum, sesame, cotton, sunflower or cucurbits [2]. *M. phaseolina*, the only species of the genus, has a wide host range, which is responsible for causing losses in more than 500 variable plant species growing across the world [3]. The fungus produces two anamorphs for various intense examinations; microsclerotial phase (black) of *M. phaseolina*, profoundly considered more pathogenic as compared to its pycnidial (hyaline) anamorph structures [2]. Mihail [4] also recognizes anchoring diversified synonyms *M. phaseolina* (Tassi) as *M. phaseoli* (Maubl.), *Macrophoma conchoci* (Swada), *Sclerotium bataticola* (Taub.) and *Rhizoctonia bataticola* (Taub.). Its morphological and pathogenic variability was widely acknowledged among isolates of different hosts and between isolates of same plant [5]. The most common diagnostic symptom for charcoal rot disease on prematurely dying or dead plants is the sloughing of cortical tissues from the lower stem and tap root and the speckled grey appearance of the infected tissues due to the formation of abundant microsclerotia in vascular, cortical and pith

tissues. The fungus population in different parts of the world was characterized based on its pathogenic variability [6], morphological characteristics [5], and molecular characteristics [7–9], but the basis of differences in pathogenicity is still unknown [10].

The term virulence (hypo/moderate/hyper) describes the ability of specific fungal isolate to cause disease. Until now, several studies on plant pathogenic fungi have reported association of the presence of dsRNA with virulence. Double-stranded RNAs (dsRNA) associated with virus-like particles is found prevalent in various fungal species [11–14]. The dsRNA molecule exists as collection of unique and discrete entities that replicates very differently from double stranded DNAs. Effects of dsRNA on plant pathogenic fungal species had studied globally and their hypovirulence to host plant is well documented [15–18]. The classified quoted example of dsRNA-virulence association is *Cryphonectria parasitica* of chestnut blight. In this, presence of dsRNA is correlated with transmissible hypovirulence in various crops [19, 20]. In contrast, *Macrophomina* the only species has not investigated extensively among cluster bean specific isolates, and no information exists on the use of hypovirulence, associated with dsRNA, to control *Macrophomina*-incited disease in cluster bean cultivars. The objectives of the present study were: (1) To detect the occurrence of extrachromosomal elements (dsRNA) amongst cluster bean infecting isolates of *M. phaseolina* grown under different eco-geographical conditions of North India and to differentiate highly pathogenic, less pathogenic, and moderately pathogenic isolates. (2) To determine if there is an association between the presence of dsRNA and virulence in *Macrophomina* isolates of cluster bean plants.

Materials and methods

Fungal isolates and culture venue

101 isolates of *Macrophomina* used in the present study were isolated from various rhizosphere soils/seeds/plants of cluster bean from the four majorly cluster bean growing states, viz., Haryana, Rajasthan, Punjab and Gujarat of North and North-West India (Nagpur, Lyallpur, Kalyani, Almora, Coimbatore, New Delhi, Assam) (Table 1). The isolates collected were used for isolation on Potato dextrose agar (PDA), pH 5.5. Culture plates were incubated in the dark at 28 ± 1°C for 4–5 days. Purified fungal cultures were preserved and maintained routinely on potato dextrose agar (Hi-media) medium (Table 2). The purified isolates were maintained as pure cultures using the single hyphal tip technique [9] for further studies. Dried mycelial disks were stored at −20°C for long-term preservation. For dsRNA extraction, fungal liquid cultures were grown in broth complete medium (PDB) for 2 weeks at 28°C to produce mycelia for the extraction and purification of dsRNA.

Extraction and purification of dsRNA

Mycelial mat was harvested by centrifugation at 8,000 rpm for 10 min from liquid cultures and the mat was washed with 10 ml TES buffer [10], prior to lyophilization process. Fungal dsRNA was extracted according to the method of Morris and Dodds [21] and Valverde et al. [22] with minor amendments. Modified CF11 cellulose packed column chromatography was employed for the extraction of dsRNA elements. Approximately 2 g lyophilized dried mycelium was used for the extraction of dsRNA from each *Macrophomina* variable. The dsRNA was precipitated and eluted by centrifugation at 10,000 rpm (15 min), and pellets were finally dissolved in 40 µl of sterile distilled water. The dsRNA species was further separated by agarose gel electrophoresis (1%) and the purified dsRNA was stored at −20°C. The presence of the dsRNA was verified by reisolating the hyphae and growing it in pure liquid culture. After several days of growth, the dsRNA was purified, and its presence, quality and relative concentration was checked by electrophoresis on a 1% agarose gel with 1× TAE buffer (40 mM Tris–Acetate, 1 mM EDTA, pH 8.0) at 6 V cm^{-1} and visualized with a UV light source by ethidium bromide staining. A fairly accurate molecular weight was determined by comparing electrophoretic mobility of the dsRNAs with molecular weight standard, 1-kb DNA ladder (Genei, Bangalore).

Confirmation of dsRNA by enzymatic digestions

Two enzymatic digestions were performed separately using DNase-I and RNase-A as described by Bogo et al. [23]. For DNase digestion, the samples were treated with 30 U of enzyme in 6 mM $MgCl_2$ (Gibco Life Technologies, USA) according to the manufacturer's instructions. For RNase-A digestion, the enzyme (Invitrogen, USA) was added to 3 mg of total nucleic acids in 0.3 M NaCl, to a final concentration of 4 µg ml^{-1} and incubated at 37°C for 1 h. The reactions were purified twice using equilibrated phenol:chloroform:isoamyl alcohol (25:24:1), and precipitated by the addition of 5.5 M ammonium acetate (0.6 vol) and 95% ethanol (2 vol). Precipitates were eluted by centrifugation at 10,000 rpm for 20 min and pellets were dissolved in 30 µl of sterile distilled water. Samples were analyzed by electrophoresis on 1.0% agarose gel in 1× TAE buffer, stained with ethidium bromide (100 ng ml^{-1}) and visualized under UV transilluminator. Isolates not hydrolyzed by DNase or high salt concentration were designated as

Table 1 Isolate number, location, host and colony characteristics w.r.t dsRNA of 101 isolates of *M. phaseolina*

Location	Host	Isolates	Phenotype
Punjab	Cluster bean	CB-1, CB-2, CB-36[a], CB-47	Dense
	Cluster bean		Feathery
	Cluster bean		Restricted
Rajasthan	Cluster bean	CB-3[a], CB-4, CB-6[a], CB-7, CB-8, CB-9[a], CB-13[a], CB-18, CB-22[a], CB-23, CB-24, CB-25, CB-27, CB-32, CB-33, CB-43, CB-45, CB-48, CB-49, CB-52[a],CB-54, CB-60[a], CB-63, CB-85, CB-86, CB-87	Dense
	Cluster bean	CB-14, CB-16, CB-19, CB-26, CB-29[a], CB-39[a]	Feathery
	Cluster bean	CB-5[a], CB-17[a], CB-34, CB-35[a], CB-37[a], CB-44, CB-100	Restricted
Haryana	Cluster bean	CB-11[a], CB-12[a], CB-20, CB-30, CB-40[a], CB-46[a], CB-50, CB-55, CB-61, CB-65, CB-67, CB-71, CB-74, CB-75, CB-77, CB-78, CB-80, CB-81, CB-88, CB-89, CB-90, CB-94, CB-95, CB-96, CB-97, CB-98, CB-99	Dense
	Cluster bean	CB-38, CB-53[a], CB-76, CB-82, CB-84	Feathery
	Cluster bean	CB-66, CB-72, CB-73, CB-79, CB-83, CB-91, CB-92, CB-93,	Restricted
Gujarat	Cluster bean	CB-21, CB-28[a], CB-31[a], CB-64	Dense
	Cluster bean		Feathery
	Cluster bean	CB-62	Restricted
Nagpur	Cotton	COT10	Dense
Haryana	Okra	OK15	Dense
Layallpur	Cotton	COT41	Dense
Layallpur	Soybean	SOY42	Dense
IARI, New Delhi	Mung bean	MB-51	Dense
Kalyani	Soybean	SOY-56	Dense
IARI, New Delhi	*Glycine hispida*	GH-57[a]	Dense
Almora	Rice	OS-58	Dense
Coimbatore	*Glycine hispida*	GH-59	Dense
New Delhi	Soybean	SOY-68	Feathery
Assam	Mung bean	MB-69	Dense
Rajasthan	Onion	AC-70	Dense

[a] Presence of dsRNA

Table 2 Summary of morphometric characteristics of isolates of *Macrophomina phaseolina* (All characteristics were observed on PDA)

Mycelial compatibility group	Color of young colony (surface)	Color of young colony (reversed)	Change in colony color at 4°C	Colony appearance	Colony growth rate
MCG-I	Yellowish to white	Butter yellow	No	Cottony, dense	0.33–0.46
MCG-II	White to brown	Yellow	Yes	Felted	0.05–0.10
MCG-III	Grey	Pale yellow	No	Glabrous, dense	0.13–0.18
MCG-IV	Green to black	Yellowish grey	No	Fluffy, feathery	0.15–0.26

dsRNA-containing isolates (+) while isolates hydrolyzed, by the activity of RNase in low NaCl concentration were designated as dsRNA free (−) isolates [24].

Virulence of dsRNA

Four dsRNA-containing *M. phaseolina* isolates (+) and four dsRNA free (−) isolates were arbitrarily selected and tested against superior varieties of cluster bean for its virulence. The isolates tested that have dsRNA were isolates CB-11 (+), CB-17 (+), CB-28 (+), GH-57 (+), while those tested that did not had dsRNA were isolates CB-14 (−), CB-38 (−), CB-62 (−) and CB-71 (−) (Table 1). Only single isolate originating from each state of North India were selected for field studies, as probably the isolates would be tolerant to the drought and heat stress commonly encountered with no significant geographic barrier in their location environment. These isolates were

Table 3 Virulence rating of susceptible *Macrophomina* isolates on the basis of virulence index and pathogenic severity

Isolate codes	Species	Source	Phenotype	dsRNA	Mean disease severity	Virulence index	Virulence rating
CB-11	*M. phaseolina*	Cotyledon	Dense	1	0.546 ± 0.04	2.93 ± 0.56	Hypovirulent
CB-17	*M. phaseolina*	Hypocotyl	Restricted	1	0.753 ± 0.08	3.11 ± 0.24	Hypovirulent
CB-28	*M. phaseolina*	Cotyledon	Dense	1	0.510 ± 0.10	1.83 ± 0.28	Hypovirulent
GH-57	*M. phaseolina*	Cotyledon	Dense	1	2.066 ± 0.11	2.37 ± 0.25	Mod. pathogenic
CB-14	*M. phaseolina*	Hypocotyl	Feathery	0	2.620 ± 0.18	7.28 ± 0.62	Mod. pathogenic
CB-38	*M. phaseolina*	Root	Feathery	0	2.823 ± 0.34	5.08 ± 0.58	Highly pathogenic
CB-62	*M. phaseolina*	Hypocotyl	Restricted	0	3.070 ± 0.40	5.10 ± 0.50	Highly pathogenic
CB-71	*M. phaseolina*	Cotyledon	Dense	0	3.043 ± 0.01	5.31 ± 0.86	Highly pathogenic

± Mean standard error

also selected according to their dense and feathery physiology in an attempt to minimize concerns associated with the prologue of new fungal isolates into areas where cluster bean is cultivated commercially.

Inoculation of seeds with virulent isolates of *M. phaseolina*

Pathogenicity of *M. phaseolina* isolates was evaluated using seeds of two superior cluster bean differential cultivars [25]. Seeds were surface sterilized with 2% sodium hypochlorite for 2 min, rinsed in sterile tap water. Isolates of *M. phaseolina* were cultured in 12 cm petri dishes at 28 ± 2°C in the dark. When PDA of the dishes was completely colonized ten seeds of each cultivar were placed on the colony of each of the *M. phaseolina* isolates. Petri dishes were incubated at 28 ± 2°C in the dark. After 5 days, virulence index and disease severity of seeds was evaluated (Table 3) based on symptoms caused by the pathogen using 0–5 scale. Where 0 = healthy seed; 1 = discoloration of a portion of the seedling in contact with the mycelium; 2 = seed teguments invaded by mycelium and sclerotia but healthy seedling; 3 = seed teguments free from the fungus, but seedling infected; 4 = seed tegument and seedling infected; 5 = seed infected and not germinated [26]. On the basis, of their virulence index and disease causing severity, isolates were eventually characterized and categorized as highly pathogenic, moderately pathogenic or hypovirulent.

Pathogenicity of the dsRNA (+) and dsRNA (−) *M. phaseolina* isolates

Pathogenicity tests were conducted on two susceptible cluster bean cultivars namely, PNB and IC-102827 to charcoal rot disease. Seeds of the tested cultivars were acquired from Forage Section, CCS Haryana Agriculture University, Hisar, Haryana. Seeds were surface sterilized with 2% sodium hypochlorite, rinsed in sterile distilled water and sown in 25 cm plastic pots filled with sand under green house conditions. Four replicate pots (each) were sown with two seeds of cluster bean for each tested isolate. Plants were watered as needed and treated according to the recommended agricultural practices. The 30-day-old plants were stem inoculated with the tested isolates CB-11 (+), CB-17 (+), CB-28 (+), GH-57 (+), CB-14 (−), CB-38 (−), CB-62 (−) and CB-71 (−) in the second internode using the stem tape inoculation technique [27]. Ten days after inoculation, developed lesions were measured in cm that may appear as the longitudinal bark necrosis at the site of inoculation (Fig. 1). Percentage of plant death was determined 21 days after inoculation [28]. Repeated reisolation was conceded to ensure the association of the tested isolates with the developed disease.

Statistical analysis

For ascertaining virulence index and pathogenicity analysis, the mean values of virulence index and pathogenicity caused by each isolate was calculated. Mean values were subjected to analysis of variance (ANOVA) using a completely randomized design where treatments were each state of origin of isolates and the replications of the isolates. Data were subjected to one-way analysis of variance, with $P < 0.05$ considered significant. Statistical analysis was performed by employing SPSS software version 1.0.

Results

Detection of dsRNA in *M. phaseolina*

The presence of dsRNA among the isolates of *M. phaseolina* was detected in 22 of the 101 isolates (21.7%) including 13 isolates from Rajasthan, 6 isolates from Haryana, 2 isolates from Gujarat and 1 from Punjab as shown in Table 1. The 22 isolates were majorly obtained from cluster bean showing dense phenotype. *M. phaseolina*

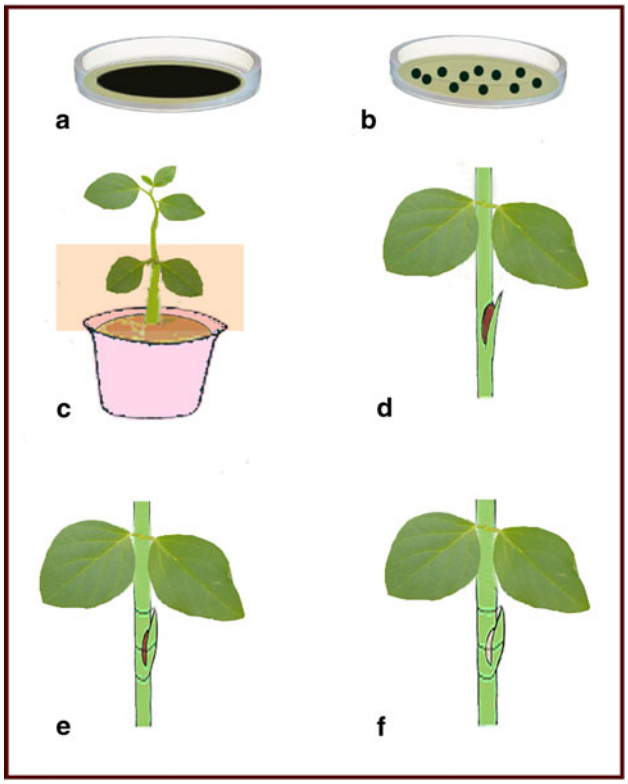

Fig. 1 Schematic representation of stem tape inoculation to check the lesion length and plant death rate under green house controlled conditions [**a** 96 h old strain of *M. phaseolina* on culture media. **b** Mycelial plugs (5 mm) cut from the mat of the colony, using a metallic cork borer. **c** and **d** A mature cluster bean plant superficially wounded and the mycelial plug was induced against the wound. **e** Brown colored wound sealed with cellophane tape. **f** Positive control of plant inoculated with sterile PDA plug, sealed with cellophane tape]

segregates were further characterized on the derivation of their electrophoretic profile. Without DNase treatment, DNA was found contaminating dsRNA, suggesting that it may compete with dsRNA for adsorption to cellulose. Low salt concentration was found vivacious in reducing DNA recovery by phenol extraction. dsRNA capitulate was also found erratic with tissue extraction. Purified dsRNA fraction collected after the phenol–chloroform treatment, and precipitated with ethanol. The size range of dsRNA varies between ~800 bp and 12 Kb (Fig. 2). The dsRNA panoramas of these molecules were further entrenched by optimizing cycles of Cellulose CF-11 chromatography and DNase and RNase treatments. Repeated sub-culturing until three cycles confirms the presence of dsRNA in isolates obtained from various eco-geographical locations, showing its stable nature. Resistance to DNase, but sensitivity to RNase-A confirmed that these bands were dsRNA. This property of dsRNA stability was used to our advantage for reducing the amount of fungal cellular RNA contamination. Two trials on each isolate were done on dsRNA and

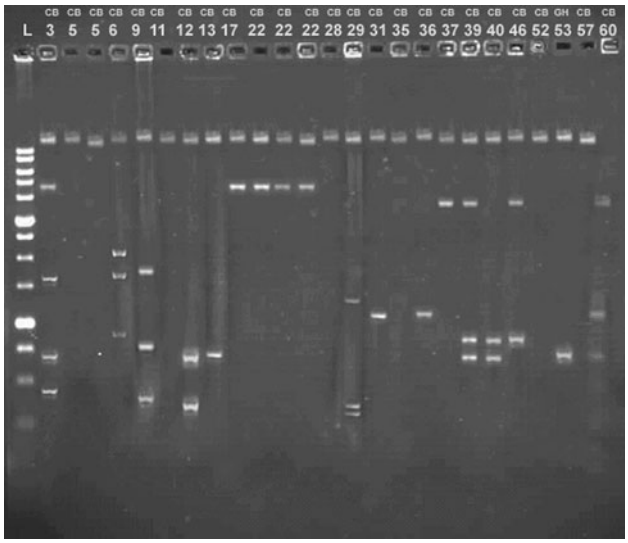

Fig. 2 Ethidium bromide stained dynamics of dsRNA from *Macrophomina* isolates on agarose gel

results were the same for each trial of *M. phaseolina* isolates. No significant differences were observed between dsRNA-containing and dsRNA-free isolates for colony morphology, mycelial growth and sporulation ability in this study (Table 1) concomitantly dsRNA was found invariably allied with virulence attributes. The present study on dsRNA stems on detection of "virus-host relationship".

Response of cluster bean seeds with virulent isolates of *M. phaseolina*

Disease severity was found prevalent in all the eight *M. phaseolina* isolates in which the seeds were randomly dispersed, its mean ranged from 0.510 to 2.06 (+) and 2.62 to 3.04 (−) in the randomly chosen germinated sections, however not much difference was observed in the promptness of necrosis symptoms in cotyledon, hypocotyls or the root parts of cluster bean. Virulence rating was generated based on mean virulence index that ranged from minimum 1.83 (+) to maximum 5.31 (−) in cluster bean seeds infected with *M. phaseolina* (Table 3).

Pathogenicity tests of the dsRNA (+) and dsRNA (−) *M. phaseolina* isolates

All the recovered *M. phaseolina* isolates tested were pathogenic to both the cluster bean varieties PNB and IC-102827, as incited total mean stem lesions ranged between 0.4 and 4.4 cm in the pathogenicity test (Table 4). The mean lesion length range varied from 0.4–2.8 cm (+) to 2.6–4.4 cm (−). Data revealed that, isolates were variable in their ability to cause plant death which was in the range of 0–100% of the control on the tested cultivars with total means of 31 and 74%

Table 4 Pathogenicity tests of dsRNA (+) and dsRNA (−) isolates on cluster bean varieties PNB and IC-102827

Isolate codes	Species	Host	Location	Phenotype	dsRNA	Mean lesion length (cm)		Mean plant death (%)
						PNB-IC	102827	
CB-11	*M. phaseolina*	Cluster bean	Haryana	Dense	1	2.433 ± 0.18	0.933 ± 0.08	70 ± 4.0
CB-17	*M. phaseolina*	Cluster bean	Rajasthan	Restricted	1	0.567 ± 0.14	2.833 ± 0.14	74 ± 2.6
CB-28	*M. phaseolina*	Cluster bean	Gujarat	Dense	1	0.467 ± 0.06	2.200 ± 0.11	55 ± 1.7
GH-57	*M. phaseolina*	*Glycine hispida*	New Delhi	Dense	1	2.333 ± 0.12	2.067 ± 0.16	68 ± 3.7
CB-14	*M. phaseolina*	Cluster bean	Rajasthan	Feathery	0	2.600 ± 0.30	3.767 ± 0.20	90 ± 1.3
CB-38	*M. phaseolina*	Cluster bean	Haryana	Feathery	0	3.533 ± 0.23	3.367 ± 0.08	31 ± 4.5
CB-62	*M. phaseolina*	Cluster bean	Gujarat	Restricted	0	4.467 ± 0.17	3.833 ± 0.20	60 ± 2.7
CB-71	*M. phaseolina*	Cluster bean	Haryana	Dense	0	3.600 ± 0.20	2.733 ± 0.17	73 ± 2.0

± Mean standard error

for the *Macrophomina* isolates, respectively. Almost means of 68–74% of the total *M. phaseolina* recovered isolates were low pathogenic and classified as hypovirulent to cause plant death on the tested cluster bean cultivars in which double stranded RNA was present (Table 4). On the other hand, with means of 31–90% of the isolates were highly pathogenic in which dsRNA was totally absent.

Discussion

Per se, presence of dsRNA has reported for a number of plant pathogenic fungi; however, this is the first report dsRNA in *Macrophomina* isolates of cluster. By numerous attempts with isolates of many different geographic origins, we were able to reveal that the presence of dsRNA is widely distributed among isolates of the fungus. The occurrence of dsRNA in isolates of *M. phaseolina* probably implies the presence of viruses [29, 30]. Sucrose gradient centrifugation to isolate and purify viruses revealed unsuccessful results [31]. Multiple dsRNA wreckage accentuates infection of virus with a segmented genome. It confirms its wide heterogeneity among the isolates as similar pro-founding's was confirmed earlier in *C. elegans* [31]; *R. solani* [32] and *M. phaseolina* isolates of various crop cultivars [10] which further confirms our findings. However, further studies are still required to elucidate the origin of dsRNA elements in *M. phaseolina*. The dsRNA profile revealed (Fig. 2) that both the number and size of the molecules in different isolates of *M. phaseolina* were heterogeneous in nature. The heterogenic pattern of dsRNA elements among the isolates was not unique, as it have been observed for several other fungal species, including *P. oryzae*, *C. elegans*, *C. parasitica*, *S. homoeocarpa*, *C. ulmi* and *R. solani* [17, 30–35]. A dsRNA molecule of ~10 kb was present in the isolates from Haryana, Rajasthan and Gujarat (Fig. 2).

We interestingly found stable dsRNA even after seven cycles of successive sub-culturing from diverse geographic locations. These results are quite analogous to those formerly recorded from stable dsRNA of *C. parasitica* [24] and *R. solani* [36], over several successions of subculture. Earlier, variation in virulence among isolates of *M. phaseolina* have been reported [37], but until now the basis for these differences is unknown. In this study, the virulence of isolates was evaluated using a commercial guar-gum producing cluster bean cultivar grown under agro-field conditions in different locations of North India.

A rain-fed kharif crop; cluster bean is primarily sown in June–July and harvested in the month of October–November. Rajasthan being a dominant shareholder of 80% is the largest guar or cluster bean producing state in the India. However, states like Haryana and Gujarat just adds-on 12 and 11%, respectively to the guar production. Guar gum exports during the 2009–2010 were around 2.2 lakh ton, which estimated to reach up to 2.5 lakh ton in 2011 year ahead. The important feature of this commodity is, it is less perishable as compared to other agro commodities and its seed can be stored for more than 3 years, which makes it most preferred goods for the stockiest and traders. According to the recent report, the Northwest province of India, a key guar-producing region received 12% above normal rainfall in the monsoon season annually. As a result, its seed cultivation area in Rajasthan, the largest producing state in India tremendously rose 10% Y/Y to 2.84 lakh ha. The trade sources reports incessant rains in late September and in early October, which causes around 20% of crop damage (http://www.karvycomtrade.com). It is a drought-tolerant annual legume crop majorly grown in arid zones of west and North West India and parts of Pakistan. So, growers that cultivate cluster bean under one cycle were exposed to a greater risk of total crop loss due to drought and high temperatures than are encountered during the spring cycle. However, not much significant

difference in lesion length between locations were observed in this study, the average mean temperature at the N. Indian field locations goes approximately from 30 to 35°C. Punia et al. [38] has reported variation in some elite varieties of cluster bean based on their peculiar morpho-physiological characteristics. We had chosen the two diversified cultivars from the elite list for studying the effectual virulence of *Macrophomina.*

The fungus produces distinctive blackish stem lesions on infected plants that can be used for quantitative evaluation of pathogenicity. Isolates with dsRNA elements were significantly less virulent than dsRNA-free isolates in both the elite cultivars (Table 3). The combined analysis of the isolates based on virulence index, disease severity and mean lesion length for both the elite cultivars indicated that the three most virulent were free of dsRNA (Table 3, 4). These results strongly suggest a strong association between the presence of dsRNA and decline in both mycelial growth rates and virulence. Similar reports of dsRNA have been observed for isolates of *C. parasitica* [21] and *S. sclerotiorum* [18].

In recent years, dsRNA-associated novel host–parasite interaction for the regulation of virulence has engrossed significant interest in plant pathogenic fungal diseases as eventual biological control agent for diseases. Transmission of dsRNA viruses occur either through cell division followed by spore or conidial production i.e. vertical transmission [39, 40] or by cell fusion between genetically compatible strains i.e. horizontal transmission [11, 13]. Because of the double-stranded nature of the dsRNAs, they are resistant to RNase treatment under high salt conditions [21]. Further studies with large number of strains, and including broad range of host plants could yield more information. The occurrence of dsRNA in Indian *M. phaseolina* isolates provides an additional means to distinguish Indian populations from those in other regions of the world. dsRNA may thus serve as another useful character for monitoring the migration of Indian isolates to other parts of the world. Confirmation of unique viral genetic elements and their genetic relatedness can be done by hybridization analysis in varying dsRNA patterns observed in the isolates in future by further following molecular ecology studies of dsRNA.

Conclusion

The data presented here will make it possible to characterize the individual viral effects in more detail in future. This could provide a viable approach for introducing phenotypic characteristics into the given fungus that would be useful for biological control of the pathogen. Further studies are required to elucidate the acquisition mechanisms of infectious elements and other factors of the host fungus to evolve additional dsRNA elements. A much better understanding of the basic population ecology of fungi is necessary before predictions of the potential transfer and escape of engineered candidate genes could be made. The present study provides information about characterization of various fungal isolates using dsRNA as potential epidemiological tool associated with virulence and pathogenicity traits.

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