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Occurrence, Disease Severity of Ginger Leaf Spot in Ginger Growing Regions of Karnataka and Morphological and Molecular Characterization of Associated Pathogen *Phyllosticta zingiberi* Ramakr.

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Abstract

Ginger (*Zingiber officinale* Rosc.) is an important tropical spice. Among the diseases of ginger, leaf spot disease caused by *Phyllosticta zingiberi* Ramakr. is considered to be one of the important foliar diseases appearing in mild or severe form in all the ginger growing tracts of the country. Investigations were carried out on disease severity, morphological and molecular characterization the pathogen causing leaf spot disease on ginger. Roving survey was conducted in four major ginger growing districts and the results showed that disease severity was highest in Hassan district (31.32 %) and lowest in Kodagu district (19.73 %).The mycelia of all the five isolates (MND, HSN, RNP, HSN and HNP) collected during the survey from different localities appeared septate and hyaline in colour. The conidia were hyaline, ellipsoidal in shape with a size ranging from 9.15-11.98 × 3.58-6.33 µm. Molecular study revealed that the HNP isolate collected from Holenarasipura taluk of Hassan district showed maximum per cent similarity (95.96%) with *Phyllosticta citricarpa*.

Keywords : Ginger leaf spot, Molecular, Morphology, *Phyllosticta zingiberi,* Roving survey

Ginger (*Zingiber officinale* Rosc.) is an important tropical spice belonging to the family Zingiberaceae. The crop is believed to be a native of Tropical Asia. Ginger is an herbaceous perennial plant and is cultivated in the tropical and sub-tropical regions of the world as a spice and medicinal crop (Mishra *et al*., 2012). In India the area under ginger cultivation is 191 million hectares (m ha), production is 2121 million tons (mt) and productivity is 11.10 (mt/ha) (Indian Horticulture Database, 2022). In Karnataka ginger production in the year 2021-2022 is 306.34 thousand tons sharing 13.80 per cent to the total ginger production in the country (National Horticulture Board).

Ginger is prone to many serious diseases which are caused by fungal, bacterial and nematode infections. Leaf spot disease of ginger caused by *Phyllosticta zingiberi* Ramakr. is considered to be one of the important foliar diseases appearing in mild or severe form in all the ginger growing tracts of the country (Singh, 2015).

Symptoms are observed on leaves as oval to elongated spots that later turn to whitish surrounded by dark brown margin with yellow halo (Ramakrishnan, 1942). Sarma *et al*. (1994) has recorded 13-66 per cent yield loss depending upon its severity. Sood and Dohroo (2005) recorded 48.3 per cent loss in mother rhizome

and 65.9 per cent in yield of fresh rhizome when the severity of *P. zingiberi* was 58.3 per cent.

In recent years, in Karnataka, the area under ginger cultivation is increasing because of its commercial value and there is an increase in leaf spot disease occurrence and severity. However, not much scientific work has been done on this disease of ginger regarding disease severity and morphology of the pathogen. Therefore, keeping all the above points in view the research was undertaken to study the occurrence, severity, morphological and molecular characterisation of the pathogen causing leaf spot disease on ginger.

Material and Methods

Survey for the Severity of Leaf Spot of Ginger

Roving method of survey was conducted to record the disease severity of leaf spot of ginger during October 2020 in ginger growing areas like Mandya, Hassan, Coorg and Mysore districts. Per cent disease severity was recorded by field key (1-9 scale) on foliage as given by Singh *et al.* (2000) and these scales were converted to percent disease index (PDI) using the formula given by Wheeler (1969).

$$PDI = \frac{\text{Sum of all disease ratings}}{\text{Total no. of ratings} \times \text{Maximum disease rating}} \times 100$$

Morphology

The disease specimen collected from the field during survey was used for isolation of the pathogen. Five isolates *viz*., MND, HSN, RNP, HNP and PYP were isolated from Mandya (Hadya village), Hassan (Agile village), Arakalgud (Ramanathapura), Holenarasipura (Gowdanahalli village) and Periyapatna (Laxmipura) taluks respectively.

Table 1

Standard evaluation system scale for leaf spot disease of ginger

Scale	Description
1	No symptom
2	1-5 spots per leaf
3	6-10 spots per leaf
5	20-25% area covered
7	26-50% area covered
9	>50% area covered

The morphological characters *viz*., colony colour, colony form, time taken for sporulation and conidial characters *viz.,* length, width, shape and colour of conidia were recorded by observing under microscope.

Isolation of DNA

The genomic DNA of five isolates *viz*., MND, HSN, RNP, HNP and PYP of *P. zingiberi*grown in a potato dextrose broth was extracted by following CTAB (Cetyltrimethyl ammonium bromide) extraction method (Dellaporta *et al*., 1983).

Polymerase Chain Reaction (PCR)

Amplification of the internal transcribed spacer regions 1 and 4 (ITS1 and 4) of the ribosomal DNA was done in a thermal cycle. A final volume of 50 µL reaction mixture was prepared with 5 µL of template DNA, 2.5 µL of each primer, 25 µL Red Taq Master Mix and 15 µL of nuclease free water.

Table 2

Internal transcribed spacer region specific primer used

Primer name	Primer sequence (5'-3')	No of base pairs (bp)
ITS1 – Forward	CTTGGTCATTTAGAGGAAGTAA	22
ITS4 – Reverse	TCCTCCGCTTATTGATATGC	20

C) PCR Amplification Conditions

Initial denaturation	95°C (05:00 min)
Denaturation	95°C (01:00 min)
Annealing	53°C (01:00 min)
Extension	72°C (01:00 min)
Final extension	72°C (10:00 min)
Final hold	4°C
No. of cycles amplified	35

Sequencing and Phylogenetic Analysis of Amplification Products

The PCR samples were sequenced (by Agrigenome labs Pvt. Ltd., Kakkanad, Kerala.) and FASTA forms of the sequences were edited manually using Basic Local Alignment Search tool (BLAST).

Results and Discussion

Survey on the Occurrence of Leaf Spot Disease on Ginger in Mandya, Hassan, Coorg & Mysuru districts

A roving survey to record the disease severity of leaf spot disease on ginger was undertaken in the farmer's field during October 2020 in ginger growing areas like Mandya, Hassan, Coorg and Mysuru districts.

The symptoms in the field were recognized by the oval to elongated whitish spots surrounded by dark brown margin with yellow halo (Plate 1 (a)). Similar symptoms were observed by Rai *et al*. (2017). Survey was conducted in Mandya, Hassan, Coorg and Mysuru districts and the disease severity in these areas ranged from 19.73 to 31.32 per cent (Table 3). The average disease severities in the various locations surveyed are as follows.

Among the taluks of Mandya district, Mandya taluk recorded the highest per cent disease severity of 27.49 per cent followed by Malavalli (24.82%) and Pandavapura (23.3%). In 3 taluks of Hassan, district highest per cent disease severity was recorded in Hassan (32.10%) followed by Arkalgud (32.06%) and Holenarasipura (29.80%). In Mysuru district per cent disease severity of 30.16 and 28.47 per cent was recorded in Periyapatna and Hunsur taluk respectively. In Somwarpet and Madikeri taluks of Kodagu district, disease severity of 19.69 and17.30 per cent was recorded respectively.

Among the districts surveyed, the results revealed that the highest average disease severity was recorded in Hassan district with 31.32 per cent followed by Mysuru district with 29.31 per cent and Mandya district with 25.20 per cent. Lowest disease severity was recorded in Kodagu district with average per cent disease severity of 19.73 per cent.

During the present study the results revealed that, the average disease severity varied from one district to another district where, the range being 19.73 to 31.32 per cent among the 4 districts surveyed. The crop age during survey ranged from 5-8 months old

Table 3

Ginger leaf spot disease severity in Mandya, Hassan, Kodagu and Mysuru districts during *kharif* 2020

District	Taluk	Village	Plantage (months)	Per cent disease index (%)	Mean disease severity of taluks (%)	Mean disease severity of districts (%)
Mandya	Mandya	Jayapura	6	32.77	27.49	25.20
		Hadya	5	34.86		
		Hullenahalli	6	25.58		
		Hulikere	6	20.45		
		Hulikerekoppalu	7	18.77		
	Pandavapura	Pattanagere	6	17.59	23.30	
		KodihalliHosur	7	26.78		
		Heggadahalli	6	23.70		
		Rangapura	6	12.76		
		Lakshmisagara	5	35.67		
	Malavalli	Hadli	5	25.87	24.82	
		Dhanuguru	6	20.54		
		Halasahalli	7	33.33		

Table 3 continued...

District	Taluk	Village	Plantage (months)	Per cent disease index (%)	Mean disease severity of taluks (%)	Mean disease severity of districts (%)
		Basavanpura	6	15.47		
		Banasamudra	7	28.88		
Hassan	Hassan	Agile	6	33.43	32.10	31.32
		Banavase	6	28.98		
		Gorur	6	35.67		
		Huluvare	7	38.47		
		Janivara	5	23.94		
	Arkalgud	KaduvinaHosalli	6	30.58	32.06	
		Ramanathapura	5	52.79		
		Mokali	7	28.69		
		Konanur	7	26.78		
		VaddaraKoppalu	7	15.67		
		Henavanahalli	6	37.85		
	Holenarasipura	HirehalliKoppalu	5	40.59	29.80	
		Muddanahalli	7	33.07		
		Gowdanahalli	8	28.95		
		Doddagavanahalli	7	19.75		
		Hodkekatte	6	26.65		
Mysuru	Periyapatna	Koppa	6	20.78	30.16	29.31
		Bylakuppe	7	33.75		
		Doddaharve	5	28.52		
		Laxmipura	6	30.68		
		Kavalu	7	37.56		
		Kampalapura	7	29.67		
	Hunsur	Chilkunda	5	30.56	28.46	
		Attikuppe	6	25.67		
		Naganahalli	6	25.95		
		Nilavagilu	7	27.57		
		Keriyuru	7	32.59		
Kodagu	Somwarpet	Hulase	6	18.67	19.69	19.73
		Kanive	6	17.90		
		Kudige	7	24.78		
		Hebbale	7	20.67		
		Sirangala	7	19.54		
		Thorenooru	8	16.55		
	Madikeri	Bettageri	8	10.76	17.30	
		Heravanadu	7	15.89		
		Makkanduru	8	18.96		
		Biligeri	7	23.57		

in various locations surveyed. The highest disease severity was recorded at 5 month old crop (52.79% in Arkalgud taluk) and lowest disease severity was recorded when the crop stage was 8 months old (10.76% in Madikeri taluk).

Bandyopadhyay *et al*. (2015) reported that the per cent disease index of leaf spot of ginger caused by *P. zingiberi* ranged from 5.38 to 38.89 per cent in the field experiment conducted in the year 2014-15 in Uttar Banga Krishi Viswavidyalaya, West Bengal.

Morphological Identification of *P. zingiberi* Ramakr.

All the five isolates were grown on PDA to study their morphological characters and the same is presented in Table 4. MND, HSN, HNP, isolates initiated mycelial growth three days after incubation and isolates RNP and PYP took two days to initiate growth after incubation. But all the isolates took 8 days after incubation to cover the complete Petri plate.

Colony colour of all the isolates *viz*., MND, HSN, RNP, HNP and PYP was whitish olivine initially, after 15 days of inoculation colony colour of all the isolates was olivaceous grey and showed flat and even growth on the media. There was complete marginal growth of all the isolates. In case of MND and RNP isolates sporulation was observed at 216 h (nine days) whereas in HSN, HNP and PYP isolates sporulation was observed at 240 h (10 days) after incubation.

The observations on morphological characters of *P. zingiberi* are in conformity with Rai *et al*. (2017) wherein they observed that the colony of the fungus when grown on PDA showed smooth surface having regular margin. The colony colour exhibited a pattern of light grey at the margins with olivaceous green zonation. Ramakrishnan (1942) also observed the thick growth, dark-olive colouration of the colony of *P. zingiberi*. Zimowska (2013) also observed olive grey aerial mycelium.

Microscopic Features of *P. zingiberi* Isolates

The microscopic features of all *P. zingiberi* isolates were observed under light microscope with 40X magnification. The shape, size and colour of conidia are recorded in Table 5. The microscopic image is shown in Plate 1.

The mycelia of all the five isolates appeared septate with hyaline in colour. The conidia were hyaline, ellipsoidal in shape with a size ranging 9.15-11.98 × 3.58-6.33 µm. The highest length of the conidia was observed in RNP isolate (11.98µm) followed by HNP (10.92 µm) and HSN (10.39 µm). The lowest conidial length of 9.15 µm was observed in MND isolate followed by PYP (9.57 µm). RNP (6.33 µm) isolate recorded highest width of the conidia followed by PYP (4.97 µm) and MND (4.93 µm). The lowest conidial width was recorded in HNP (3.58 µm) isolate followed by HSN (4.45 µm).

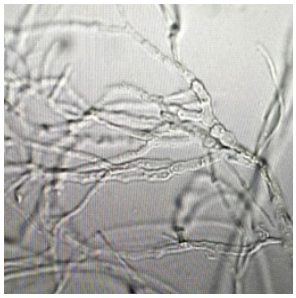
TABLE 4

Morphological characteristics of *P. zingiberi* isolates

P. zingiberi isolates	Mycelial initiation (days after incubation)	Time taken to cover the Petri plate (days after incubation)	Colony colour	Colony form	Time taken for sporulation (h)	Zonations	Margin
MND	3	8	Whitish olivine	Flat and even growth	216	Olivaceous green	Complete
HSN	3	8	Whitish olivine	Flat and even growth	240	-	Complete
RNP	2	8	Whitish olivine	Flat and even growth	216	-	Complete
HNP	3	8	Whitish olivine	Flat and even growth	240	-	Complete
PYP	2	8	Whitish olivine	Flat and even growth	240	Olivaceous grey	Complete

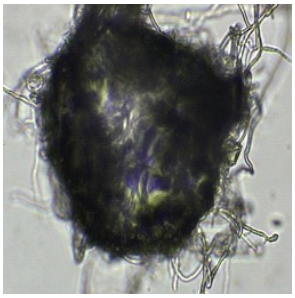
TABLE 5
Conidial characteristics of *P. zingiberi* isolates

P. zingiberi isolates	Conidia				
	Length (L) in µm	Width (W) in µm	L/W ratio	Shape	Colour
MND	9.15	4.93	1.84	Ellipsoidal	Hyaline
HSN	10.39	4.45	2.33	Ellipsoidal	Hyaline
RNP	11.98	6.33	1.89	Ellipsoidal	Hyaline
HNP	10.92	3.58	3.05	Ellipsoidal	Hyaline
PYP	9.57	4.97	1.93	Ellipsoidal	Hyaline

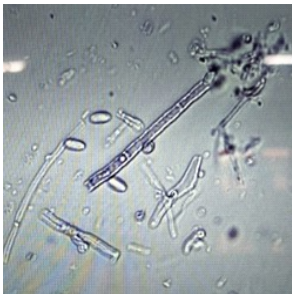


a) septate and hyaline mycelSeptium

a) Pure culture of *P. zingiberi*



a) Pycnidia under 40x



a) Hyaline conidia under 40x

Plate 1 : Morphological characters of *P. zingiberi*

The length/width (L/W) ratio of the conidia ranged from 1.84 to 3.05. Highest was recorded in HNP (3.05) followed by HSN (2.33) and PYP (1.93). Lowest L/W ratio was recorded in MND 1.84) followed by RNP (1.89).

Results on conidial characters are in conformity with Zimowska (2013) where in he reported that the conidia are aseptate or single celled, hyaline, oval to ellipsoidal in shape, with a size of 5.2-5.5 µm × 1.8-2.2 µm *P. plantaginis* in *Plantago lanceolata*. Ravikumara (2011) also reported that in *P. musarum* causing leaf spot of banana the conidia were hyaline, one celled, sub globose to ellipsoidal and measure 7.5-15 µm × 2.5-5 µm.

Molecular Studies of *P. zingiberi* Ramakr.

DNA Extraction from P. zingiberi Isolates : DNA from all five isolates of the pathogen was success fully extracted using Cetrimide Tetradecyl Trimethyl Ammonium Bromide (CTAB) extraction method (Crowhurst *et al*., 1995).

PCR Amplification

The DNA extracted from *P. zingiberi* isolates were amplified using primers ITS1 and ITS4. The PCR region amplified the ITS rDNA sequence of region 1 and 4, which include 5.8S rRNA and 28S rRNA gene. The PCR produce was approximately 600 bp (Plate 2). The results were in concordance with Arafat (2018) who studied the identification and characterization of *P. capitalensis* causing black spot disease on Guava. He isolated DNA from *P. capitalensis* and amplified using primers ITS1 and ITS4. The PCR produce was 626 bp.

Sequence Based Species Identification using BLAST

The sequence similarity search was performed using web interface of NCBI BLAST N program and the results are presented in Table 6. In BLAST sequence similarity search with target nucleotide database, MND isolate showed 93.00 per cent similarity with *Phyllosticta citricarpa* which causescitrus black spot

TABLE 6

Sequence similarity of the *P. zingiberi* isolates

Source	Target data base	Total no. of hits	No. of hits having >90% similarity	Similarity, coverage	Species
MND	Nucleotide	100	100	93	*Phyllosticta citricarpa*
				95.95	*Guignardia citricarpa*
		100	100	95	*Phyllosticta capitalensis*
		45	44	95.38	*Phyllosticta ampelicida*
				95.38	*Guignardia bidwellii*
		17	17	95.58	*Phyllosticta citriasiana*
HSN	Nucleotide	100	100	93.1	*Phyllosticta citricarpa*
				93.65	*Guignardia citricarpa*
		100	100	95.17	*Phyllosticta capitalensis*
		45	45	95.38	*Phyllosticta ampelicida*
				95.38	*Guignardia bidwellii*
		17	17	95.58	*Phyllosticta citriasiana*
RNP	Nucleotide	100	100	95.40	*Phyllosticta citricarpa*
				95.95	*Guignardia citricarpa*
		100	100	95.95	*Phyllosticta capitalensis*
		45	45	95.38	*Phyllosticta ampelicida*
				95.38	*Guignardia bidwellii*
		17	17	95.58	*Phyllosticta citriasiana*
HNP	Nucleotide	100	100	95.96	*Phyllosticta citricarpa*
				93.65	*Guignardia citricarpa*
		100	100	95.95	*Phyllosticta capitalensis*
		45	44	95.38	*Phyllosticta ampelicida*
				95.38	*Guignardia bidwellii*
		17	17	95.58	*Phyllosticta citriasiana*
PYP	Nucleotide	100	100	93.65	*Phyllosticta citricarpa*
				93.65	*Guignardia citricarpa*
		100	100	95.95	*Phyllosticta capitalensis*
		45	45	95.38	*Phyllosticta ampelicida*
				95.38	*Guignardia bidwellii*
		17	17	95.58	*Phyllosticta citriasiana*

disease, 95.95 per cent with *Guignardia citricarpa* which is the telomorphic stage of *Phyllosticta citricarpa*, 95.00 per cent with *Phyllosticta capitalensis* causing black spot disease on Guava, 95.38 per cent *Phyllosticta ampelicida* which causes black rot of grapevine and *Guignardia bidwellii* is the telomorphic stage of *Phyllosticta ampelicida*, 95.58 per cent similarity with *Phyllosticta citriasiana* which is an pathogen of citrus causing a tan spot on fruit.

HSN isolate showed 93.10 per cent similarity with *Phyllosticta citricarpa*, 93.65 per cent with *Guignardia citricarpa*, 95.17 per cent with *Phyllosticta capitalensis*, 95.38 per cent

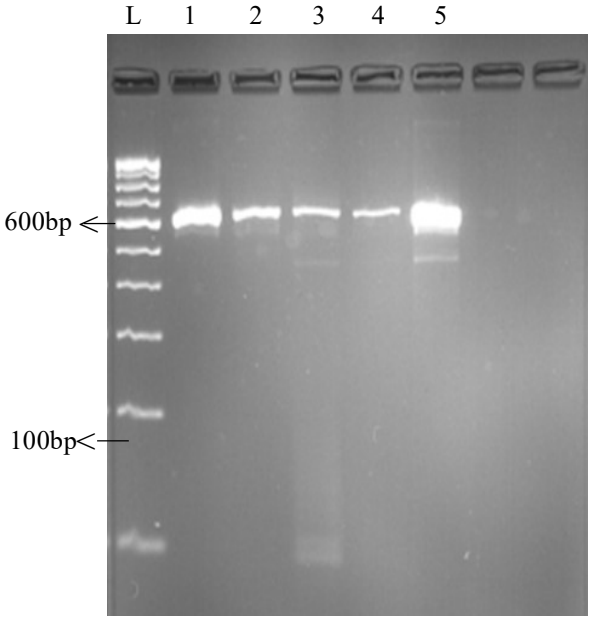


Plate 2 : Gel electrophoretic profiles of ITS1 and ITS4 of amplicon from *P. zingiberi* isolates (Wells: L: 100bp ladder, 1 - MND, 2 - HSN, 3 - RNP, 4 - HNP, 5 - PYP)

Phyllosticta ampelicida and *Guignardia bidwellii*, 95.58 per cent similarity with *Phyllosticta citriasiana*. RNP isolate showed 95.40 per cent similarity with *Phyllosticta citricarpa*, 95.95 per cent with *Guignardia citricarpa*, 95.95 per cent with *Phyllosticta capitalensis*, 95.38 per cent *Phyllosticta ampelicida* and *Guignardia bidwellii*, 95.58 per cent similarity with *Phyllosticta citriasiana*.

HNP isolate showed 95.96 per cent similarity with *Phyllosticta citricarpa*, 93.65 per cent with *Guignardia citricarpa*, 95.95 per cent with *Phyllosticta capitalensis*, 95.38 per cent *Phyllosticta ampelicida* and *Guignardia bidwellii*, 95.58 per cent similarity with *Phyllosticta citriasiana*. PYP isolate showed 93.65 per cent similarity with *Phyllosticta citricarpa*, 93.65 per cent with *Guignardia citricarpa*, 95.95 per cent with *Phyllosticta capitalensis*, 95.38 per cent *Phyllosticta ampelicida* and *Guignardia bidwellii*, 95.58 per cent similarity with *Phyllosticta citriasiana*.

The genomic data is not available for *P. zingiberi* in NCBI GenBank and genomic data of only four species *viz., P. citricarpa, P. citriasiana, P. capitalensis and P. ampelicida* is available till date in NCBI GenBank. Therefore, the sequence data of five isolates were compared for similarity with the available genomic data of *Phyllosticta* species. The results are in line with Arafat (2018), where he isolated the pathogen from infected guava fruit and DNA was isolated from the pathogen and was sequenced. The sequence was compared with known homologous sequences of *Phyllosticta* and *Guignardia* in databanks.

From the above investigation it was reported that MND isolate showed highest per cent similarity of 95.95 with *Phyllosticta citricarpa*, HSN isolate showed 95.58 per cent with *Phyllosticta citriasiana*, RNP isolate showed 95.95 per cent with *Phyllosticta capitalensis*, HNP isolate showed 95.96 per cent with *Phyllosticta citricarpa* and PYP isolate showed highest per cent similarity of 95.95 with *Phyllosticta capitalensis*.

This study reveals the occurrence, disease severity of ginger leaf spot in ginger growing regions of Karnataka. To our knowledge, there is no evidence of scientific work in Karnataka regarding the morphology and molecular characterization of the pathogen. The combination of morphological and molecular characterization has strengthened our work to identify the isolated pathogen as *Phyllosticta zingiberi* Ramakr. from the diseased ginger leaf.

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