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Research Article

FIELD SURVEY, MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF *PHYTOPHTHORA CAPSICI*, CAUSES FOOT ROT DISEASE OF BLACK PEPPER IN KARNATAKA, INDIA

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A B S T R A C T

India is one of the exporters of black pepper (*Piper nigrum L.*) but the production of this crop is in the decline stage, because of the foot rot disease. The objective of this study was to determine the morphological and molecular characters of *Phytophthora capsici*, the causal agent of foot rot disease of black pepper in Karnataka. Six major pepper growing areas were surveyed and confirmed for the incidence of foot rot disease. Chickmagalur district (Boothanakadu village) had the highest disease incidence (18%) followed by Shivamogga (Kunjavalli village) (16%) and the lowest incidence at Belgaum district (5%). Based on the morphological characterization, the foot rot pathogen exhibited chlamydospore, torulose hyphae and lemon-shaped sporangia with long pedicel.

INTRODUCTION

Black pepper (*Piper nigrum* L.) the "King of Spices" is medicinally very important and considered as a commercial crop in India. Black pepper is a vine and the cultivation of black pepper is mainly confined to India and has gained popularity in the world pepper market. Foot rot is one of the destructive diseases of pepper in India. Foot rot is debilitating disease and death of plants can occur at anytime including very older vines (Ravindran, 2000). Field survey is the estimation of crop loss in field conditions which is very much important in agricultural sector to explore the plant diseases in different agroclimatic situations as well as to demarcate the disease free areas for quality seeds and seedling productions. Field survey helps in providing geographical information about the status, location of a disease and economic loss. One of the reasons is that the quantitative severity loss relationships have no conceptual framework for crop loss assessments. Furthermore, disease incidence, disease severity, crop loss, virulence analysis and genetic variations among the pathovars will be identified (Roberts *et al*., 2004). Field survey plays an important role in assessing the relation of pathogen with weather condition depending upon soil and variety. Survey suggests the role of pathogens in the yield loss. Pathogens causes wilt, seedling infections, foot rot, root-rot, leaf spot and rust, in which pathogens plays an important role in disseminating the diseases (Anandaraj and Sarma, 1995). Black pepper is known to harbor a large number of both bacterial and fungal diseases. Among them, foot rot disease caused by *Phytophthora capsici* is a very dangerous disease in pepper. This framework would allow for a distinction between measurements of severity and measurement of loss.

Molecular markers are new potential genetic tools to enhance selection efficiency and are advantageous, since these have no effect in themselves on the phenotype. The number of availability of molecular markers is enormous and also has the advantage that the heterozygote of these can be identified. Hence, this enables rapid screening of disease and saves on time and effort. DNA markers have proved to be an efficient tool for the molecular characterization of the plant pathogens. PCR based molecular, random amplified polymorphic DNA (RAPD), inter simple sequence repeat (ISSR), simple sequence repeats (SSR), restriction fragment length polymorphism (RFLP) and amplified fragment length polymorphism (AFLP) are useful in various applications. Among them, RAPD and ISSR are the arbitrary markers in which only one primer is used (Meng and Chen, 2001).

Hitherto survey reports showed a steady increase in the number of affected sites. In the present study, emphasis has been made to fulfil the following objectives, which involves the survey of farmer's fields for the incidence and prevalence of foot rot disease. Based on the cultivars and the percentage of disease incidence will help farmers to manage foot rot disease in pepper. This study was undertaken in order to assess

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the factors influencing the incidence of foot rot disease of pepper, its distribution in Karnataka and the implications of regulation in the spice industries for the management of foot rot disease. The main purpose of this was to obtain a list of places affected by *P. capsici* and to screen the commonly used varieties of pepper for resistance and genetic variability using molecular markers RAPD and ISSR which are reproducible and more specific than other molecular markers. Presently, this technique was used to study genetic variability among the isolates from different regions of Karnataka.

MATERIALS AND METHODS

Field survey in pepper growing areas in Karnataka

Field survey was conducted in pepper growing areas in six districts in Karnataka. Sudisha *et al.* (2006) has explained and classified the agro climatic zones of Karnataka into different regions, which includes 10 zones such as North eastern transition zone (Bidar), North eastern dry zone (Gulbarga and Raichur), North dry zone (Bijapur, Bagalkote, Belgaum and Koppala), Central dry zone (Chitradurga and Davanagere), Eastern dry zone (Bangalore, Tumkur, and Kolar), South dry zone (Chamarajanagara, Mysore, Mandya and Hassan), South transition zone (Shivamogga), Northern transition zone (Belgaum, Dharwad, Gadag and Haveri), Hilly zone (Karwar, Madikeri and Chickmagalur) and coastal zone (Dakshina Kannada). Out of which field survey has been carried out in only pepper growing areas namely Madikeri, Shivamogga, Chickmagalur, Hassan, Dakshina Kannada and Belgaum which comes under different zones. The presence of foot rot of pepper (percentage of infected vines/field) and its severity with typical symptoms was recorded. Early infection shows the lesions with minute spots on leaves near the ground, becoming specific fimbriate edge leaf lesions. The disease is usually undetected by farmers until the upper part of pepper vine shows symptoms of leaf yellowing, wilting and dropping. Most of the symptoms were observed at this stage.

Field survey to assess the disease incidence of foot rot in pepper growing regions in Karnataka

Totally 100 field survey was carried out during 2014-16 in six districts, namely, Madikeri, Shivamogga, Chickmagalur, Hassan, Dakshina Kannada and Belgaum. Taking the nearest pepper fields of each district, 250 vines were evaluated for the disease in one hectare of pepper growing field. Field survey were taken in pre monsoon and peak monsoon seasons, in May-June and second survey in the month of August-September respectively, at this time weather condition can support shading and provides a good environment for the growth and development of pepper vine as well as infections of *P. capsici* (Nguyen, 2004). Disease was measured through observation of disease incidence and severity of overall field symptoms of foot rot disease. Disease severity was determined using 0-4 score scale which represents 0=0%, 1=1-30%, 2=31-50%, 3=51-75%, 4=76-100% (Kim and Hwang, 1992).

Isolation of P. capsici from pepper plants

The samples collected during the survey were cut into small pieces from infected stem and leaf part. Later small pieces were surface sterilized with 4% sodium hypochlorite for 1 min under aseptic conditions. The samples were then rinsed in distilled water in a beaker for 1 min. The samples were blotted to dry using sterile filter paper. Then the tissue segments were transferred into Petri dishes containing Oat meal agar (OMA) containing antibiotics (streptomycin). After inoculation plates were incubated at 23 ± 2°C for 7 days under 12 h dark and 12 h night condition according to standard tissue isolation technique (Tuite, 1969). Growth of mycelial growth of *Phytopthora* was started observing after 7 days of incubation, later the mycelia growth was sub cultured to Petri plates containing OMA medium. Morphological characteristics observation such as mycelia structure and types of conidia produced were performed using compound microscope.

Study of pathogenicity

Sporangial suspension was prepared and used to check for its pathogenicity. The method was followed by Riberio (1978) for the production of sporangia. The sporangial suspension (2×10^5 sporangia/ml) was prepared in a known amount of sterile distilled water and was used for inoculation to prove its pathogenicity. Plants of black pepper were inoculated with 1 ml of sporangial suspension by making little injury to them. Then they were kept in humid chamber raised in the earthen pots. Then they were observed for symptoms up to 15-25 days.

Screening of resistant varieties for foot rot infection under green house condition

In 2014-15, the greenhouse experiments were conducted in nursery Mysore to find the resistant varieties against foot rot disease on different varieties of pepper vines Sreekara, Subhakara, Panchami, Pournami, PLD-2, Thevam, Girimunda, Malabar, Shakthi and Arkacoorg which were collected from Appangala Spice Research Center, Madikeri district. In this experiment, totally 10 varieties with three replicates were raised in earthern pots (6 × 10") filled with 1 kg of nursery mixture (Sand + Soil + cow dung 2:2:1). Vines with 5-6 leaves (six months old), were used for inoculation.

Six month old pepper vine was inoculated with sporangial suspension of *P. capsici* (2×10^5 sporangia/ml) and was used for inoculation to prove the resistant characters. Ten varieties of pepper vines were inoculated with 1 ml of sporangial suspension by making little injury to the leaves. Then they were kept in humid chamber raised in polythene bags and kept for disease evaluation. Foot rot disease incidence was evaluated up to 15-25 days after inoculation the plants were graded according to a standard rating scale of 0-4, in which 0, 1, 2, 3 and 4 respectively indicates immune resistant, moderately resistant, moderately susceptible lines and highly susceptible as suggest by Abraham *et al*. (1996), which refers to 0=0%, 1=1-30%, 2=31-50%, 3=51-75% and 4=76-100% of the disease.

Percent disease incidence (PDI) was assessed based on the formula

$$\text{PDI} = \frac{\text{No. of disease incidence} \times 100}{\text{Total No. of plants observed}}$$

Isolate variability using molecular markers collected from different regions of pepper in Karnataka using molecular markers

DNA extraction

Fungal mycelia were grown in potato dextrose broth (PDB) and incubated at 23 ± 2°C for 7 days under 12 h dark and 12 h night condition. The mycelia were then harvested in

microcentrifuge tube. DNA was extracted according to Esselman *et al*. (1999). DNA was visualized by agarose gel electrophoresis for estimation of amount and quality. The DNA was further used for RAPD and ISSR according to Joshi *et al.* (2000).

Quantification of DNA

The isolated DNA was quantified in a nanodrop (Thermo Scientific, 2000C, Wilmington, USA). DNA sample of 1µl was placed in the slot and the absorbance was read at 260 nm and 280 nm. The purity of DNA was calculated using A_{260}/A_{280} ratio, which should be around 1.8. The concentration was calculated using the formula:

1 OD unit at 260 nm = 50 µg/ml of DNA

Random amplified polymorphic DNA (RAPD)

Ten primers from OPR, OPM, OPS and OPB series (Operon Technologies Inc. USA), were used for assessing genetic diversity among the isolates of *P. capsici*. PCR amplification was performed in a total reaction volume of 25 µl containing 1X PCR buffer, 10 mM of dNTPs, 10 pmole of each primer, 1U *Taq* DNA polymerase (Fermentas, Hanover, MD, USA) and 100 ng of template DNA. The RAPD-PCR amplification were performed in a Gradient thermal cycler (BioRad, California) with an initial denaturation 94°C for 3 min, denaturation 94°C for 1 min, annealing 36°C for 1 min, extension 72°C for 2 min final extension 72°C for 10 min and hold for 4°C and repeated for 45 cycles.

Table 1 List of RAPD Primers

No.	Primer	Sequence
1	OPR 1	5'-TGCGGGTCCT-3'
2	OPR 2	5'-CACAGCTGCC-3'
3	OPR 3	5'-ACACAGAGGG-3'
4	OPR 4	5'-CCCGTAGCAC-3'
5	OPS 3	5'-CAGAGGTCCC-3'
6	OPS 7	5'-TCCGATGCTG-3'
7	OPM 1	5'-GTTGGTGGCT-3'
8	OPM 2	5'-ACAACGCCTC-3'
9	OPM 3	5'-GGGGGATGAG-3'
10	OPB 13	5'-TTCCCCCGCT-3'

Agarose gel electrophoresis

The 10 primers used were from Kit of Operon technologies. The amplified RAPD products were analysed by electrophoresis using 1.2% agarose gel in 1X TBE buffer along with little ethidium bromide. The gels were photographed and documented using BIO-RAD Molecular Imager, Gel Doc ™ XR + and Imaging System.

Inter simple sequence repeat (ISSR)

Ten primers of ISSR as shown in table 2. Custom synthesized from Sigma-Aldrich (USA) were used. PCR amplifications for ISSR analysis were performed in a total reaction volume of 25 µl containing 1X PCR buffer, 10 mM of dNTPs, 10 pmole of each primer, 1U *Taq* DNA polymerase (Fermentas, Hanover, MD, USA) and 100 ng of template DNA. ISSR PCR amplifications were performed in a Gradient thermal cycler (BioRad, California) with an initial denaturation at 94 °C for 3 min, followed by 40 amplification cycles of 94°C for 1 min, 42°C for 1 min and 72°C for 2 min and a final extension step of 72°C for 10 min.

Table 2 List of ISSR primers

No.	Primers No.	Sequence
1	ISSR 5	5'- (AG)8C-3'
2	ISSR 6	5'- (AG)8T-3'
3	ISSR 8	5'- (GACA)4-3'
4	ISSR 12	5'- (GTG)3GC-3'
5	ISSR 13	5'- (CTC)3GC-3'
6	ISSR 14	5'- (GAG)3GC-3'
7	ISSR 18	5'- (GA)6CC-3'
8	ISSR 19	5'- (CA)6GC-3'
9	ISSR 20	5'- (CA)6AG-3'
10	ISSR 21	5'- (CA)6GT-3'

Agarose gel electrophoresis

The amplified ISSR products were analyzed by electrophoresis using 1.2% agarose gel in 1X TBE buffer along with little ethidium bromide. The gels were photographed and documented using BIO-RAD Molecular Imager, Gel Doc ™ XR + and Imaging System.

Statistical analysis

The data from the laboratory and green house experiments were analyzed separately for each experiments were subjected to arcsine transformation and analysis of variance (ANOVA), SPSS, version (10) significant effects of treatments were determined by the magnitude of 'F' value (p=0.05). Treatment means were separated by tukeys HDS test. The RAPD data and ISSR data from all amplifications were recorded by scoring polymorphic DNA bands and compiled in a binary matrix in which 1 indicated the presence and 0 the absence of the marker. The data was converted to distance matrices based on Nei (1978) unbiased minimum distance. The distance matrices were used to construct a dendrogram by the unweighed pair-group method with arithmetic mean (UPGMA) using tools for population genetic analysis (TFPGA, ver. 1.3) (Miller, 1997).

RESULTS

Field survey to assess the disease incidence of foot rot in pepper growing regions in Karnataka

In Madikeri districts (Hilly regions) highest disease incidence of 16% in Cherambane village which belongs to Madikeri taluk. Bhagamandala showed 14%, Somwarpet showed 12 % and Virajpete showed 7%, respectively, foot rot disease infections and least disease incidence was recorded in Bylakuppe village with 4% as shown in figure 1. Leaves yellowing symptom was observed on both upper and lower leaf canopies at all the fields surveyed.

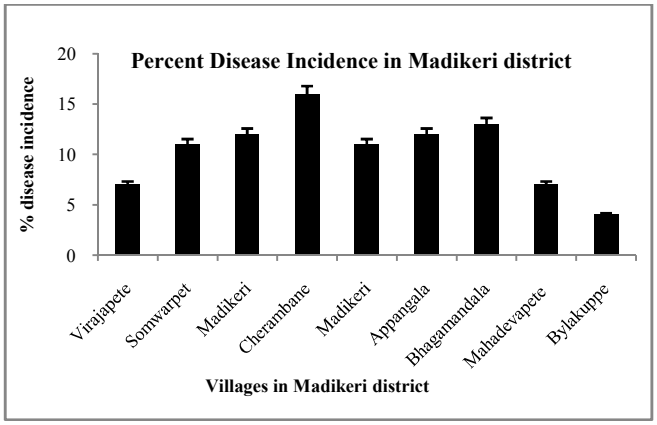


Fig 1 Survey for foot rot disease in Madikeri district

In Chickmagalur district, Boothanakadu showed highest of 18%, Asagudu showed 16% followed by Medapur 11%, Shankarapura, Umbalibailu, Hirekere, Mavinahalli with 8% disease incidence, respectively. Very least disease incidence of 3% was observed in Koppa taluk as shown in figure 2.

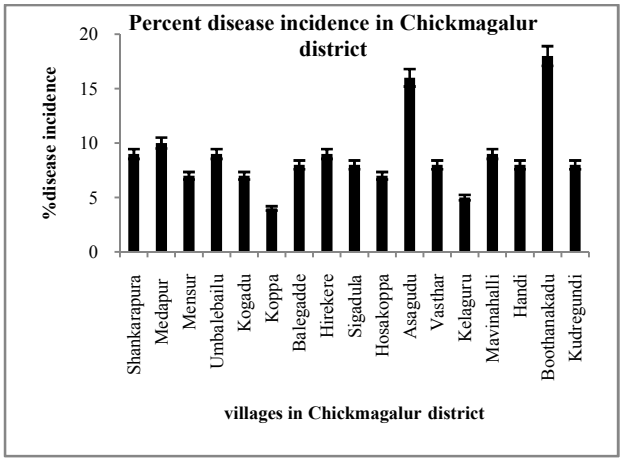


Fig 2 Survey for foot rot disease in Chickmagalur district

In Shivamogga (South transition zone) district, Kunjavalli village showed highest disease incidence of 14% followed by Balagatte with 10% and Kutthi College of Malnad and least disease incidence was observed with 2% as shown in figure 3.

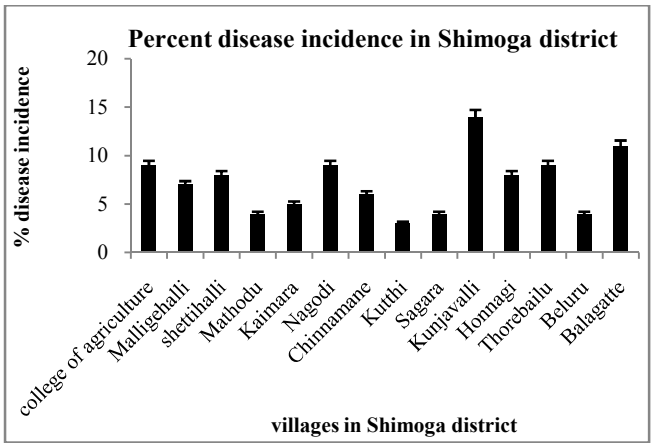


Fig 3 Survey for foot rot disease in Shivamogga district

In South dry zone (Hassan), highest disease incidence was recorded in Belagodu with 11% followed by Moovala and Arehally 8% each and lowest disease incidence was observed in Uchangi and Malalur with 4% and 5%, respectively, as shown in figure 4.

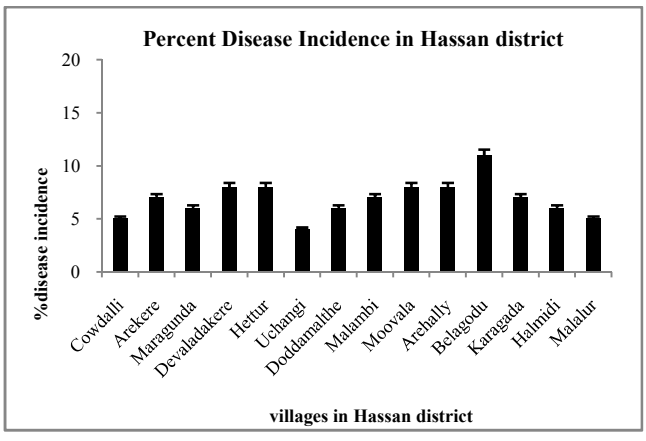


Fig 4 Survey for foot rot disease in Hassan district

In Dakshina Kannada (coastal zone) district, highest disease incidence was observed in Bellare with 8%, followed by 7% disease incidence in Sullia, Kalmadka, Venoor and Adyanadka, respectively, and very least disease incidence was recorded in Puttur with 3% disease incidence as shown in figure 5.

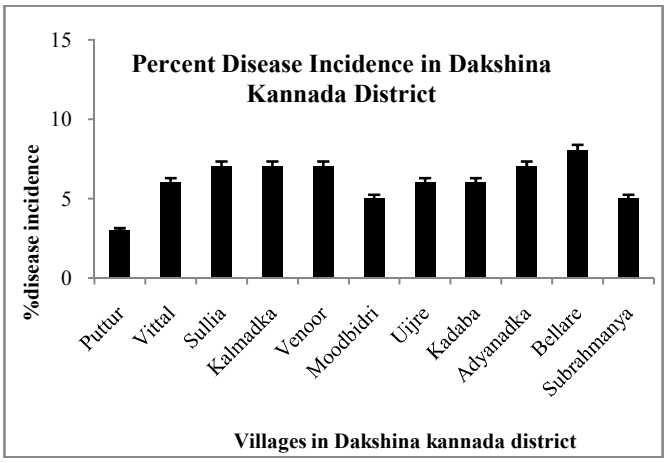


Fig 5 Survey for foot rot disease in Dakshina Kannada district

In Belgaum districts (Northern transition zone), more disease incidence was recorded in Hukkeri and Gotur with 5% followed by Gokaka, Hidkal, Bellambi and Kurani with 4%, respectively. Least disease incidence was recorded in Jinaral village with only 3% respectively as shown in figure 6.

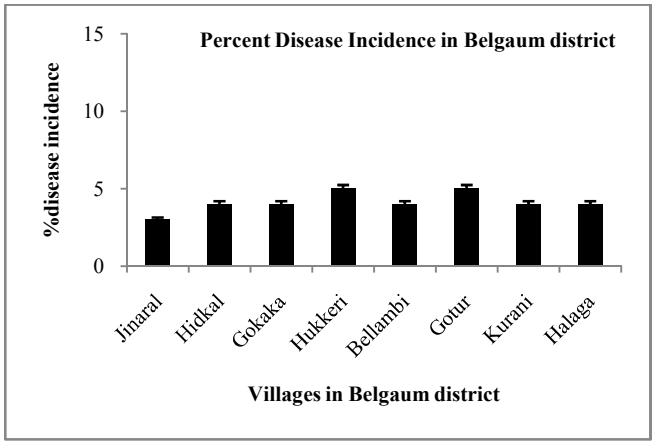


Fig 6 Survey for foot rot disease in Belgaum district

The infection at initial stage starts as water soaked, which is recorded more in leaf infection. The lesions later turn to brown to dark brown in color and within 2-3 days it appears as slimy dark patches. Young leaves become flaccid followed by yellowing and defoliation.

Isolation of P. capsici from pepper plants

The fungus was identified by observing colony characters such as linear growth, colour and sporulation. Based on the morphological characterization, the foot rot pathogen exhibited chlamydospore, torulose hyphae and lemon-shaped sporangia with long pedicel as shown in figure 8 and as described by Mchau and Coffey (1995).

Fig 8 Petriplates showing mycelia growth and lemon-shaped sporangia with long pedicel

List of isolates collected from different regions of Karnataka

Totally 68 isolates were isolated from the infected material and are listed in table 3. The strains were revived on OMA for morphological and virulence tests.

Table 3 List of 68 isolates collected from different region

Sl. No.	Place of collection	Variety	Pathogenicity test
01	Hukkeri, Belgaum, district	Panchami	Positive
02	Hidkal, Belgaum district	Panchami	Negative
03	Gotur, Belgaum district	Panchami	Positive
04	Hakathuru, Madikeri district	Sreekara	Negative
05	Hondli, Madikeri district	Panchami	Negative
06	Madikeri, Madikeri district	Subhakara	Positive
07	Madikeri, Madikeri district	Sreekara	Negative
08	Appangala, Madikeri district	Panchami	Negative
09	Mahadevapete, Madikeri district	Arkacoorg	Negative
10	Bylakuppe, Madikeri district	Arkacoorg	Negative
11	Kodlipet, Madikeri district	Subhakara	Negative
12	Cherambane, Madikeri district	Arkacoorg	Positive
13	Shanivarasante, Madikeri district	Pournami	Negative
14	Shirangala, Madikeri district	Subhakara	Negative
15	Areyuru, Madikeri district	Sreekara	Negative
16	Kushalanagara, Madikeri district	Arcacoorg	Negative
17	Kushalanagara, Madikeri district	Arcacoorg	Negative
18	T. Settigeri, Madikeri	Arcacoorg	Negative
19	Birunanui, Madikeri	Pournami	Negative
20	Srimangala, Madikeri district	Panchami	Negative
21	Srimangala, Madikeri district	Shakti	Negative
22	Balale, Madikeri district	Malabar	Negative
23	Hudikeri, Madikeri district	Arcacoorg	Negative
24	Hudikeri, Madikeri district	Panchami	Negative
25	Hudikeri Madikeri district	Panchami	Negative
26	Virajpete, Madikeri district	Arkacoorg	Negative
27	Virajpete, Madikeri district	Arkacoorg	Negative
28	Virajpete, Madikeri district	subhakara	Positive
29	Virajpete, Madikeri district	Panchami	Negative
30	Virajpete, Madikeri district	Panchami	Negative
31	College of Agriculture, Shivamogga	Arcacoorg	Negative
32	Malligahalli, Shivamogga district	Panchami	Negative
33	Shettihalli, Shivamogga district	Panchami	Negative
34	Mathodu, Shivamogga district	Arkacoorg	Negative
35	Kaimara, Shivamogga district	Arkacoorg	Negative
36	Nagodi, Shivamogga district	Subhakara	Negative
37	Chinnamane, Shivamogga district	Panchami	Negative
38	Kauthi, Shivamogga district	Panchami	Negative
39	Chinnamane, Shivamogga district	Arcacoorg	Negative
40	Sagara, Shivamogga district	Panchami	Negative
41	Panniyur, Shivamogga district	Panchami	Negative
42	Kunjavalli, Shivamogga district	Panchami	Negative
43	Honnagi, Shivamogga district	Panchami	Negative
44	Honnagi, Shivamogga district	Panchami	Negative
45	Thorebailu, Shivamogga district	Panchami	Negative
46	Thorebailu, Shivamogga district	Panchami	Negative
47	Beluru, Shivamogga district	Pournami	Negative
48	Balgatte, Shivamogga district	Panchami	Negative
49	Hirikere, chickmagalur	Shakti	Negative
50	Madikeri	Shakti	Negative
51	Koppa, chickmagalur	Subhakara	Negative
52	Medapura, Chickmagalur	Malabar	Negative
53	Mensur, Chickmagalur	Arcacoorg	Negative
54	Kudregundi, Chickmagalur	Panchami	Positive
55	Asagudu, Chickmagalur	Pournami	Positive
56	Shankarapura	Panchami	Positive
57	Mavinahalli	Shakti	Positive
58	Medapur	Malabar	Positive
59	Boothanakadu, Chickmagalur	Arcacoorg	Positive
60	Nagodi, Shivamogga district	Panchami	Positive
61	Koppa	Pournami	Positive
62	Balagatte	Panchami	Positive
63	Kasargod, Dakshina kannada	Shakti	Positive
64	Shettihalli	Malabar	Positive
64	Belagodu	Arcacoorg	Positive
66	Sullia, Dakshina Kannada	Panchami	Positive
67	Bellare, Hassan	Panchami	Positive
68	Kunjavlli	Panchami	Positive

Pathogenicity

After inoculation, the pathogen produced typical foot rot symptoms on vines. Infected vines showed symptoms on upper part of pepper vine resulting in leaf yellowing, wilting and dropping. The pathogen was re-isolated successfully from infected leaf pieces on selected medium. Identification of the pathogen was made on the basis of morphological features as described previously and with reference to original cultures collected during the survey. Isolates varied in their ability to cause disease, among 68 isolates only 20 isolates confirmed pathogenicity (Koch's Postulates) as shown in table 4. Symptoms produced were similar to those previously observed on pepper plants in the field during the survey. Moreover, isolates recorded after the termination of the experiment on selective media were identical to those isolated from the infected plants collected during the survey.

Table 4 List of *P. capsici* used in the study

Isolate ID	Place of collection	Soil type
Pc1	Hukkeri	Red soil
Pc 2	Gotur	Black soil
Pc 3	Cherambane	Red soil
Pc 4	Virajapete	Black soil
*Pc*5	Madikeri	Black soil
Pc 6	Kudregundi	Black soil
Pc 7	Asagudu	Black soil
*Pc*8	Shankarapura	Red soil
*Pc*9	Mavinahalli	Red loamy soil
*Pc*10	Medapur	Red soil
Pc11	Boothanakadu	Red loamy soil
Pc12	Nagodi	Red soil
Pc13	Koppa	Black soil
Pc14	Balagatte	Red soil
Pc15	Kunjavalli	Red soil
Pc16	Shettihalli	Black soil
Pc17	Belagodu	Red soil
Pc18	Sullia	Red soil
Pc19	Bellare	Black soil
Pc20	Kadaba	Black soil

Screening of resistant varieties for foot rot infection under green house condition

Among 10 genotypes tested foot rot disease incidence under green house condition highest disease incidence was recorded from Subhakara variety with 24% followed by Sreekara with 23%, Pournami with 20%, Malabar with 19%, Panchami with 18% , Arkacoorg with 17%, Girimunda with 16% and PLD-2 with 16%, respectively, and least disease incidence was observed in Thevam and Shakthi with 5% and 4% as shown in figure 7.

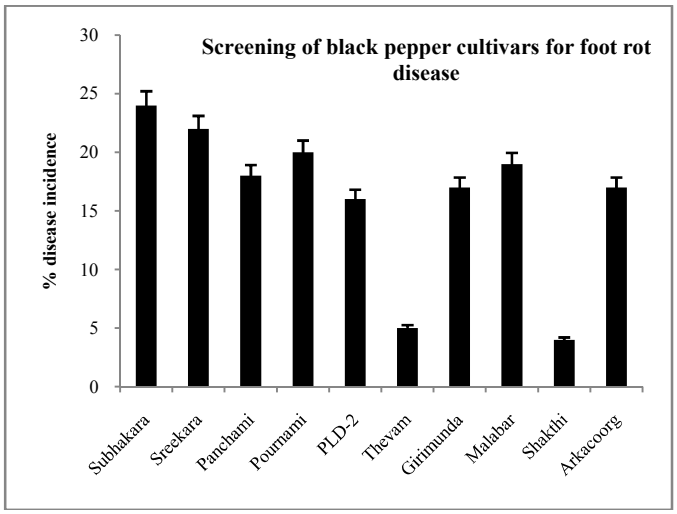


Fig 7 Screening of resistant varieties for foot rot disease

Random amplified polymorphic DNA (RAPD) analysis

RAPD profiles were generated using 20 isolates of *P. capsici* and using 10 primers (Table 1). A total of 149 bands were scored, with a polymorphic of 147 bands. Out of these 147 bands, the percentages of polymorphism generated by all the primers were 98.09%. Each amplification was repeated twice to confirm the reproducibility and only reproducible bands were considered for scoring and analysis. Among the 10 primers, OPR1, 2, 4, OPS3, 7, OPM1, 3 and OPB 13 showed 100% polymorphism with 149 bands. Less polymorphism was observed in OPR 3 and OPM2 with 90 and 90.90% polymorphism. The band size generated by all the primers ranged from 100 to 1500 bp. Gels showing the amplification products are shown in figure 9.

Table 5 Total number of polymorphic bands of *P. capsici* isolates amplified using 10 RAPD primers

Sl. No.	Operon Primers	Bands scored	Polymorphic bands scored	Polymorphism %
1	OPR1	20	20	100
2	OPR 2	13	13	100
3	OPR 3	10	9	90.00
4	OPR 4	19	19	100
5	OPS3	18	18	100
6	OPS 7	11	11	100
7	OPM 1	09	09	100
8	OPM 2	11	10	90.90
9	OPM 3	18	18	100
10	OPB 13	20	20	100
	Total	149	147	98.09

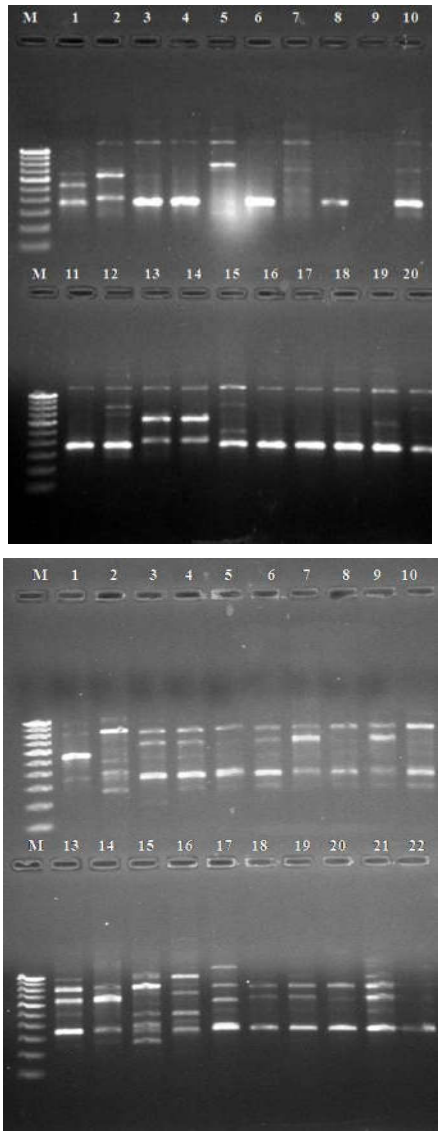


Fig 9 Agarose gel showing RAPD of *P. capsici* (Lane 20) isolates obtained by amplifying 100 ng of DNA using the primers OPM 1 (A) and OPS 7 (B). M, DNA ladder; Lane 1 to 20 corresponds to the isolates in Table 5.

RAPD cluster analysis

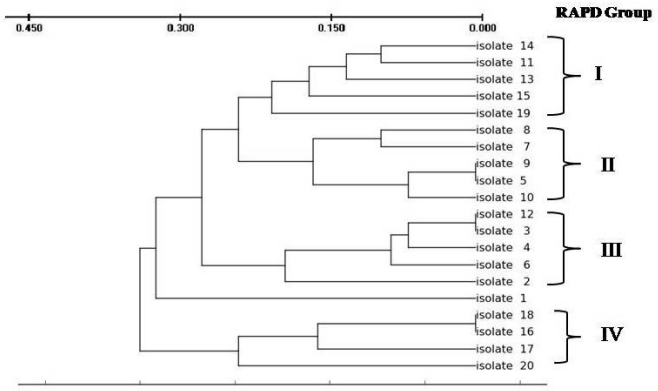


Fig 10 UPGMA dendrogram based on the similarity index illustrating the genetic relationship among the isolates of *P. capsici* based on RAPD profile on OPS7 primer. Distance based on Nei (1978)

Cluster analysis with UPGMA using genetic distances was done to generate a dendrogram. The isolates of *P. capsici* divided into four groups by RAPD analysis. Group 1 contains five isolates of *P. capsici* (Pc14, Pc11, Pc13, Pc15 and Pc19) which belongs to South transition and Hilly zones. Group II

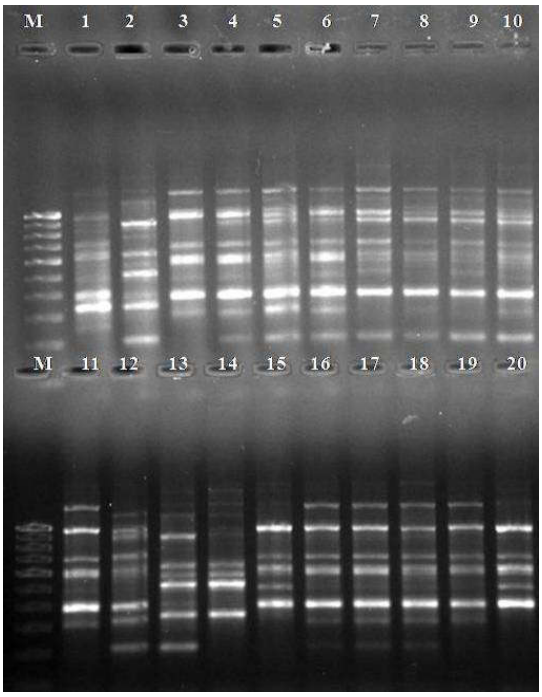
contains five isolates of *P. capsici* (Pc8, Pc7, Pc9, Pc5 and Pc10) belongs to hilly and coastal zone. Group III contains five isolates of *P. capsici* (Pc12, Pc3, Pc4, Pc6 and Pc2) belongs to hilly, Southern transition, Northern transition and coastal regions and Group IV contains four isolates (Pc18, Pc16, Pc17 and Pc20) which belongs to Southern transition, and coastal zones. Isolate Pc1 placed as an out group in the dendogram which represents Northern transition zone. This shows that the diversity is host specific. The genetic distance values generated from RAPD markers varied between 0.01 and 0.370.

Inter simple sequence repeat (ISSR)

ISSR profiles were generated using 20 isolates of *P. capsici* using 10 primers (Table 2). A total of 143 bands were scored, out of these, 141 bands were polymorphic and percentage of polymorphism of all the primers were 98.60% and ISSR20 showed 92.85% polymorphism. Each amplification was repeated thrice to confirm the reproducibility and only reproducible bands were considered for scoring and analysis. Out of 10 primers, ISSR5, 6, 12, 13, 14, 18,19, 20 and ISSR 21 showed maximum polymorphism with 100% polymorphism and less polymorphism was observed in ISSR8 with 94.44% as shown in table 6. The band size generated by all the primers ranged from 100 to 1500 bp. Gels showing the amplification products are shown in figure 11.

Table 6 Total numbers of polymorphic bands of *P. capsici* isolates amplified using 10 ISSR primers

Sl. No.	Operon primers	Bands scored	Polymorphic bands scored	Polymorphism %
1	ISSR 5	12	12	100
2	ISSR 6	18	18	100
3	ISSR 8	18	17	94.44
4	ISSR 12	19	19	100
5	ISSR 13	13	13	100
6	ISSR 14	15	15	100
7	ISSR 18	09	09	100
8	ISSR 19	19	19	100
9	ISSR 20	14	13	92.85
10	ISSR 21	06	06	100
Total		143	141	98.60



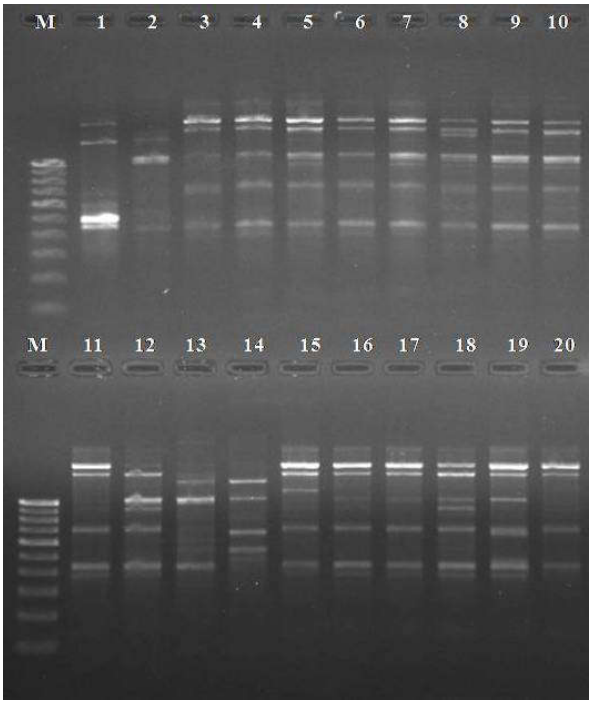


Fig 11 Agarose gel showing ISSR products of *P. capsici* (Lane 20) isolates obtained by amplifying 100 ng of DNA using the primers ISSR19 (A) and ISSR 20 (B). M, DNA ladder; Lane 1 to 20 corresponds to the isolates in table 6.

ISSR cluster analysis

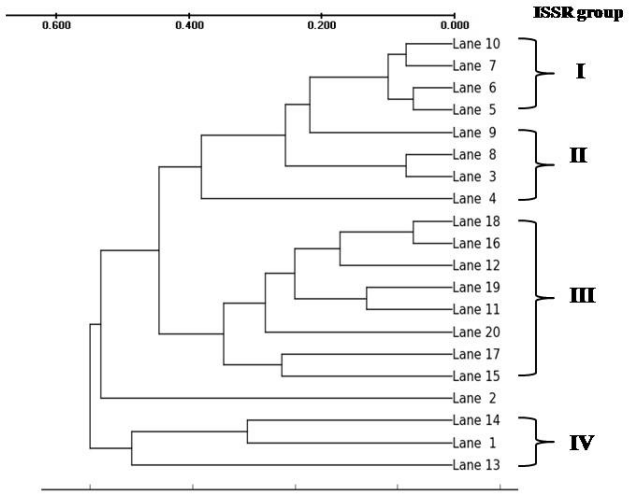


Fig 12 UPGMA dendrogram based on the similarity index illustrating the genetic relationship among the isolates of *P. capsici* based on ISSR 20 primer (Distance based on Nei (1978))

Cluster analysis with UPGMA using genetic distances was done to generate a dendrogram (Fig. 12). Five major groups were identified; group I contains Pc 10, Pc7, Pc6 and Pc5 belongs to hilly region zone, Group II contains Pc9, Pc8, Pc3 and Pc4 all belongs to hilly region zone. Group III contains Pc 18, Pc16, Pc12, belongs to Coastal, Southern transition and hilly regions. Pc19 and Pc11, belongs to coastal and hilly regions, Pc20, Pc17 and Pc15 which belongs to coastal, southern dry and Southern transition zone. Group IV contains Pc14, Pc1 and Pc13 belongs to Southern transition, and hilly region Pc2. Isolate Pc2 placed as an out group in the dendogram which represents Northern transition zone. The genetic distance values generated from ISSR markers varied between 0.01 and 0.57.

DISCUSSION

Out of six districts surveyed in Karnataka, Chickmagalur and Madikeri districts showed highest disease incidence of 18% and 16%. Most of the pepper fields were surveyed in Madikeri, Shivamogga, Chickmagalur, Hassan, Dakshina Kannada and Belgaum districts in different regions namely Hilly zone, Southern transition zone, South dry zone Coastal and Northern transition zone, respectively. Survey indicated the cultivation of popular varieties such as Pournami, PLD-2, Girimunda, Malabar and Arka coorg. Overall, incidence and severity of foot rot was greatest at hilly zone and south dry zone. George *et al*. (2015) also reported that incidence of foot rot disease in Madikeri, Shivamogga and Chickmagalur district with disease incidence of 14%, 17% and 20% of foot rot disease infections. Thomas *et al*. (2017) reported a highest disease incidence (65%) of the foot rot disease in Mathodu village of Shivamogga district and 50% disease incidence have been recorded in Chickmagalur taluk and Mudigere taluk. In our observation, Asagud and Boothanakadu showed the highest disease incidence of 16% and 18%, respectively and the least disease incidence was recorded in Belgaum district with only 5% disease incidence. Several districts *viz*., Hassan, Shivamogga, Dakshina Kannada and Belgaum showed less disease incidence when compared to Chickmagalur and Madikeri districts. Such reports on losses due to heavy incidence of foot rot have been reported from Dakshina Kannada, Shivamogga and Hassan districts of Karnataka (Jahagirdar, 1998). So this survey helped to identify the major pepper growing areas infected by foot rot disease in six major pepper growing areas. During the fortnight, preceding the appearance of the disease, the average daily maximum temperature ranged between 25±2°C, with a minimum temperature 18-20°C, maximum relative humidity 80-90% and 10-12 rainy days required for the initial appearance of the disease and the prevailing weather parameters of maximum temperature, minimum temperature, relative humidity and total rainfall. However, there was a strong indication that these weather parameters appear to be favorable for the build up of inoculums and initial infection of the crop. Similar observation was observed with 50% disease incidence in Kabilaseathve village of Chickmagalur district (Thomas and Naik, 2017).

Field survey indicated the yield losses and per cent incidence of diseases under suitable environment. The survey results showed that *P. capsici* was present in pepper in all areas surveyed from the seedling to the fruiting stages. There was a high incidence of foot rot disease in several districts of Karnataka. The term foot rot is used to describe the symptoms that occur when leaf infection spreads from foot to the petiole and to the stem often causing it to break in cloudy weather, results in the loss of fruiting branches. This severe symptom of foot rot was common in the hilly zone, South transition zone and South dry zone which includes, Chickmagalur, Madikeri, Shivamogga and Hassan districts but rarely seen in Dakshina Kannada (coastal zone) and Belgaum districts (Northern transition zone).

Foot rot was severe during August, heavy precipitation during July-August combined with optimum temperature of 22-24°C and more than 78% relative humidity helped in the development of foot rot diseases. Besides these factors, with poor soil drainage and alkaline pH > 8.2 favored the rapid development of diseases (Anandaraj *et al*., 1996). Relatively high rainfall during the monsoon with high soil moisture (>25%) and conducive temperature (22°C to 29°C) and stable relative humidity (80%) are favorable for multiplication of the fungus. Based on field inspection, the collar rot infection occurs either at the collar or just above or below the soil level. Collar and root infection go unnoticed until the foliar yellowing symptom is recognized. The lesions later turns to brown to dark brown in color within two to three days and later it appears as slimy emitting foul smell (Nambiar and Sarma, 1977; Siti *et al*., 2013).

PCR-based techniques have been adopted as a convenient and powerful means of detecting genetic differences among closely related organisms. Molecular markers can demonstrate similarities and dissimilarities between different isolates of same species even when a morphological description is severely limited (Esselman *et al*., 1999; Truong *et al*., 2010). RAPD and ISSR markers proved to be efficient and inexpensive way to generate molecular data. They have been used successfully in determining genetic relationship (Gilbert *et al.,* 1999).

Accurate and rapid identification of a pathogen is necessary for the appropriate management of the disease (Jana *et al*., 2003). Morphological characteristics are key for the identification and taxonomy of many fungal species including *P. capsici* (Sahar *et al*., 2012).). In the present study, out of 10 RAPD primers, total bands scored were 149; among them 147 w polymorphic and the percentage of polymorphism was 98.09%. Most of the primers showed polymorphic except OPR3 and OPM2 showed 90% and 90.90% of polymorphism. The genetic coefficient values generated varied between 0.01 and 0.370 and an average of 0.120 coefficient. Domyati *et al*. (2011) reported 914 amplicons from 17 ISSR primers, 67% were polymorphic and 33% were monomorphic. Saleh (2011) stated that, a total of 88 DNA fragments with an average of 12.57 fragments/ primer were revealed with seven RAPD primers.

Pradeepkumar *et al*. (2003) reported that RAPD analysis on 22 cultivars of *P. nigrum* from South India by using 24 RAPD markers, generated 372 bands among them 367 were polymorphic. Jaccard's similarity ranged between 0.11 and 0.66 with a mean of 0.38.

The present study obtained polymorphism amounting 98.60%of polymorphism among the 20 isolates from 10 ISSR primers which produced 143 bands out of which 141 are polymorphic with similarity co-efficient ranging from 0.01 to 0.57. UPGMA analysis showed four groups in both RAPD and ISSR primers which produced 143 in ISSR and 149 bands in RAPD. Among them, ISSR showed 98.09% of polymorphism in ISSR and 98.60% of polymorphism in RAPD. This is mainly due to the use of number of randomly selected pre-screened highly informative primers. The amount of polymorphism obtained and the discriminate bands recorded in the study is higher in RAPD markers.

In the dendogram, it is important to note that the *P. capsici* isolates collected from North transition zone were placed as an out group in both RAPD and ISSR dendograms which support the temperature, soil condition and relative humidity that is favorable for multiplication of the *P. capsici*. In hilly, south transition, south dry and coastal zones distributed equally in all

the group but Belgaum which belongs to North transition zone has been placed in separate group. Our result corroborate with Mahmodi *et al*. (2013), who reported that ISSR markers were used to estimate genetic diversity in *C. truncatum* from Malaysia. A set of three ISSR primers revealed on 26 isolates from the amplified products. Cluster analysis with UPGMA method clustered *C. truncatum* isolates into two main groups, which differed with a distance of 0.64. He showed the correlation between genetic and geographical distribution of the isolates in different climatic regions as environment might be influencing the pathogenecity of the pathogens.Many researchers have compared RAPD and ISSR methods, found that ISSR markers can exhibit higher level of polymorphism or reproductivity (Parsons *et al.,* 1997; Esselman *et al.,* 1999). In our study, both RAPD and ISSR showed the similar results, but ISSR marker showed higher levels of polymorphism. This was also supported by Kakani *et al*. (2011). . Phylogenetic relationship among different isolates of *P. capsici* could be well established based on RAPD and ISSR markers.

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