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SEED BORNE NATURE AND INFECTIVITY OF *FUSARIUM SOLANI* CAUSING CHILLI WILT: ITS EARLY DETECTION FROM SEEDS AND SOIL USING CONVENTIONAL AND MOLECULAR METHODS

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ABSTRACT
Chilli wilt is most destructive emerging disease in south India and causing huge damage to the crop production. The pathogen remains in soil and seed for long time and causes damage year after year. Seed samples of seven popularly growing chilli varieties and rhizosphere soil from hot spots were collected from south Indian states and analysed for the frequency of microflora associated with seeds and causation of wilt disease. The maximum disease incidence during was recorded at Bellary district of Karnataka (29.48% PDI) and Kurnool district of Andhra Pradesh state. Maximum population (13.2 x 10 $^{5/}$ cfu/g of soil) of *F solani* was recorded from the rhizosphere soils of these regions. Among the microflora associated with seeds, the frequency of *F. solani* was more (10-56.50 %) which also varied with cultivars with maximium infection on Byadgi dabbi which is the highly susceptible variety. Further, a quick and early detection for the *Fusarium solani* from seed and infected soil was made by using conventional methods *viz*., standard blotter, rolled towel, seedling syptom and transmission test and polymerase chain reaction (PCR) based molecular method. A significant reduction in germinationa and vigour index of infected seeds were recorded when compared with healthy control. Translation elongation factor 1*á* gene (TEF-1α) was used for detection of pathogen from seed and rhizosphere soil and an amplified product of 658 bp was observed confirming *F. solani* infection in seed and soil. PCR based detection method was found to be the most sensitive and highly useful in early and quick detection of *F. solani* from seed and rhizosphere soil which can be used in disease management strategies.

INTRODUCTION

Chilli (*Capsicum annuum L.*), despite its fiery "hotness" is one of the very popular spice and vegetable crop grown worldwide known for its medicinal and health benefiting properties. During past few years the insect and diseases are increasing in chilli cultivation and taking a heavy toll. Among the diseases, wilt caused by fungal pathogen *Fusarium solani* is emerging as a serious problem in major chilli growing areas of south India. The disease causes a loss up to 2-87% especially in some of the popular varieties *viz*., byadgi dabbi, byadgi kaddi and other hybrids developed by public and private sectors (Madhukar *et al*., 2002; Devika Rani, *et al*., 2007; Raghu, *et al*., 2013). The disease causes seedling death in nursey if the infected seeds are sown even in non-infected soil and reduces germination and seedling vigoure. In main field the plants start wilting gradually and all the leaves become yellow and drooping occurs but leaves will not detach from the plants. Gradually whole field is infected and plants wilt followed by death (Fig.1 & 2). The disease is caused by *Fusarium solani* which is seed and soilborne fungi (Ascomycota: Sardariomycetes: Hypocreales: Nectriaceae). Which is a cosmopolitan soil inhabiting fungus which is known to be phylogenetically diverse. Infected (or) contaminated seeds serve as major source of inoculum for large number of plant pathogens which may infect the seeds and survive as spore (or) resting structures on (or) within the seeds (Neergaard, 1977).

Some of the earlier works revealed the importance of seed borne detection of plant pathogens. Sajeesh *et al*. (2014) reported the pathogenic ability of seed borne fungi and proved through transmission studies by seedling symptom test and pot culture studies. The histopathological studies revealed the location of seed borne fungi in the infected soybean seeds. Most of the observations the fungal pathogens were occupied in the pericarp, hilum and endosperm regions. Boyer *et al*. (2014) detected eight different fungal species namely, *A. alternata*, *A. triticina*, *B. sorokiniana*, *C.lunata*, *D. tetramera*, *A. flavus*, *A. niger* and *F. semitectum* from twenty four wheat cultivars seeds by standard blotter paper method. Among them, incidence of *A. alternata* (41.5%) was dominant. Ellis *et al* .(2011) conducted a study on *F. graminearum* causes seed decay and damping-off of soybean. This study evaluated the effect of inoculum density of *F. graminearum,* temperature, and fungicide seed treatments on disease development .

There is a need to develop tools which permit rapid, sensitive and specific diagnosis in mixed contaminated samples for timely management of chilli wilt. Polymerase chain reaction (PCR) which has emerged as a major tool for the diagnosis of phytopathogenic fungi and contributed to the alleviation of

some of the problems associated with the detection and containment of plant pathogens can be used in these detection studies (Henson and French 1993; Martin *et al*., 2000; Ghignone and Migheli, 2005). The objectives of the present study is to identify the hot spots of fusarium wilt based on disease incidence, estimation the frequency of micfroflora associated with chilli seed and rhizosphere soil to study the relation between pathogen population and disease severity. To develop an early and quick detection technique for the *F. solani*, from seed and soil by emplyoing both conventional and molecular techniques.

MATERIALS AND METHODS

Source of isolates and collection of seed and soil samples

A roving survey was conducted for two seasons (2012-13& 2013-14) in major chilli growing areas of Karnataka, Andhra Pradesh (Present Andhra pradesh and Telangana), and Maharashtra states. Seeds from harvested/stored lots (heaps), infected plant and soil samples from the rhizosphere of naturally infected soil were collected. The disease incidence was calculated by the following formula.

$$\text{Percent disease incidence}(\%) = \frac{\text{Number of plant infected}}{\text{Total number of plants examined}} \times 100$$

All samples were brought to the laboratory and stored at -80ºC. The samples were further processed for isolation and identification of different microflora by following different seed health testing methods given by ISTA. Isolation of *F. solani* from soil was carried out by serial dilution technique on specific media. The pathogen population (cfu/g of soil) was corelated with disease incidence.The pathogen pure cultures were stored at 4 ºC for further use.

Detection methods

Blotter Method

The collected seed samples of were analyzed for the presence of seed borne fungal microflora by blotter method given by International rules for Seed Testing (ISTA, 1999). Four hundred seeds were tested for each variety with three replications. Chilli seeds were placed on three layers of moist blotting paper in each glass petridis and incubated at 27 ± 1ºC under 12 hrs light and 12 hrs darkness for 7 days. Each seed was observed under stereo binocular microscope in order to record the presence of fungal colony based on growth habit. The pathogens associated wirth seeds were detected by following the keys given by Mathur and Kongsdal (2003) based the growth characters. Temporary slides were prepared for proper identification of fungi under compound microscope at 10x and 40x and identified with the help of Keys suggested (Booth, 1971). The results were presented as percent incidence of individual pathogen. The fungi from the incubated seeds were transferred to PDA for further confirmation and estimation of frequency of the microflora. The culture was incubated at 27 ± 10ºC for seven days. Per cent frequency (PF) of occurrence was calculated by applying the following formula (Javaid *et al*., 2006). PF = (No. of seeds on which fungus appear/ Total No. of Seeds) x100

Rolled Paper Towel Method

Germination ability of the seeds was determined in the laboratory at room temperature (27 ± 1ºC) by following the method developed by Warham (1990). 400 seeds were randomly taken from each variety and 100 seeds were placed between a pair of moist paper towels by maintaining three replication. The towels were rolled and the ends were closed by threads and covered by polythene paper to prevent drying. After 10 days of incubation, the observations like germination percentage, non germinated seed (hard seed and rotten seed), post-emergence death, shoot length, root length, vigor index and disease incidence were recorded. Vigour of the seedling was determined by using the formula given by Abdul Baki and Anderson (1973).

Vigour Index = (Mean of root length + Mean of shoot length) × Percentage of seed germination

Seedling symptom and transmission studies

Chilli seeds collected from various surveyed areas were further examined by seedling symptoms test. Test tubes (100 x 16 mm) were filled with 5-6 ml of 2 percent water agar and prepared slants. Infected seeds were placed in test tubes (one seed/tube) and incubated at 25 ± 1ºC with 12 h of alternate light and dark conditions. When the germinated seedling reaches the rim of test tube, cotton plug was removed and observation was recorded based on the symptom (Khare, 1996. Both naturally infected and artificially inoculated seed were used for this study.

For transmission studies, 60 seeds (variety: Byadgi dabbi) were sown in pots (20 seeds/pot) containing sterile soil. Plants were kept at glass house with automatic temperature and humidity control system. The pots were watered and observed daily for 15 days and percent germination, seed rot and seedling death were recorded and compared with control. The pathogen from the infected seedling was re-isolated on PDA for the confirmation.

Extraction of Genomic DNA

The isolates of *Fusarium solani* were cultured on potato dextrose agar and subsequently on potato dextrose broth (PDB) at 27 ± 1ºC for 7 days. After incubation, the mycelial mats were harvested by filtration and stored at -80ºC for genomic DNA extraction by following the protocol of Murray and Thompson (1980) with slight modifications. Filtered mycelium (3 g) was ground to a fine powder in liquid nitrogen, transferred to 1 ml of CTAB extraction buffer (0.1M Tris, 1.5 M NaCl, 0.01M EDTA and 2 % CTAB) in a 2-ml tube and kept at 65ºC for 90 minutes with occasional stirring. An equal volume of chloroform: isoamyl alcohol (24:1) was added to all and the centrifuged at 10,000 rpm for 20 min at 4ºC. The upper supernitant was pippetted out and transferred to a fresh tube and mixed with 100 μl of sodium acetate (3 M) and 600μl of ice-cold isopropanol and centrifuged at 10,000 rpm for 10 min at 4ºC. The pellet was washed with 70 % ethanol, dried at room temperature and finally dissolved in $T_{10}E_1$ (10 mM Tris–Cl, pH 8.0; 1 mM EDTA) for further use. The DNA was quantified using spectrophotometer and quality analysis was done on 1.0 % agarose gels.

Primers and PCR conditions.

The TEF-1*á* gene was amplified using specific primers Fs4-f (5′ATCGGCCACGTCGACTCT3′) and Fs4 (5′GGCGTCTGTT GATTGTTAGC3′) repored by Abd-Elsalam *et al.,* 2006 and

Arif *et al.,* 2012. The primers were synthesized by Bangalore Genie Pvt Ltd. Bangalore and supplied as lyophilized products of desalted oligos. PCR amplification was carried out in a final volume of 20 ì l, which consisted of 2X PCR assay buffer with 15 mM $MgCl_2$ - 2 μl, 2.5 mM dNTPs mixture 1μl, , Primer (5PM/µl) 1 μl forward and reverse each, *Taq* DNA polymerase (6.0U μl^{-1}) (Bangalore Genei, India) -0.5 μl , and Template DNA (25 ng/µl)-1 μl . All the reactions were carried out in a Corbett Research gradient thermocycler (96 well system) supplied by M/s JH BIO Innovation Pvt. Ltd R.T. Nagar, Bangalore. The PCR reactions were carried out with an initial denaturation at 95ºC for 5 min, followed by 40 cycles of denaturation at 90ºC 1 for min, annealing at 58ºC for 1 min, 72ºC for 2 min, and final extension at 72ºC for 20 min. The amplified products were resolved by electrophoresis in 1.2 % agarose gels with 1X TBE (Tris Borate EDTA) buffer, 0.5 μg ml^{-1} of ethidium bromide and loading buffer (0.25% Bromophenol Blue in 40% sucrose). Four μl of the loading dye was added to 20 μl of PCR product and loaded to the agarose gel. Electrophoresis was carried at 75 V for 1 h. The gel was visualized under UV light, and photographed with a gel documentation unit (Syngene Inc., Cambridge, UK).

RESULTS AND DISCUSSION

Survey and identification of hot spots for the chilli wilt

Seedborne pathogens posses a serious threat to better crop health and establishment. The pathogens in close association with seeds making it niche for its long-term survival, introduction into new areas and widespread dissemination of pathogens. Survey during 2012-13 and 2013-14 for chilli wilt disease is presented in Table 1. The data indicated that, the maximum disease incidence was recorded in Bellary district of Karnataka (PDI: 29.48%) and Kurnool district of Andhra Pradesh (PDI: 34.90%) (Fig-5). A significant disease incidence and crop losses also recorded from Raichur, Koppal, Haveri and Belgaum districts of Karnataka, Guntur and Ananthpur districts of Andhra Pradesh states. These areas are identified as hot spots for disease incidence. The disease incidence also varied with variety to variety with maximum disease incidence was recorded in byadgi dabbi (range: 30-80%) followed by sitara (20-60 %). A possitive correlation between disease incidence and pathogen population was observed in all the surveyed places with maximum average population 13.2 x 10^{-5}/g of soil was recorded from these hot spots indicating the direct relation between disease incidence and pathogen population

Identification of microflora associated with chilli seeds and detection of seed borne *F. solani*.

Chilli fusarium wilt is becomimg more and more serious in traditional chilli growing areas of south India. Its early and exact detection and diagnosis is very first step to ensure efficient disease management. Careful examination of uprooted, plantsmust be confirmed for the absence of external root symptoms and presence of dark-brown discoloration in xylem tissues of roots and stem will help in disease diagnosis.

Standard blotter method

A total of 40 seed samples were analysed in this study to asses the frequency of microflora associated with seeds by employing conventional seed heath testing methods as decribed in material and mathods. In all the samples the common microflora observed was *Fusarium solani, Collectotrichum* spp., *Aspergillus* spp., *Mucor* and *Rhizopus* spp. The frequency of *F. solani* was more with 10-56.50 percent followed by *Colletotrichum* spp. with 5-15 per cent (Table 2). *Fusarium solani* caused white mycelium growth on the seed and the microscopic observations reveal that, it produced microconidia with single cell ovoid shape, macroconidia with 3-4 septa, sickle shaped with blunt ends. Chlamydospores were formed either solitary or in chain at the middle or end of the mycelium. These morphological characters confirmed the infection of *F. solani* in seeds.

Rolled paper towel method

The germination percentage and vigoure index was significantly reduced when tested against *F.solani*. The germination percentage of *F. solani* infected seeds was significanly low (10 %)when compared with hot water treated seeds (97.50%) which was taken as control (Table 3). The seeds which show low incidence of *F. solani* shown higher incidence of *Aspergillus* and *Penicillium* spp. a significant frequence of *Colletotrichum* spp. aslo observed resulting in poor germination and seedling vigour index (Table 3). The decline in the seed gemination and seedling vigoure are due to the toxins produced by these pathogens. Many workers

Figure 1: Disaese free field

Figure 2: Completely infected field.

Table 1: Average disease incidence of chilli wilt disease in major growing states of south India.

State	District	Taluk	Per cent disese incidence		
			2012-13	2013-14	Pooled
	Bagalakot	Kaladgi	-	8.5	8.5
		Mean	-	8.5	8.5
Karnataka	Belagavi	Saundatti	6.76	8.65	7.7
		Bailahongal	17.07	24.82	20.94
		Mean	11.91	16.73	14.32
	Bellary	Hospet	12.25	13.38	12.81
		Siraguppa	34.42	49.93	42.18
		Bellary	28.31	46.49	37.4
		Kampli	21.73	29.38	25.55
		Mean	24.18	34.79	29.48
	Chickmagaluru	Tarikere	7.23	-	7.23
		Kadur	5	-	5
		Birur	6	-	6
		Mean	6.08	-	6.08
	Chitradurga	Hosdurga	8.58	7.82	8.2
		Chitradurga	8.36	10.23	9.29
		Hiriyur	11.33	13.75	12.54
		Mean	9.42	10.6	10.01
	Davangere	Davangere	6.02	8.5	7.26
		Jagaluru	8.49	10.75	9.62
		Mean	7.25	9.62	8.44
	Dharwad	Dharwad	11.3	14.23	12.76
		Hubli	6.44	10.82	8.63
		Kundgol	10.07	11	10.53
		Kalghatgi	5.88	13.25	9.56
		Navalagund	5.8	7.31	6.56
		Mean	7.9	11.32	9.61
	Gadag	Gadag	7.18	8.19	7.68
		Nargund	9.67	13.09	11.38
		Mean	8.42	10.64	9.53
	Haveri	Haveri	20.07	27.94	24
		Ranebennur	11.98	22.63	17.3
		Byadgi	25.57	37.62	31.6
		Hirekerur	13.38	18.88	16.13
		Hanagal	8.5	10.25	9.38
		Shiggaon	9.51	16.88	13.19
		Mean	14.83	22.36	18.6
	Koppal	Koppal	20.3	27.08	23.69
		Mean	20.3	27.07	23.69
	Raichur	Sindnur	9.38	19.13	14.25
		Manvi	13.86	26.75	20.31
		Raichur	26.69	38.49	32.59
		Mean	16.64	28.12	22.38
	Uttara Kannada	Halyala	25.25	-	25.25
		Joida	20	-	20
		Mean	22.63	-	22.63
Andhra Pradesh	Kurnool	Mantralaya	26.01	34.88	30.44
		Emmiganur	29.12	41.15	35.13
		Adoni	32.52	45.72	39.12
		Mean	29.21	40.58	34.9
	Guntur	Sattanapalli	25.33	34.03	29.68
		Guntur	21.97	28.85	25.41
		Mean	23.65	31.44	27.54
	Ananthpur	Ananthpur	19.34	27.88	23.61
		Mean	19.34	27.88	23.61
Maharashtra	Solapur	Pandrapur	6.13	9.57	7.85
		Sangola	5.81	8.07	6.94
		Mean	5.97	8.82	7.39
	Grand mean		14.75	21.06	17.36
	Standard deviation		8.65	12.66	10.47

carried out investigations and detected various pathogen microflora associated with chilli seeds such as, *Collectotrichum capsici*, *Curvularia lunata*, *Fusarium solani*, *Alternaria* spp. *F. oxysporum* (Mridha and Siddique, 1989; Hasmi, 1989). Similar reports were given by the studies of Solanke (2001) and Asamol (2001) along with other pathogens such as *Aspergillus flavus*,

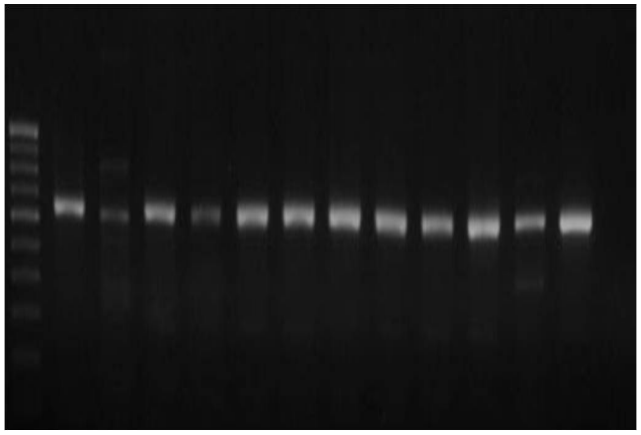

Figure 3. Amplification pattern of TEF 1a gene specific to Fusarium solani from seed samples of different cultivars. Lane1-Marker 100 kb, 1-Byadgi dabbi, 2-Sitara, 3-Nayanatara, 4-Gayathri, Guntur local, 6-Byadgi kaddi, 7-Uttal

collected from surveyed area were sown in pots (20 seeds/ pot) containing sterile soil. Plants were kept at glass house with automatic temperature and humidity control system. The pots were watered and observed daily for 15 days and percent germination, seed rot and wilt incidence were recorded and compared with control. The infected seeds showed lower germination (10.25-50.25) and higher seedling mortality (5-60%). Where as the germination (98.50%) was more and seedling death (2.50%) was very low in healthy control treatment.

Detection of *F. solani* in chilli seeds and rhizospheric soil by Specific amplication of *Fusarium solani* using TEF 1α.

Earlier it was believed that seed borne pathogens could be only detected only by conventional methods like, plating the seeds on blotters or on PDA. But with the advent of PCR based methods, it became very easy to detect seed borne pathogens in a mixed and contaminated infections by designing the specific markers. This early and quick detection methods are more amd more useful in quarantine stations in order to check the entry of new pathogen thereby minimise the losses due to possible outbreaks.

Table 2 : Incidence and frequency of *Fusarium solani* in seed samples collected from different parts of south India by Standard Blotter method

Sl No	Place of collection	Sources	Variety	Germi nation (%)	% incidence of *F. solani*
1	Kurgod (Bellary, KAR)	Farmer's field	Byadgi kaddi/ B. dabbi	16.5	60
2	Nelahal (Raichur, KAR)	Farmer's field	Byadgi kaddi/ B. dabbi	10.25	52.25
3	Basavapura(Koppal, KAR)	Farmer's field	Byadgi kaddi/ B. dabbi	25.75	35.25
4	Shidenur (Haveri, KAR)	Farmer's field	Byadgi kaddi, B. dabbi, Sitara, Nayanatara	40.25	30.25
5	Inchal (Belgaum, KAR)	Farmer's field	Sitara, Nayanatara	15.1	25.25
6	Amminabavi(Dharwad, KAR)	Farmer's field	Sitara, Nayanatara	26.25	4.025
7	UAS Dharwad (KAR)	UAS horticulture field	Byadgi Dabbi, Byadgi Kaddi, GPM-9	25.5	10.25
8	Betageri (Gadag, KAR)	Farmer's field	Byadgi kaddi/ B. dabbi	30.25	40.5
9	Kattavaripalem (Guntur, AP)	Farmer's field	Guntur local, Gayathri	65.5	13.25
10	Madire (TS)	Farmer's field	Dabbi, Hybrids, Guntur, Brahma	50.25	12.25
11	Emmiganur (TS)	Farmer's field	Dabbi, Hybrids, Guntur	25.52	30.52
12	Pandrapura (MH)	Farmer's field	Dabbi, Hybrids, Guntur	75.5	5.5

Table 3 : Germination percentage of chilli seeds infected by *Fusarium solani*

Treatment	Seed rot	Root rot	Healthy seedlings	Germination percentage	Seedling Vigoure index
Infected	160	20	20	10	180
Control (hot water treated)	0	0	195	97.5	9750
percentage	80	10	10		

A. niger, *Rhizopus stolonifer*. Varied frequency of microflora associated with infected chilli seeds were reported by Padaganur and Naik (1991). They reported *Colletotrichum capsici* (75.5 %), *Fusaium* spp (16.25%) and *Alternaria* spp. (5 %).

Seedling symptom and transmission test.

Seedling syptom test

Selected byadgi dabbi seed samples collected from surveyed locations subjected to seedling syptom test under two percent water agar in test tubes. The germinated seedling shown wilting symptom 30 days after inoculation. The syptoms were observed as gradual wilting and drooping of leavs. Later the plants died. The dead plants were cut into small bits and placed on PDA to confirm *F. solani* infection.

For transmission studies, seeds of variety byadgi dabbi

Based on survey data for the disease incidence and severity, seven popularly growing genotypes and 12 rhizospheric soil samples from hot spots were collected and analysed for seed and soil borne nature of *F. solani* using *Fusarium solani* species specific primer sets Fs4-f (5'ATCGGCCACGTCGACTCT3') and Fs4 (5'GGCGTCTGTTGATTGTTAGC3').The total genomic DNA from the pathogen isolated from infected seed and soil was used as a template. An amplification product of A 658 bp region of TEF1α gene specific to *Fusarium solani* (Fig. 3 & 4) confirmerd that the pathogen is seed as well as soil borne. This PCR based detection will healp in early detection of the pathogen and avoids further losses. Detection of *Fusarium solani* based on morphological discrimination requires special

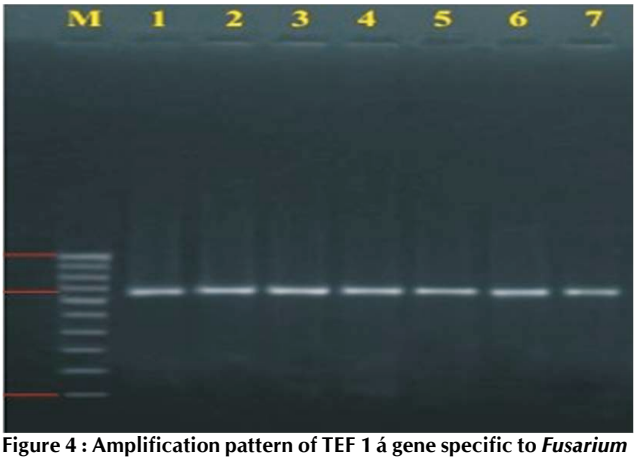

Figure 4 : Amplification pattern of TEF 1 á gene specific to *Fusarium solani* from Rhizosphere soil of infected field. Lane 1: Marker 100 KB, 1 Kurgod (Bellary, KAR), 2 Nelahal (Raichur, KAR), 3 Basavapura, (Koppal, KAR), 4 Shidenur (Haveri, KAR), 5 Inchal (Belgaum, KAR), 6 Amminabavi(Dharwad, KAR), 7 UAS Dharwad (KAR), 8. Betageri (Gadag, KAR), 9.Kattavaripalem (Guntur, AP), 10 Madire (TS), 11 Emmiganur (TS), 12 Pandrapura (MH).

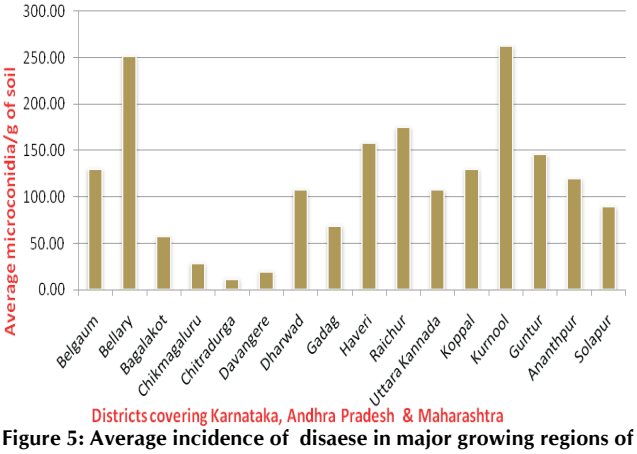

Figure 5: Average incidence of disaese in major growing regions of karnataka, Andhra Pradesh, Telangana state and Maharashtra

skill and the expertise of taxonomists or specialists. Arif *et al.* (2012) carried out the detection of *F. solani* causal agent of wilt and rots in many plant species using PCR based methods by amplification of target genes such as, internal transcribed spacer (ITS), rDNA and transcription elongation factor (TEF-1α). As there is a saying in sanskrith *"Subheeje sukshetre sampadhe jayathe"* which means healthy seeds in disease free soil will germinate and establish well and ultimately gives good yield. So right selection of seeds become very important. Confirmation of disease free seeds before sowing by conventional and molecular detection will minimises further crop losses.

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