Pathogenicity and Molecular analysis of *Fusarium* isolates in Nendran and Grand Naine

ABSTRACT

Fusarium wilt, a devastating disease, poses a significant global threat to the banana industry. This study is aimed to assess the host susceptibility and pathogenicity of *Fusarium* isolates in tissue-cultured Nendran and Grand Naine banana varieties. Eight isolates were acquired from the Department of Plant Biotechnology at Tamil Nadu Agricultural University, Coimbatore. Among the eight isolates tested, S-6, S-32G, S-56 and S-67 produced disease symptoms in both Nendran and Grand Naine varieties. Subsequently, the pathogen was reisolated from infected tissues displaying vascular discoloration. While the isolate S-6 produced 66.67% of disease incidence in both the varieties, S-32G, S-56 and S-67 produced 100% disease incidence in Nendran and Grand Naine. Molecular analysis confirmed the presence of the *Fusarium oxysporumf.sp. cubense*pathogen, with an amplicon observed at 260 bp. Furthermore, non-pathogenic isolates may potentially exert direct or indirect effects on pathogenic strains, offering prospects for disease control.

Keywords: *Fusarium wilt, disease incidence, tissue culture plants of Grand Naine and Nendran, non-pathogenic Fusarium strains, disease control.*

1. INTRODUCTION

Banana is an important cash and food crop in tropical and subtropical regions. India is the largest banana producer in the world and it produced about35.365 million metric tonnes [1]*Fusarium* wilt, a highly destructivedisease caused by *Fusarium oxysporumf.sp. cubense (Foc)*presents a significant global threat to the banana industry.*Foc* can be categorized into four distinct physiological races, namely race 1, 2, 3, and 4. Race 1 is known to target banana cultivars such as 'Gros Michel' (*Musa* sp. AAA group), 'Pome,' 'Silk,' and 'Pisang Awak' (Musa sp. AAB group). Race 2 primarily affects the 'Bluggoe' cultivar and those closely related to it. In contrast, race 3 does notinfect any *Musa* species. Conversely, race 4 having wide range of host compatibility, affecting almost all cultivars, such as 'Dwarf Cavendish' (*Musa* sp. AAA group), along with the cultivars vulnerable to races 1 and 2 [2].The pathogen invades the roots of banana plants, occupies and blocks the xylem vessels leading to reddish-brown discoloration in the rhizome and pseudo stem. As the infection progress, the leaves of banana plants will show yellowing, wilting and collapsing around the pseudo stem.Banana plants affected by disease frequently perish prior to the formation of bunches, leading to a decline in overall yields ultimately [3].Plants produced through tissue culture methods are typically disease-free, having consistent growth patterns, and yield more and earlier compared to plants propagated from suckers or rhizomes [4]. The use of pathogen-free propagation material,

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such as that obtained through tissue culture, in uncontaminated soil allows for the cultivation of susceptible clones. The primary approaches for controlling *Fusarium* wilt involve the use of chemical soil fumigation and the cultivation of resistant cultivars.Currently, there are no viable long-term chemical or physical methods for disease control. While there is knowledge of soils that naturally suppress the disease, there have been no reports of converting disease-prone soil into a disease-suppressive state. Efforts in biological and cultural control studies have yielded unsatisfactory results thus far [5]. The elite cultivar namely Grand Naine and variety Nendran are popularly cultivated in the Western Ghat regions of Tamil Naduas Nendran being used in culinary(chips making) purpose and Grand Naine having a great export potential in the banana market.Its reaction against wilt disease in the tissue culture plants are required to understand the host susceptibility and its pathogenicity. Therefore, the present investigation was made to study the *Fusarium* isolates against tissue culture banana plantlets of Nendran and Grand Naine.

2. MATERIALS AND METHODS

2.1. *Fusarium* isolates

Eight*Fusarium oxysporumf.sp. cubense*(*Foc)*isolates were obtained from Department of Plant Biotechnology, Centre for Plant Molecular Biology and Biotechnology, Tamil Nadu Agricultural University, Coimbatore for the study of host susceptibility and its pathogenicity.

2.2. Morphological characterization

Pure cultures of *Fusarium* isolates grown in PDA medium were observed for the phenotypic characters including colony characteristics such as growth, colour of mycelium and pigmentation if any. For microscopic characteristics such as presence of macroconidia and microconidia, three to four 9 mm discs of pure culture of *Fusarium* isolates were transferred to 100 ml of PDA broth and incubated at28 ± 2°C. After 10 days of incubation, 10 µl of *Fusarium* cultures were placed over the glass slide andobserved under phase contrast microscope(iScopeEuromex) for the presence of macroconidia and microconidia at 10x and 40x magnification.

2.3. Pathogenicity assay

2.3.1. Plant material

Two months old primary hardened tissue cultured plants ofGrand Naine (AAA) and Nendran (AAB)varietieswere procured from SPIC(Southern Petrochemical Industries Corporation Limited), Coimbatore. The obtained plants were maintained under greenhouse conditions for secondary hardening for a week.

2.3.2. Inoculum preparation

Pure cultures of *Fusarium* isolates grown in PDA medium were inoculated into 250 ml conical flask consisting of 150 ml Potato Dextrose broth followed by incubation cum shakingat 25±2° C and 130

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rpm for 8 days[6]. After incubation period, the conidial (micro and macro) load in the PD broth was observed under microscope using Haemocytometer slide and then adjusted to 1 x10^6 conidia/ml.

2.3.3. Inoculation procedure of *Fusarium* in banana plants

The plant roots were trimmed and injury was made by using sterile tooth pick sticks in the rhizome part and root hairs of the banana plants. Then the plants were dipped in *Fusarium*spore suspension containing conidial concentration of 10^6 spores/ml for 30 minutes so that the spores can attach to the roots and further the entry of spores into the plants will be facilitated through the injury made in the roots. Then the plants were replanted into the pots containing sterile coir pith[6].6 plants per isolate (3 plants for Grand Naine and 3 plants for Nendran) were inoculated for each trial and the experiment was repeated thrice. After 3 hours of replanting, watering was done using rose can and then the plants were watered on alternate days. To avoid nutrient deficiency, all 19 (N:P: K: 19:19:19) was given at concentration of 8grams/litre of water one day prior to inoculation and then followed at one week interval from the day of inoculation. Disease assessment was done at 15 and 22 DAI (Days After Inoculation) i.e., after the occurrence of first visible symptom(yellowing). During the assessment, the plants wereobserved for the *Fusarium* wilt symptoms including yellowing, wilting andvascular discolouration of rhizome and pseudo stem.

2.4. Wilt disease reaction and incidence

The*Fusarium*infected plants were observed for the appearance of first visible symptom (yellowing), wilting of base leaves and the percentage of disease incidence and the area of rhizome discolouration. The discolouration was evaluated as 25%, 50%, 70% and 100% for the infected plants. The disease incidence was calculated using the formula [7],

$$\text{Disease incidence (\%)} = \frac{No.\,of\ seedlings\ exhibited\ symptoms}{\text{No. of seedlings inoculated}}\, x\ 100$$

2.5. Re-isolation of *Fusarium* pathogen

The *Fusarium* pathogen was reisolated from the infected tissues of banana plantlets showing wilting symptom. The roots along with the pseudo stem of banana plants showing wilting symptoms were collected and washed thoroughly to remove the soil debris. Then the small pieces(5-8mm long) of infected tissues are surface sterilized using 1% sodium hypochlorite for 30 seconds and washed thoroughly thrice in series of sterile distilled water to remove the residues of sodium hypochlorite for 1 minute. Sterilized tissues were placed on PDA medium petri plates containing Streptomycin (50µg/ml). The plates were incubated at 28±2ºC for 4 days and observed periodically for the growth of colonies. The single hyphal tip method was employed to purify the pathogen.

2.6. Molecular confirmation of *Fusarium* isolates from wilted plants

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2.6.1. Isolation of genomic DNA from *Fusarium* isolates

Genomic DNA from *Fusarium* strains was obtained from single-spore cultures during the late log phase(5-7days), which were grown in PDB (Potato Dextrose Broth) at 25 ± 2 °C in the absence of light and shaking [8].Mycelia were collected in sterile filter paper and powdered using pestle and mortar using liquid nitrogen. The extraction was carried out by CTAB method [9],using phenol-chloroform-isoamyl alcohol mixture, subsequent to digestion with proteinase K and RNase. The DNAsamples were suspended in low TE buffer (10 mM Tris-HCl - pH 8.0; 0.1 mM EDTA).

2.6.2. Molecular confirmation of *Fusarium* isolates

The extracted genomic DNA from the purified cultures of reisolated pathogen was used as a template for PCR reaction.For PCR amplification, 2X Master mix (Smart Prime) was used in 20 µL reaction mixture (10 µL of 2X Master mix, 7 µL of nuclease free water, 1 µL of forward primer(1µM), 1 µL of reverse primer (1 µM)and 1 µL of template DNA (50 ng)).The primer used for the confirmation of *Fusarium oxysporumf.sp. cubense*was SIX9_Foc_F (5'ATCGCTGAAGCCCAGAACAA'3)andSIX9_Foc_R (5' TTCTGTCCGTCGATCGTTCC'3).PCR cycling conditions included an initial denaturation step for 3 min at 95ºC followed by 30 cycles of denaturation (95ºCfor 15 s) - annealing (58ºC for 15 s) - extension (72ºCfor 10 s) followed by a final extension of 72ºC for 10 min[10]. The presence of PCR amplicon size of 260 bp was considered as positive for *Foc* infection.

2.7. Statistical analysis

Thisgreenhouse experiment follows a completely randomized design (CRD).The disease incidence was calculated using the formula mentioned above (2.4.). The One-way analysis of variance (ANOVA) was conducted using R software (version 4.3.1) and the significant difference between means were identified by Tukey HSD test.

3. RESULTS AND DISCUSSION

3.1. Colony morphology

The colony appearance of 8 *Fusarium* isolates were observed as sparse and complete, flat or raised, white fluffy mycelial growth varied with light pinkish and violet tinges (Fig.1) (Table.1). Aerial white cottony mycelial growth was reported in *Foc* isolate [11]. Microscopic investigation of these *Fusarium* isolates confirms the presence of oval/elliptical shaped microconidia and cylindrical, elongated to sickle shaped macroconidia in all the eight isolates (Fig.1) (Table.1).

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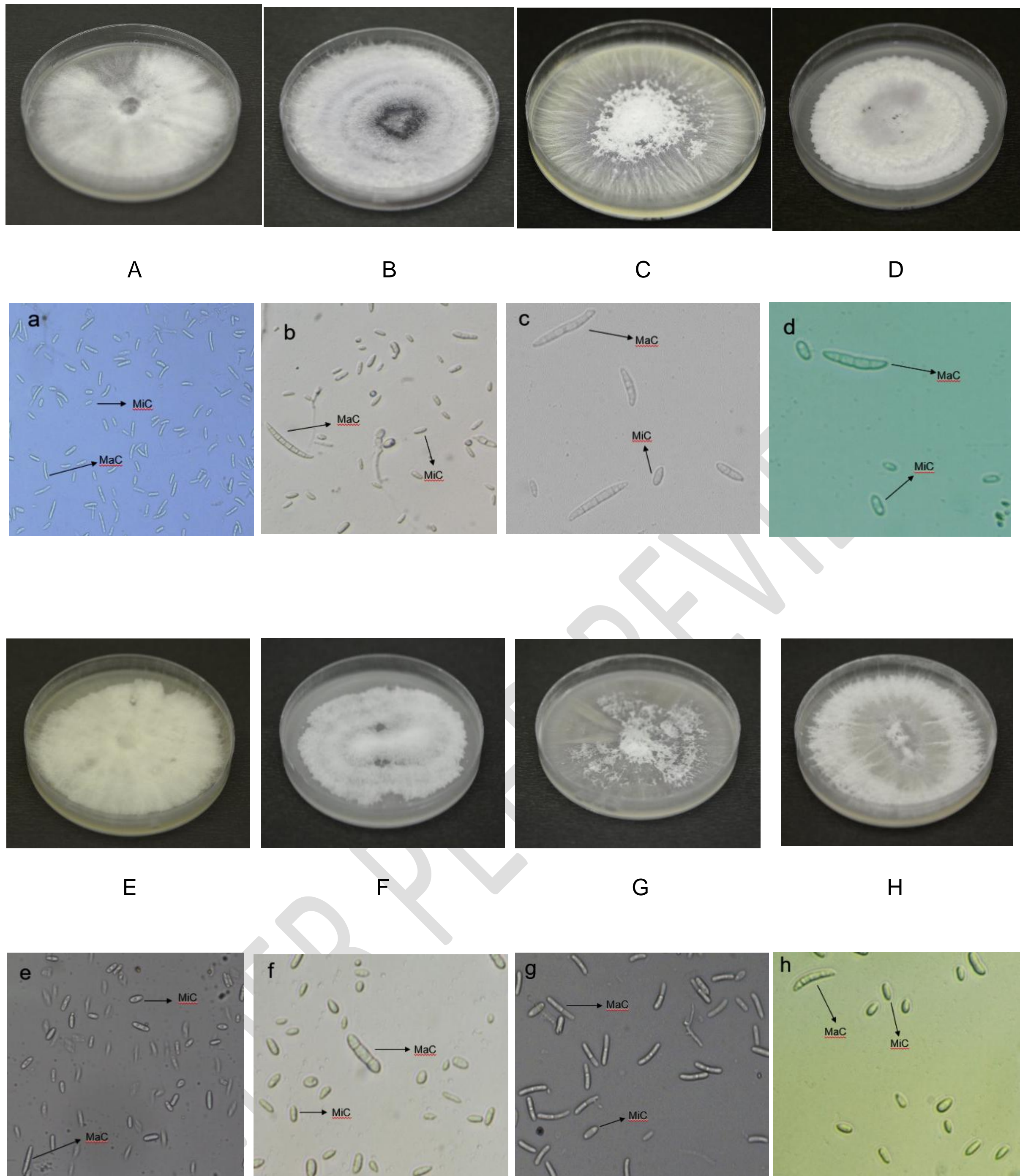

Fig.1. Colony morphology of different *Foc* isolates

A, B, C, D, E, F, G, H – colony appearance of Foc isolates S6, S32G, S56, S67,S35, S39, S53 and S63 respectively; a,b,c,d, e, f, g, h – Microscopic spore image of Foc isolates S6, S32G, S56, S67, S35, S39, S53 and S63 respectively; MiC – microconidia;MaC – macroconidia

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Table 1. Colony morphology of *Foc* isolates

S.no.	Isolates	Colony appearance	Spore characters	
			Microconidia	Macroconidia
1.	S-6	White fluffy aerial mycelial growth with light pinkish tinge	Oval/Elliptical	Curved and cylindrical shaped macroconidia with single septation
2.	S-32G	White fluffy aerial mycelial growth with violet tinges	Oval/Elliptical	Sickle shaped macroconidia with three septations
3.	S-35	White aerial cottony and fluffy mycelial growth	Oval/Elliptical	Cylindrical and elongate shaped macroconidia with single septation
4.	S-39	White fluffy and cottony aerial mycelial growth with violet tinges	Oval/Elliptical	Cylindrical shaped macroconidia with three septations
5.	S-53	Sparse white aerial mycelial growth with pinkish tinge	Oval/Elliptical	Curved and cylindrical shaped macroconidia with single septation
6.	S-56	Sparse white aerial mycelial growth with pinkish tinge	Oval/Elliptical	Fusiform and elongate shaped macroconidia with single septation and blunt ends
7.	S-63	white sparse cottonyaerial mycelial growth	Oval/Elliptical	Curved and elongateshaped macroconidia with three septation
8.	S-67	Raised white cottony and fluffy aerial mycelial growth with violet tinge at the centre	Oval/Elliptical	Fusiform shaped macroconidia with three septations

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Slightly curved and fusiform shaped macroconidia were reported in *Foc* TR4 isolate [12].Falciform macroconidia with average of 4-5 septations and elliptical to cylindrical shaped microconidia with 2-3 septations are reported in the isolates of *Fusarium spp*.[13] which were identified as *Fusarium oxysporum*[14].

3.2. Pathogenicity of *Foc* against Grand Naine and Nendran

The first pathogen infected symptom (yellowing) was observed 15 DAI(Days After Inoculation) in both Grand Naine(Table 2) and Nendran(Table 3). The days taken for the wilting of base leaves was 22 DAIin both Grand Naine(Table 2) and Nendran(Table 3). In this experiment, the wilting symptoms developed after 3 weeks which is earlier by one week, as against 4 weeks period reported for the development of infection by Thangavel et al. [15].The disease severity was measured in susceptible banana variety after 50 percent yellowing symptoms occurred and the duration ranged from 6-12 weeks post inoculation [16].In our study, we assessed the severity of the disease in 3 weeks post inoculation.

The *Fusarium* isolates inoculated in banana plants of Grand Naine and Nendran were assessed based on the percentage of disease incidence. Out of 8 isolates, only 4 *Fusarium* isolates *viz.*, *Foc* S-6, *Foc* S-32G, *Foc* S-56 and *Foc* S-67 have shown wilt symptoms(yellowing, wilting and vascular discolouration)(Fig.2).In these banana varieties, the isolates S-35, S-39, S-53 and S-63 did not produce any disease symptom which was validated after three trials. So, these isolates may be non-pathogenic to these varieties. This non-infection may also be due to random mutagenesis that might have occurred during repeated subculturing of the pathogen. But this needs to be validated by sequencing.

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Fig.2. Pathogenicity assay of different *Foc* isolates in Grand Naine and Nendran

A1, B1, C1, D1 – yellowing and wilting symptoms of Foc isolates S6, S32G, S56, S67 in Grand Naine respectively; A2, B2, C2, D2 – yellowing and wilting symptoms of Foc isolates S6, S32G, S56, S67 in Nendran respectively; GN- control plants of Grand Naine; N- control plants of Nendran; a,c,e,g – vascular discolouration of Foc isolates S6, S32G, S56, S67 in Grand Naine respectively; b,d,f,h - vascular discolouration of Foc isolates S6, S32G, S56, S67 in Nendran respectively.

Nel B *et.al.*[17] reported that from the isolated 60 *Fusarium* isolates, only one isolate proved to cause wilt symptoms and others reported as non-pathogenic isolates. The area of rhizome discolouration was evaluated based on the percentage of discoloured area. S-56 showed 50% discolouration in 2 replicate and 100% discolouration in one replicate while S-67 have shown 50% discolouration for all the three replicates in Grand Naine. In Nendran, the isolate S-56 exhibited, 25%, 50% & 75% for 3 replicates and the isolate S-67 imparted 50%(2 replicates) & 70%(1 replicate). S-32G has exhibited 25%, 50% & 75% in three replicates in Grand Naine and 50%, 75% & 100% for 3 replicates in Nendran. Out of 3 replicates, S-6 has shown50%(1 replicate) and 75%(1 replicate) discolourationin Grand Naine and in one replicate of Grand Naine, no rhizome discolourationoccurred. For S-6, about 25%(2 replicates)discolouration was observed in Nendran and in 1 replicate, no rhizomediscolouration observed(Table 2&3).The percent disease incidence of *Foc*S-6 and *Foc* S-32G, *Foc* S-56 and *Foc* S-67 in banana plants of Grand Naineand Nendran variety were recorded as 66.67% and 100% respectively (Table 2& Table 3). No yellowing and wilting symptoms were noticed in Foc S-35, Foc S-39, Foc S-53 and Foc S-63(data not shown).

Table 2. Assessment of *Fusarium* isolates for pathogenicity in Grand Naine

S.No.	Culture ID	Days taken & Incidence (%)			Area of Rhizome discolouration/no. of seedlings			
		For the 1st symptom appearance	For the wilting of leaves	% incidence at 22 DAI	25% cut surface	50%	75%	100%
1	S-6	15 DAI	22 DAI	66.67(59.45)[b]	0	1	1	0
2	S-32G	15 DAI	22 DAI	100(88.35)[a]	1	1	1	0
3	S-35	NS	NS	0(1.65)[c]	0	0	0	0
4	S-39	NS	NS	0(1.65)[c]	0	0	0	0
5	S-53	NS	NS	0(1.65)[c]	0	0	0	0
6	S-56	15 DAI	22 DAI	100(88.35)[a]	0	2	0	1
7	S-63	NS	NS	0(1.65)[c]	0	0	0	0
8	S-67	15 DAI	22 DAI	100(88.35)[a]	0	3	0	0
9	Control	NS	NS	0(1.65)[c]	0	0	0	0

Figures in parentheses are arcsine transformed values of percentages. Values followed by the same letters in a column are not significantly different by the Tukey Honestly Significant Difference (HSD) test with α = 0.05 (F = 68.61; df = 8, 18; P< 0.001); NS - No symptom

Table 3. Assessment of *Fusarium* isolates for pathogenicity in Nendran

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S.No.	Culture ID	Days taken & Incidence (%)			Area of Rhizome discolouration/no. of seedlings			
		For the 1st symptom appearance	For the wilting of leaves	% incidence at 22 DAI	25% cut surface	50%	75%	100%
1	S-6	15 DAI	22 DAI	66.67(59.45)[b]	2	1	0	0
2	S-32G	15 DAI	22 DAI	100(88.35)[a]	0	1	1	1
3	S-35	NS	NS	0(1.65)[c]	0	0	0	0
4	S-39	NS	NS	0(1.65)[c]	0	0	0	0
5	S-53	NS	NS	0(1.65)[c]	0	0	0	0
6	S-56	15 DAI	22 DAI	100(88.35)[a]	1	1	1	0
7	S-63	NS	NS	0(1.65)[c]	0	0	0	0
8	S-67	15 DAI	22 DAI	100(88.35)[a]	0	2	1	0
9	Control	NS	NS	0(1.65)[c]	0	0	0	0

Figures in parentheses are arcsine transformed values of percentages. Values followed by the same letters in a column are not significantly different by the Tukey Honestly Significant Difference (HSD) test with α = 0.05 (F = 68.61; df = 8, 18; P< 0.001); NS - No symptom

The pathogen reisolated from the infected tissues of wilted banana plants were used for confirmation. The primers employed targeted the homolog 'a' of gene *SIX9*. The amplification of 260 bp confirmed as that the isolates were *Fusarium oxysporumf.sp. cubense*(Fig.3) which further proved the Koch's postulates.Based on the data currently at hand, it appears that homolog 'a' of SIX9 gene is found within *Foc* (*Fusarium oxysporum f.sp.cubense*) and absent in other formaespeciales of*F. oxysporum* [18].

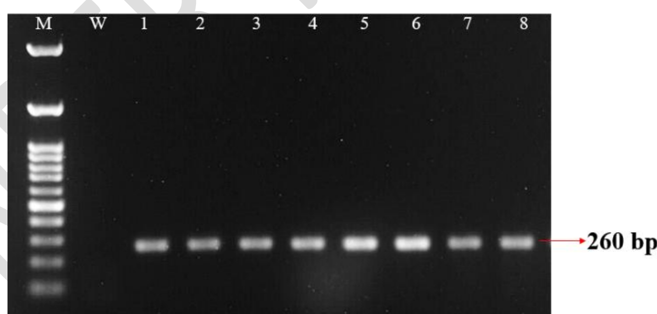

Fig.3.Amplification of *Fusarium* isolates with *Foc*-specific primer

Lane M: 100bp ladder,LaneW: Negative control, Lane1-8: Foc S-6, S-32G, S-35, S-39, S-53, S-56, S-63, S-67 respectively.

4. CONCLUSION

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The pathogenicity test conducted with the eight isolates revealed that only four of themwere pathogenic as evidenced by the occurrence of the disease symptoms. Among these four pathogenic isolates, variation was observed in the severity of the expression of the symptoms.The strains S-32G, S-56 and S-67exhibited severe symptoms while in S-6strain, the expression of symptom was less. The variation in the ability of the *Foc* strains to cause infection and the severity of the infection can be deciphered through genome analysis of the pathogen, host and pathogen-host interactions. The non-pathogenic strains are increasingly utilized as a biocontrol and they exhibit diverse modes of action that contribute to their biocontrol capabilities. They engage in nutrient competition within the soil, influencing the chlamydospore germination rate of the pathogen. Additionally, these strains compete for infection sites on the root and can instigate plant defense reactions, leading to the induction of systemic resistance. The significance of these mechanisms is strain specific [19].Co-infection studies of the identified non-virulent strains along with a virulent strain is ongoing to assess the feasibility of employing these non-virulent strains for biocontrol agent in Grand Naine and Nendran.

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