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PHENOTYPING ASSAYS FOR PATHOGENICITY DETERMINATION OF *FUSARIUM OXYSPORUM* F. SP. *CUBENSE* TROPICAL RACE 4

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ABSTRACT

Fusarium oxysporum f. sp. *cubense* (*Foc*) is a lethal pathogen affecting banana plants. To manage this pathogen, resistance exploration, along with exclusion and eradication, is crucial. A simple and reproducible method of assessing pathogenicity is essential for identifying resistant candidate genotypes. In this study, we evaluated the pathogenicity of *Foc* tropical race 4 (TR4) on tissue-cultured banana plants (cv. NIGAB-1 and Grand Naine) using three inoculation methods: the pouring method (PM), the chlamydospore method (CM), and the kernel method (KM). The latency period for both varieties inoculated by the chlamydospore method was two weeks after inoculation (wai), whereas plants inoculated using the pouring and kernel methods showed symptoms after three weeks. Disease severity findings revealed significant differences at the end of the assessment period among the different inoculation methods. The pouring method proved to be more effective than the other two methods, yielding consistent, repeatable results with dose-dependent final disease severity. This method can be employed to evaluate the pathogenicity of *Foc* (TR4) and assess various disease control methods to identify effective solutions.

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INTRODUCTION

The fungus *Fusarium oxysporum* f. sp. *cubense*, which is responsible for Panama wilt of banana, is widely regarded as one of the most destructive pathogens in the world (Maymon *et al.*, 2020). By the 1950s, a disease eradicated the banana industry based on CV. Gros Michel which was extremely susceptible to widely prevailed Race 1 of *Foc*. This resulted from the switch of growers from Gros Michel cultivations with the resistant Cavendish, having somehow inferior characters. This was the end of the Panama disease with the end of the Gros Michel industry. Unfortunately, after 3 decades a new race named as *Foc* (TR4) was reported that begin to infect previously non-susceptible Cavendish banana varieties (Marquardt, 2001). The disease continues its spread and is considered a major constraint in the global production of bananas (Ploetz, 2006a; Viljoen *et al.*, 2020).

The *Foc* causes infection of banana plants by penetration of the roots and colonization of the xylem vessels (Ploetz, 2006b). The infected plants express yellowing of older leaves which progresses towards younger leaves. Internally plants develop vascular discoloration in roots, rhizome, and pseudostem (Moore *et al.*, 1995). The *Foc* is divided into three physiological races such as race 1, 2,

and 4 based on its pathogenicity to differential host cultivars. The *Foc* race 1 (R1) is known to attack 'Gros Michel' (AAA), 'Lady Finger' (AAB), and 'Pisang Awak' (ABB) bananas. The *Foc* race 2 (R2) causes disease in 'Bluggoe' (ABB) and closely related ABB clones. The *Foc* race 4 (R4) is known to cause disease in the 'Cavendish' banana subgroup (AAA) as well as most cultivars that are susceptible to *Foc* R1 and R2 (Ploetz, 2015; Bai *et al.*, 2020; Buddenhagen, 2007; Siamak and Zheng, 2018). Based on the ability to infect Cavendish bananas under various environments, the *Foc* R4 has been separated into 'subtropical' race 4 (SR4) and 'tropical' race 4 (TR4) (Ploetz, 2015; Viljoen, 2002). Cavendish bananas in both the tropics and subtropics are reported to be attacked by *Foc* TR4, whereas, *Foc* SR4 attack and produce disease in the subtropics only (Buddenhagen, 2007; Ploetz, 2006a). In comparison to other strains of *Foc* races, the *Foc* TR4 (VCG 01213/16) is regarded as the most damaging due to the broad host range and high virulence (Thangavelu *et al.*, 2020).

Fusarium wilt is difficult to manage (Were *et al.*, 2022) as previously different management strategies such as chemical, cultural, and physical measures have shown inadequate effectiveness (Ploetz, 2015), therefore, the use of rigorous quarantine measures and strategies to prevent further spread of *Foc* from the areas where incursion has occurred used to contain the disease (Thangavelu *et al.*, 2020). Planting disease-resistant varieties is considered the most effective management practice for this lethal disease of banana, a reliable and standardized inoculation protocol is necessary to speed up the strategies involved in the development of varieties resistant to this disease as well as to evaluate different disease control methods (Wu *et al.*, 2010; García-Bastidas *et al.*, 2019; Dita Rodriguez *et al.*, 2021). Previously several inoculation protocols for *Foc* under a greenhouse environment have been described. The majority of these inoculation methods result in the infection of plants, however, the methods employed are distinct, and often reproducible results are not obtained (Sun and Su, 1984; García-Bastidas *et al.*, 2019; Costa and da Silva Costa, 2004; Smith *et al.*, 2008). Therefore, a research experiment was conducted to determine the efficiency of different inoculation methods of *Foc* (TR4) causing Panama wilt of banana. The results of our study will help determine the pathogenicity of *Foc* (TR4) as well as evaluate different disease control methods to find out effective control.

MATERIALS AND METHODS

Isolation and identification of *Foc* (TR4)

Pseudostem and corm samples, both exhibiting clear vascular discoloration, were extracted from banana plant showing Panama wilt symptoms. Samples were kept in paper bags and brought to the laboratory. Small sections (3-6 mm long) of discolored vascular strands (Figure 1) extracted from the specimens and after surface sterilization with commercial bleach were placed on a half-strength PDA medium (Ingredients per liter of distilled water: 100 g peeled potatoes, 10 g dextrose and 20 g agar) amended with an antibacterial agent (eg. streptomycin solution @ 1.2 mL/240 mL PDA) (Ainsworth, 1971). Single-spore (monoconidial) cultures of the isolated fungal organism were prepared and identification of *Fusarium oxysporum* isolate was carried out based on cultural and morphological characteristics as described by Leslie and Summerell (2006).

The *Fusarium* isolate was grown on potato dextrose agar (PDA, Oxoid) at 25 °C for 7 days. DNA material was extracted CTAB method (Manickkam and Moses, 2023). PCR-based molecular diagnostics using primer set SIX9_Foc_F/ SIX9_Foc_R (Carvalhais *et al.*, 2019) to identify the isolate as *Foc* was carried out. PCR-specific primers Foc-1/Foc-2 (Lin *et al.*, 2009) were used to confirm the isolate as *Foc* race 4. Similarly, PCR-specific primers FocTR4-F/FocTR4-R (Dita *et al.*, 2010) were used for the identification of the isolate as *Foc* tropical race 4 (TR4). The PCR product was amplified using ITS1/ITS4 primers. The PCR products were purified via a DNA purification kit (Thermo Scientific) sequencing of PCR products outsourced from Macrogen, Inc. Seoul, a South Korean biotechnology company. The results were compared with blast search sequences in the National Center for Biotechnology Information (NCBI) database. Using Mega X software, a phylogenetic tree was built using the maximum likely hood approach, and tree topology was replicated using a bootstrap study of 1000 replicates (Khan *et al.*, 2017).

Pathogenicity test

Inoculum production

Pouring method

Mung bean medium was prepared for the production of macro and microconidia of the isolated organism. 500 ml of water was poured into 1 L Erlenmeyer flasks and 2 g of fresh mung beans were added to them, flasks were sealed with a cotton plug and sterilized in an autoclave. The sterilized mung-been-medium was filtered through

cheesecloth under sterile conditions and inoculated with five mycelial plugs of the isolated organism from a freshly grown PDA plate. Inoculated flasks were incubated at 25 ± 2℃ for 6 days on rotary shaker (130 rpm). After 6 days post inoculation content of flask was filtered through double layers of sterilized cheesecloth to eliminate hyphae. The required conidial concentration was prepared by using a hemocytometer. The plants were inoculated with 200 ml/Kg soil of conidial suspension @ 10^4 conidia/ml (Low dose), @10^5 conidia/ml (Medium dose), @ 10^6 conidia/ml (High dose) (García-Bastidas *et al.*, 2019).

Figure 1. Section of the pseudostem of wilted banana plant infected with *Fusarium oxysporum* f. sp. *cubense* (TR4). Red arrows show discolored vascular strands.

Chlamydospore method

The mycelial plugs of *Foc* were combined with a sterilized medium composed of sandy soil, corn powder, and distilled water in a flask for the production of chlamydospores. Flasks were then incubated for 12 days at 25℃. The fresh sterilized sandy soil was taken in the flask and infected with the infected substrate with a ratio of 1:12. The flasks containing the resulting mixture were then incubated for a period of 6 weeks. Plants were inoculated with 5 g/Kg soil (Low dose), 10 g/Kg soil (Medium dose), and 20 g/Kg soil (High dose) (García-Bastidas *et al.*, 2019; Costa and da Silva Costa, 2004).

Kernel method

Five mycelial plugs of the isolated organism obtained from seven-day-old culture were mixed with 100 g of sterilized grains in a flask to produce infected maize kernels. Flasks were kept in the dark for 10 days at 25℃ and the plants were inoculated with 5 kernels/Kg soil (Low dose),10 kernels/Kg soil (Medium dose), and 20 kernels/Kg soil (High dose) (García-Bastidas *et al.*, 2019). *Foc* (TR4) was inoculated on tissue-cultured banana plants of cv. NIGAB-1 and Grand Naine are grown in earthen pots in a greenhouse containing 5 kg of sterilized soil. Hardened two and half month old tissue-cultured banana plants were planted in earthen pots and after fifteen days plants were inoculated by the pouring method, chlamydospore method, and kernel method at low, medium, and high doses as described above. Control plants were treated with 200 ml distilled water/Kg soil, an equal quantity of sterilized soil substrate, and an equal number of sterilized maize kernels for pouring, chlamydospore, and kernel method, respectively.

The plants were observed every week for external symptoms and progression. The time passed between inoculation and expression of symptoms in 3 out of 5 plants was considered a latency period. The disease

severity (%) based on leaf chlorosis was recorded. For internal evaluation, plants were internally inspected at weekly intervals. The rhizome of banana plants was washed to remove soil particles after uprooting from pots and was cut longitudinally. Disease severity (%) based on rhizome discoloration was recorded.

Statistical Analysis

Statistical analyses were performed using Statistix 8.1 software. All data were analyzed by two-way Analysis of Variance (ANOVA), whereas significant differences among means at $P < 0.05$ were determined by the Least Square Difference (LSD) test.

RESULTS

Isolation and Identification of Foc (TR4)

Morphological identification

Isolation from disease samples produced typical *Fusarium* colonies. A single-spore (monoconidial) culture of an isolated fungal organism was prepared and identification of *Foc* isolate was carried out based on cultural and morphological characteristics. It produced a white colony and abundant macro and micro conidia and chlamydospores. Macro conidia were 3–5 septate slightly sickle-shaped with an attenuated apical cell and foot-shaped basal cell. Microconidia were one- or two-celled and oval to kidney-shaped. Chlamydospores were round and thick-walled, produced singly or in pairs.

Molecular identification

The isolate was subjected to PCR-based molecular diagnostics using primer set SIX9_Foc_F/ SIX9_Foc_R (Carvalhais *et al*., 2019) and a 260 bp amplification product was obtained, which is the expected band size for all *Foc* isolates. Similarly, we used *Foc* race 4 specific primers Foc-1/Foc-2 (Lin *et al*., 2009) and 242 bp amplification product was obtained, which revealed the identity of the isolate as *Foc* race 4. In addition, we conducted PCR-based molecular diagnostics using *Foc* TR4-specific primers FocTR4-F/FocTR4-R (Dita *et al*., 2010), and PCR amplification products of 463 bp were obtained, which is the expected band size for *Foc* TR4 isolates.

The sequence obtained from the amplification of ITS1/ITS4 primers products were matched from NCBI database using BLAST search. The sequence was identified and deposited in NCBI Genbank (Genbank Accession No. OQ691956.1). The isolate was identified as *Fusarium oxysporum FOC TR4 2p.* Phylogenetic analysis by MEGA X showed in Figure 2.

Pathogenicity Test

Foc (TR4) was inoculated on two banana varieties i.e., NIGAB-1 and Grand Naine in the greenhouse using the pouring method (PM), chlamydospore method (CM), and kernel method (KM). All the methods of inoculation remained successful in producing symptoms, however, disease symptoms were not produced in control treatment and were excluded from statistical analysis. Typical leaf chlorosis and brown discoloration of the rhizome were observed in inoculated plants (Figure 3). The leaf chlorosis results showed a highly significant difference among the methods used on NIGAB-1and Grand Naine ($F = 1094.64$; $P < 0.05$ and $F = 5262.05$; $P < 0.05$ respectively) at various doses ($F = 142.12$; $P < 0.05$ and $F = 855.90$; $P < 0.05$, respectively). Similar to leaf chlorosis, rhizome discoloration in both varieties also exhibited a highly significant difference among the three methods ($F = 4208.12$; $P < 0.05$ and $F = 6251.67$; $P < 0.05$, respectively) at three different doses ($F = 442.61$; $P < 0.05$ and $F = 671.67$; $P = 0.05$, respectively). Moreover, the latency period in both varieties inoculated by the chlamydospore method was two weeks after inoculation (wai) and expressed symptoms before the pouring and kernel methods where symptoms appeared after three weeks of inoculation. The disease severity increased with time which is also reflected in the Area Under Disease Progress Curve (AUDPC).

The pouring method yielded consistent, dose-dependent final disease severity as the highest disease severity calculated based on leaf chlorosis (%) and rhizome discoloration (%) in both the varieties i.e., NIGAB-1 (Figure 4a & 4b) and Grand Naine (Figure 5a & 5b) was recorded at the high dose applied. Banana plants of the NIGAB-1 variety developed 79% leaf chlorosis and 48% rhizome discoloration whereas the Grand Naine variety developed 78% leaf chlorosis and 47% rhizome discoloration inoculated by pouring method at a high dose. The pouring method at a low dose resulted in the lowest disease severity as the plants of the NIGAB-1 variety developed 43% leaf chlorosis and 12% rhizome discoloration whereas the Grand Naine variety developed 42% leaf chlorosis and 11% rhizome discoloration. Consistent with the results of leaf chlorosis and rhizome discoloration, in pouring method highest AUDPC was recorded with highest dose followed by low and medium dose of both varieties.

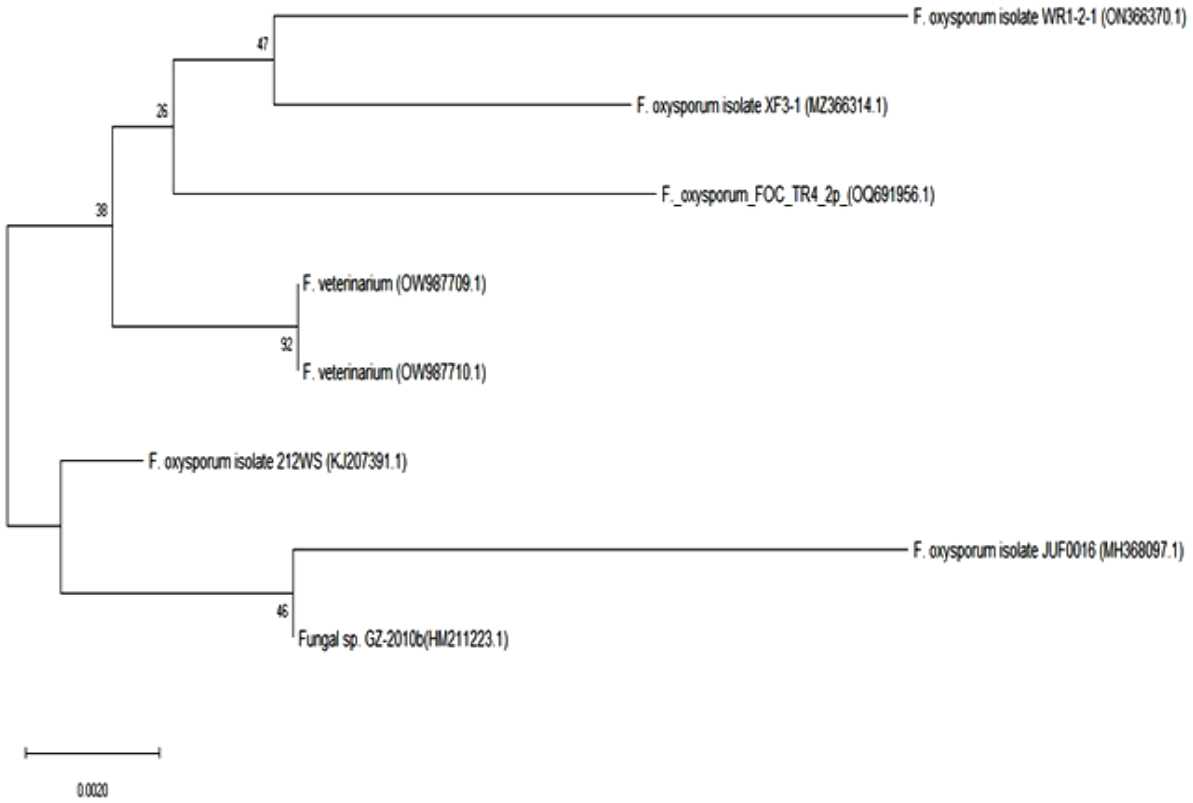

Figure 2. A phylogenetic tree generated using nucleotide sequence information of the ITS region of the conserved ribosomal DNA of the *Foc* (TR4) isolate.

Figure 3. Symptoms of Panama disease of banana caused by *Fusarium oxysporum* f. sp. *cubense* (TR4). a) Un-inoculated plant; b) inoculated plant showing chlorosis; c) un-inoculated rhizome and d) inoculated rhizome showing brown discoloration.

The chlamydospores method resulted in inconsistent final disease severity and AUDPC corresponding to dose. As dose-dependent results in leaf chlorosis and rhizome discoloration were not observed in both varieties. The NIGAB-1 variety developed 100% leaf chlorosis and 62% rhizome discoloration whereas the Grand Naine variety developed 84% leaf chlorosis and 53% rhizome discoloration inoculated by chlamydospore method at a high dose. The chlamydospore method at medium dose resulted in 85% leaf chlorosis and 53% rhizome discoloration in plants of the NIGAB-1 variety whereas the Grand Naine variety developed 90% leaf chlorosis and 60% rhizome discoloration. The NIGAB-1 variety inoculated by the chlamydospore method at a low dose developed 90% leaf chlorosis and 58% rhizome discoloration whereas the Grand Naine variety developed 71% leaf chlorosis and 46% rhizome discoloration.

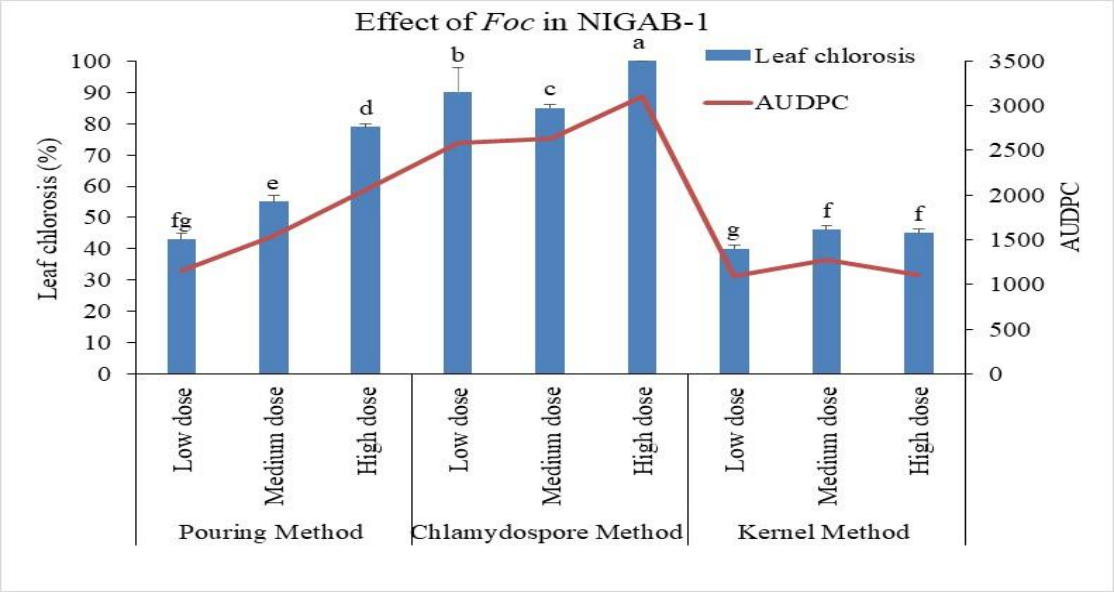

Figure 4 (a). Effect of different inoculation methods on leaf chlorosis (%) and AUDPC in plants of the NIGAB-1 variety, 10 weeks after inoculation with *Foc* (TR4).

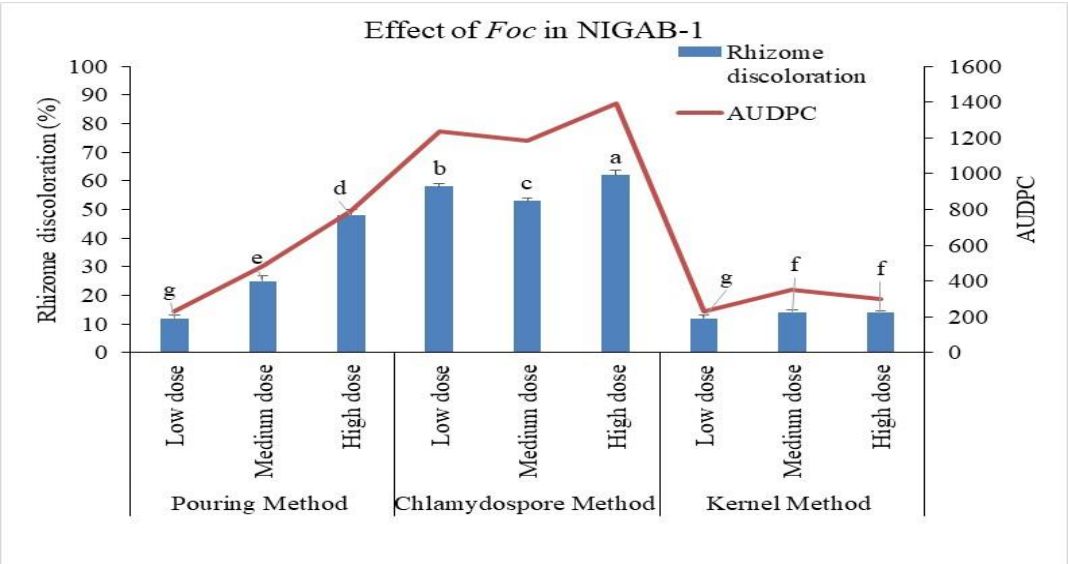

Figure 4 (b). Effect of different inoculation methods on rhizome discoloration (%) and AUDPC in plants of the NIGAB-1 variety, 10 weeks after inoculation with *Foc* (TR4).

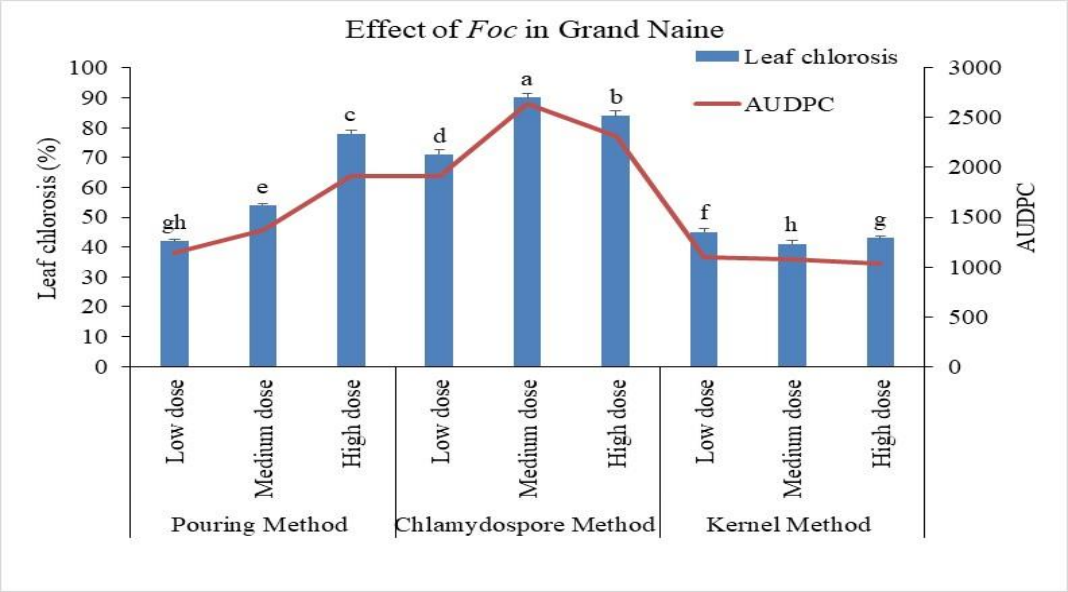

Figure 5 (a). Effect of different inoculation methods on leaf chlorosis (%) and AUDPC in plants of Grand Naine variety 10 weeks after inoculation with *Foc* (TR4).

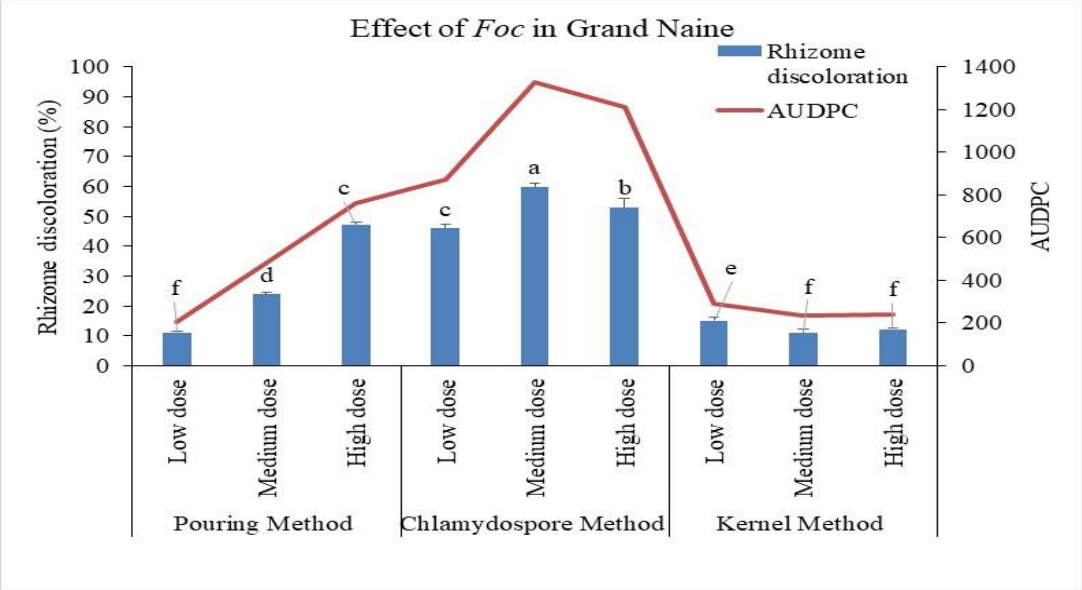

Figure 5 (b). Effect of different inoculation methods on rhizome discoloration (%) and AUDPC in plants of Grand Naine variety 10 weeks after inoculation with *Foc* (TR4).

Similarly, the kernel method at low, medium, and high doses used in both varieties resulted in variable final disease severity. Banana plants of the NIGAB-1 variety developed 45% leaf chlorosis and 14% rhizome discoloration whereas the Grand Naine variety developed 43% leaf chlorosis and 12% rhizome discoloration inoculated by kernel method at a high dose. The kernel method at medium dose resulted in 46% leaf chlorosis and 14% rhizome discoloration in plants of the NIGAB-1 variety whereas the Grand Naine variety developed 41% leaf chlorosis and 11% rhizome discoloration. Plants of the Grand Naine and NIGAB-1 varieties inoculated by kernel method at low doses developed 45% and 40% leaf chlorosis and 15% and 12% rhizome discoloration, respectively. In leaf chlorosis, the highest AUDPC was recorded with medium dose followed by highest and lowest inoculum dose in NIGAB-1. In contrast to the highest AUDPC, in rhizome discoloration was recorded

with medium inoculum dose followed by lowest and highest dose. Similarly inconsistent pattern of AUDPC value was observed in Grand Naine variety.

DISCUSSION

Based on the morphological characteristics as described by Leslie and Summerell (2006) isolated organism were identified as *F. oxysporum*. However, to properly separate the isolate into formae speciales and physiological race, the identification based only on morphology is inadequate. On the other hand, use of species-specific primers in molecular diagnostics offer another method for quickly identifying and differentiating them (Dita *et al.*, 2010; Lin *et al.*, 2009).

Therefore, in our study, the isolate identified as *F. oxysporum* based on cultural and morphological characteristics was subjected to PCR-based molecular diagnostics using primer set SIX9_Foc_F/ SIX9_Foc_R (Carvalhais *et al.*, 2019) and a 260 bp amplification product was obtained, which is expected band size for all *Foc* isolates. Our findings comply with those of Carvalhais *et al.* (2019), who conducted PCR-based molecular diagnostics of *Foc* strains by using primer set SIX9_Foc_F/ SIX9_Foc_R which resulted in a 260 bp amplification product which is expected band size for all *Foc*.

Similarly, we used *Foc* race 4 specific primers Foc-1/Foc-2 (Lin *et al.*, 2009) and 242 bp amplification product was obtained, which revealed the identity of the isolate as *Foc* race 4. In addition, we conducted PCR-based molecular diagnostics using *Foc* TR4-specific primers FocTR4-F/FocTR4-R (Dita *et al.*, 2010), and a PCR amplification product of 463 bp was obtained, which is the expected band size for *Foc* TR4 isolates. Our results are following those of Thangavelu *et al.* (2019), who demonstrated the identification of Foc race 4 by employing PCR-based molecular diagnostics using Foc race 4 specific primers (Foc-1/Foc-2) described by Lin *et al.* (2009). A 242 bp amplification product was obtained showing that the isolate belonged to Foc race 4. Additionally, they also used Foc TR4 specific primers (FocTR4-F/FocTR4-R) developed by Dita *et al.* (2010) which resulted in a PCR amplification product of 463 bp, which is the expected band size for Foc TR4 isolates.

Foc (TR4) was inoculated on two banana varieties i.e., NIGAB-1 and Grand Naine in the greenhouse using the pouring method (PM), chlamydospore method (CM), and kernel method (KM). All the methods of inoculation remained successful in producing symptoms. Typical leaf chlorosis and brown discoloration of the rhizome were observed in inoculated plants. These results comply with previous results reported by García-Bastidas *et al.* (2019), who evaluated five different Panama disease phenotyping assays. Banana plants inoculated by all tested methods expressed disease symptoms within 10 weeks, however, the inoculum concentration had a considerable impact on the final disease scores. In addition, efficiency, dependability, and discriminative capacity are required for phenotyping procedures (García-Bastidas *et al.*, 2019).

The pouring method yielded consistent, dose-dependent final disease severity as the highest disease severity calculated based on leaf chlorosis (%) and rhizome discoloration (%) in both the varieties i.e., NIGAB-1 and Grand Naine was recorded at the high dose applied. The outcomes of our research work are in agreement with García-Bastidas *et al.* (2019), who reported that banana plants inoculated with *Foc* by pouring method produced reliable and reproducible outcomes as evidenced by external symptoms, internal symptoms, and real-time PCR assays on complete rhizomes. Furthermore, this technique enables the inoculation of two hundred and fifty plants in sixty minutes by a single person, making it easier to phenotype sizable mutant and breeding populations. Further, the susceptible chickpea varieties have been reported to express wilt symptoms early in response to enhanced inoculum concentration, and in response to low inoculum concentration, late expression of symptoms and slow development of the disease have been observed (Sugha *et al.*, 1994; Zote *et al.*, 1996; Pande *et al.*, 2007). The chlamydospores method resulted in inconsistent final disease severity and did not give dose-dependent results at low, medium, and high doses used in both varieties. Likewise, García-Bastidas *et al.* (2019) have also reported previously that chlamydospores are very aggressive even at low doses, but their formation can take up to three months, and it can be difficult to accurately quantify them. Similarly, the kernel method at low, medium, and high doses used in both varieties resulted in variable final disease severity. Our findings comply with the results of García-Bastidas *et al.* (2019), who reported that the findings of the maize kernel method were too inconsistent across the various amounts of inoculum that were employed. This was most likely caused by the limited dispersion of kernels or inoculum propagules in the pots, as well as their proximity to roots. Furthermore, the composition of the inoculum, which

may include micro and macroconidia, chlamydospores or mycelium can influence the infectivity (Smith *et al.*, 2008; Costa and da Silva Costa, 2004).

CONCLUSION

The pouring method of inoculation was found more effective than the other two methods since consistent, repeatable results were obtained by this method with dose-dependent final disease severity. It can be utilized for the evaluation of the pathogenicity of *Foc* (TR4) as well as to evaluate different disease control methods to find out effective control.

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