Pathogenic, Phenotypic and Genetic Characterization of *Fusarium oxysporum* f. sp. *cubense* Isolates Affecting Banana (*Musa* spp.) Cultivars in Puerto Rico

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ABSTRACT

Panama disease, or Fusarium wilt as it is also know, caused by *Fusarium oxysporum* f. sp. *cubense* (FOC) is one of the most destructive and widely disseminated diseases of banana (*Musa* spp.). The objectives of this study were to (1) assemble a collection of FOC isolates from symptomatic banana plants present in Puerto Rico; (2) characterize the fungal isolates in terms of their pathogenicity, phenotype and molecularly; and (3) screen isolates by the vegetative compatibility groups (VCG) using known testers. A total of 28 monosporic isolates were recovered from symptomatic banana plants from the municipalities of Isabela, Aguada, Mayaguez and Gurabo. Tissue-culture-derived plantlets of cultivars '2-R-2, 500' (Gros Michel, race 1 differential), 'Dole' (Bluggoe, race 2 differential) and '5-A' (Cavendish, race 4 differential) were used in pathogenicity assays. Inoculations were carried out by immersion of roots in a 10^6 conidia mL^{-1} aqueous suspension. Two months post-inoculation, disease severity was measured on internal rhizome discoloration and symptoms. The isolates were characterized phenotypically based on colony appearance and microscopic morphological traits produced on potato dextrose agar (PDA) and carnation leaf agar (CLA). For VCG complementation tests, nitrate non-utilizing (*nit*) mutants from wild-type isolates were generated in PDA amended with 1.5% (30g/L) chlorate and pairings were carried out on minimal media (MM). Testers from VCGs 0120, 0124, 0125 and 01210 were used to screen the isolate collection. VCG analysis showed that 16 of the isolates belonged to VCG 0124, one of the isolates to VCG 0120 and nine did not form a stable heterokaryon with any of the testers used. Three separate primers sets were used for species and race determination of isolates. A portion of the translation elongation factor 1α (TEF-1α) gene was amplified with primers EF-1/EF-2 and their sequence information used for species identification by comparison to published *Fusarium* spp. Primer set PBL/PBR, specific for race 1, was used to determine if any

of the isolates recovered belonged to this race. Lastly, the specific primers to the tropical race 4 (TR4) strain of FOC, was used to determine if any of the isolates recovered from Puerto Rico belonged to this race. All isolates, including several identified as *F. sacchari*, caused pathogenic reactions on bananas following inoculation. In addition, pathogenicity tests were useful in determine race structure based on the differential reaction on the set of plants used in inoculations. Twenty two of the isolates were identified as *F. oxysporum* and six as *F. sacchari* based on the phenotypic, VCG and molecular tests. Although most of the isolates identified as FOC amplified when using the specific primer set for FOC TR4, the resulting fragment appeared to be larger when compared to control fragment, so the result was associated with a false positive. Based on our results the genetic composition and race structure of the FOC population in Puerto Rico is comprise by race 1 and 2 isolates within the VCG 0124 and it is safe to say that FOC TR4 is absent in the island. The research carried out creates awareness about the plant pathogen, the need to be vigilant in case of outbreaks and suggests that strong quarantine measures are the best approach in keeping out foreign FOC strains

RESUMEN

La enfermedad conocida como el mal de Panamá o marchitez por Fusarium, causada por *Fusarium oxysporum* f. sp. *cubense* (FOC), es una de las enfermedades más destructivas y ampliamente distribuidas de bananos (*Musa* spp.). Los objetivos de este estudio fueron: (1) recuperar aislados de FOC de plantas de bananos sintomáticas en Puerto Rico; (2) caracterizar los aislados recuperados patogénica, fenotípica y molecularmente; y (3) determinar los grupos de compatibilidad vegetativa (GCV). En este estudio, se recuperaron 28 aislados monospóricos de plantas sintomáticas de bananos presentes en los municipios de Isabela, Aguada, Mayagüez y Gurabo. Para las pruebas de patogenicidad se utilizaron plantas propagadas mediante cultivo de tejido pertenecientes a los cultivares 2-R-2, 500 ('Gros Michel', diferencial para raza 1), 'Dole' ('Bluggoe', diferencial para raza 2) y '5-A' ('Cavendish', diferencial para raza 4). Las inoculaciones se llevaron a cabo utilizando una suspensión de esporas 10^6 ml^{-1}. Dos meses luego de la inoculación, se determinó la severidad de la enfermedad tomando en consideración síntomas internos. Los aislados se caracterizaron fenotípicamente basado en la apariencia de la colonia y características morfológicas microscópicas producidas en agar de papa (PDA) y agar de hojas de clavel (CLA). Para las pruebas de complementación de GCV, los mutantes *nit* se generaron en PDA enmendado con clorato al 1.5% (30g/L) y el apareamiento se llevó a cabo en medio mínimo (MM). Para determinar los GCV que componen la colección de aislados de FOC, se utilizaron los GCV conocidos 0120, 0124, 0125 y 01210. Se amplificó una porción del gen "translation elongation factor 1-α" utilizando los cebadores EF1/EF2 y las secuencias de esta región se utilizó para la identificación a nivel de especie los aislados. Los cebadores PBL/PBR, específicos para la raza 1, se utilizaron para determinar la presencia de esta raza entre nuestros aislados. Por último, los cebadores específicos para la raza 4 tropical (TR4), se utilizaron como método diagnóstico para

detectar la presencia de la raza 4 dentro de nuestros aislados. Todos los aislados, incluyendo a los identificados fenotípica y molecularmente como *F. sacchari*, resultaron patogénicos. Veintidós de los aislados fueron identificados fenotípica y molecularmente como *F. oxysporum* y seis como *F. sacchari*. Dieciséis de los aislados formaron un heterocarión robusto con el VCG 0124, mientras que seis de los aislados no formaron un heterocarión con ninguno de los GCV conocidos utilizados. Todos los aislados identificados como FOC amplificaron cuando se utilizaron los cebadores Foc TR4-F/Foc TR4-R, sin embargo el fragmento generado resultó de mayor tamaño que el fragmento esperado, por lo que se consideró un falso positivo. De acuerdo con los resultados obtenidos, la composición genética y estructura de razas de la población de FOC en Puerto Rico está compuesta por aislados pertenecientes a la raza 1 y 2 dentro del VCG 0124. Además se podría concluir que FOC TR4 está ausente en la isla.

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1. INTRODUCTION

Bananas (*Musa acuminata* Colla and *M. balbisiana* Colla and hybrids) are large, perennial, monocotyledonous herbs (Simmonds and Sheperd, 1955). Taxonomically, they are classified within the Family *Musaceae* and in the Order Zingiberales (Simmonds and Sheperd, 1955). The greatest banana diversity can be found in Southern and Southeast Asia, where the crop evolved (Perrier et al., 2011). Since archeological evidence supports that is domestication begun approximately 12,000 B.P. (Denham et al., 2004), bananas are considered to be one of the first cultivated crops (Perrier et al., 2011). Today, the crop is widely grown in the tropics/sub-tropics and consumed in nearly all countries around the world, providing crucial nutrition for millions of people (Perrier et al., 2011).

The vast majority of cultivated and edible types of bananas were natural selections of two diploid species, *M. acuminata* and *M. balbisiana* or interspecific natural hybrids of these two species (Perrier et al., 2011). A system developed by Simmonds and Shepherd (1955) was devised to indicate the haploid contributions, or genomes, of the two species mentioned above in the cultivated bananas: *M. acuminata* (A) and *M. balbisiana* (B). Currently, there are over 1,000 known banana cultivars comprising 50 different subgroups (Ploetz, 2005). Despite the wide range of genetic diversity in Musa germplasm, a limited number of cultivars with a narrow genetic base accounts for most of the global production (Perrier et al., 2011). Nearly 43% (15% export trade and 28% locally consumed) of the total production is based on the Cavendish (AAA) subgroup, whereas the AAB plantain subgroup is responsible for 21%. These two combined account for 64% of all production (FAOSTAT, 2013).

Bananas and plantains (a type of banana) are within the world's most valuable primary agricultural commodities (Ploetz, 2015a; Ploetz et al. 2015). According to Ploetz (2015b), "the exported fruit result in high paying jobs, valuable community services and significant influxes of

foreign currency in the producing countries." In 2011, global production reached 145 million metric tons with a gross production value of USD 44.10 billion. Although only about 15% reaches international markets, export trade produced USD 8.9 billion in 2011 (FAOSTAT, 2013). The remaining 85% of the world's bananas are consumed or sold in local or regional markets mostly in developing countries (FAOSTAT, 2013). India, Uganda, China, Philippines, Ecuador, Brazil, Indonesia, Colombia, Cameroon and Tanzania (in that order) are the ten countries in the world with most banana production (FAOSTAT, 2013). In the Americas the top five exporting countries are Ecuador, Costa Rica, Panama and Belize, with Ecuador being the largest banana exporter in the world (FAOSTAT, 2013). In the Caribbean, the export trade is dominated by the Dominican Republic although other subgroups contribute significantly to the regional production (FAOSTAT, 2013).

Economically and nutritionally, bananas and plantains are among the most important starchy crops in Puerto Rico (Cortes and Gayol, 2006). This industry provides an important source of income and employment in the central and coastal plains of the island (Cortes and Gayol, 2006). On the island, plantains and bananas rank first and second in production area for starchy crops with a production of 258,999 and 197,346 thousand, respectively (Puerto Rico's Department of Agriculture, 2014). Furthermore, collectively they are the third most important agricultural industry in Puerto Rico, after milk and poultry (Puerto Rico's Department of Agriculture, 2011). Bananas and plantains are integrally involved in Puerto Ricans' history and cultural cuisine with "tostones", "mofongo" and "pasteles" being part of the daily diet and some of the most popular dishes. In 2010, both bananas and plantains were the most consumed farinaceous crops on the island with an annual per capita consumption of close to 13.5 and 30.0 kg, respectively (Puerto Rico's Department of Agriculture, 2010).

The banana export industry and smallholder production systems are both affected by a number of limiting factors in two broad categories, biotic and abiotic factors. Some of the factors which impact yield significantly in both export and smallholder production include soils and nutrition, drainage and irrigation, type of cultivar, planting densities and rhizome management, and black leaf streak disease management (Stover, 1986). Labor and environmental issues, international trade regulations and tariffs, and production imbalances are a few of the major abiotic factors that affect principally the export sector (Ploetz et al., 2015). Plant pathogenic diseases are the most important biotic constraints in banana production, especially those caused by fungi (Ploetz and Evans, 2015).

Although many pests and diseases affect bananas and plantains, those caused by fungi are some of the most important in limiting production (Ploetz, 2004). Among fungal diseases reported in Puerto Rico, yellow Sigatoka (*Mycosphaerella musicola* Leach) and black leaf strike (*M. fijiensis* Morelet) are well distributed throughout the island (Cortes et al., 2009). These two diseases are considered 'major' due to the constant fungicide applications needed in order to maintain yields (Irish et al., 2006). According to Ploetz (2001) the annual cost of fungicide applications in export plantations is approximately $1,000 per hectare, which is 15-20% of the final retail price of the fruit in importing countries. Cordana leaf spot (*Cordana musae*) (Diaz andAlmodovar, 2007), cigar-end rot (*Verticillium theobromae* and *Trachysphaera fructigena*) (Diaz andAlmodovar, 2007), Botryodiplodia finger rot (*Botryodiplodia theobromae*) (Ploetz, 2004), sooty mould (*Cladosporium cladosporioides*) and anthracnose (*Colletotrichum musae*) (Ploetz et al., 1994) are other fungal diseases affecting banana cultivars on the island.

In addition to fungal diseases, bacterial diseases like bacterial soft rot caused by *Erwinia* spp. has been previously reported in Puerto Rico (Diaz and Almodovar, 2007). Also plant parasitic

nematodes such as *Radopholus similis*, *Pratylenchus coffeae*, *Rotylenchus reniformis*, *Helicothylenchus* spp. and *Meloidogyne* spp. (Diaz and Alvarado, 2007) are serious pests in *Musa* spp. production. Other biotic limiting factors are banana streak virus (BSV) (Diaz and Alvarado, 2007) and insects like the banana corm weevil (*Cosmopolites sordidus* Germar).

Although the diseases and insect pests previously mentioned negatively affect yield and if not managed properly (increasing production costs), none are considered as serious a threat when compared to Fusarium wilt or Panama disease. Fusarium wilt is caused by the plant pathogenic fungus, *Fusarium oxysporum* f. sp. *cubense* (FOC) (E.F. Smith) Snyder & Hansen. Fusarium wilt was first reported in Australia in 1876 and since then it has spread throughout almost all banana producing regions (Ploetz, 2006). Currently, the disease is considered one of the most destructive in the history of agriculture (Perez-Vicente, 2004). More recently, in countries such as Malaysia and Indonesia, where the aggressive race (tropical race 4 - TR4) of the disease has been reported in Cavendish plantations, the annual losses have exceed 75 million USD (Masdek et al., 2003). One of the main reasons this disease is considered a major threat is due to the lack of effective chemical, biological and cultural control measures to manage the pathogen and epidemic once it becomes established in the field (Ploetz, 2006).

Aside from a 1919 report by Brandes describing FOC from symptomatic plants and from soil in banana plantations, there have been no subsequent reports or research conducted on this important plant pathogen in Puerto Rico. No detailed information exists concerning the organism's distribution and population structure on the island. (e.g., pathogenicity/race structure, phenotype, VCG, race genetic diversity). Therefore, considering the lack of recent studies, the emergence of new strains of this important plant pathogen and the cultural and economic importance of bananas in Puerto Rico were the reasons that justified our research. So with that,

this study intends to: 1) conduct a survey of the island and assemble a collection of FOC isolates from symptomatic plants in the island; and 2) perform a detailed characterization of the isolate collection. Detailed analyses will include pathogenic, phenotypic and vegetative compatibility group (VCG) combined with molecular characterizations. One of the project's goals is to increase awareness among farmers and the general public about the importance of preventing the introduction of emerging races of the disease not found on the island (e.g., TR4).

2. LITERATURE REVIEW

THE HOST

Bananas (*Musa acuminata* and *M. balbisiana* hybrids) belong to the Musaceae family, one of the eight families in the order Zingiberales. Banana plants are large (2-9 m) perennial monocotyledonous herbs that develop from subterranean rhizomes (**Fig. 1A**). The aboveground portion of the herb consists of leaves and their fused petiole bases, which together form the pseudostem. A dozen pinnately veined leaves emerge from the pseudostem as rolled cylinders to form the canopy. At the moment of the flowering, a stem rises from the top and through the center of the pseudostem and its growth becomes geotropic (Ploetz et al., 1994; Ploetz, 2003). Shoots that arise laterally and in a radial fashion from the base of the rhizome are known as suckers (**Fig. 1C**) (Ploetz et al., 1994). Either the suckers or the rhizome are used to vegetative propagate the plant, although plantlets from meristem culture are now widely used (Ploetz et al., 1994; Ploetz, 2003). Flowers on the stem are arranged in nodal clusters in a radial fashion, where those located in the basal portion are female, whereas those in the upper portion are male (**Fig. 1B**) (Ploetz et al., 1994; Ploetz, 2003). For fruit development, pollination is not required since most of the cultivars are parthenocarpic (Ploetz et al., 1994; Ploetz, 2003). The fruits which are considered botanical berries are known as fingers, a cluster of fruit as hands and the total of fruit in the inflorescence as the bunch (Ploetz et al., 1994; Ploetz, 2003).

Bananas are grown and produced in a wide range of subtropical and tropical environments (Ploetz et al., 2015). According to the Banana Production Manual (2001) the environmental requirements of bananas in order to grow best include: (a) temperature range of 12-37°C, with 27°C being the optimal mean monthly temperature; (b) high water demand, with 25 mm per week being the minimum and an average annual rainfall of 1500-2500 mm; (c) deep, well drained loam

soil with high humus content and a pH range of 5.6-7.5; and (d) large amounts of nitrogen and potassium to maintain high yields.

In export production, monocultures prevail, but in smallholders different banana cultivars and crops are often interplanted (Ploetz et al., 2015). Either micropropagated plants, rhizomes or suckers can be used as vegetative propagation material. The recommended planting distance is 2 m between and 2 m within row for a planting density of 1,175 plants per acre. Planting arrangement may vary depending on whether the field is flat or sloping. In flat fields rows should be straight, whereas in sloping fields rows should follows the contour lines to avoid or decrease soil erosion. Holes should be proportional to the seed (i.e. rhizome) or vegetative propagation material used. During the rainy seasons the rhizome should be placed protruding out of the hole, while in drought seasons the rhizome should be completely covered by the soil. Planting to harvest interval during the first cycle of banana is typically 14-18 months (Ploetz, 2015b)

THE PATHOGEN

Fusarium oxysporum belongs in the Eukaryota Domain, Fungal Kingdom, Ascomycota Phylum, Ascomycete Class, Sordariomycetidae Subclass, Hypocreales Order, and Nectriaceae Family. *F. oxysporum* species complex is comprised of a suite of anamorphic, filamentous, morphologically similar, pathogenic and non-pathogenic strains that affect a wide variety of plants, animals and humans (O'Donnell et al., 1998). Based on the agricultural and economic impact it is the most important taxon of *Fusarium* (Ploetz, 2005). According to Ploetz (2015a), plant pathogenic forms of the *F. oxysporum* species complex affect a single or limited set of host plants. Panama disease or Fusarium wilt of banana is caused by *F. oxysporum* f. sp. *cubense* (FOC) (E.F. Smith) Snyder & Hansen, a *forma specialis* within the *F. oxysporum* species complex (O'Donnell et al., 2009).

The disease was first reported in Australia in 1876 (Bancroft, 1876). In the late 19th century the pathogen was observed in tropical America affecting 'Gros Michel' plantations in Costa Rica and Panama (Stover, 1962). It wasn't until 1910, in Cuba, that FOC was identified as the causal agent and where the name of the *forma specialis* emerged (Ploetz 2005). In the 1960s the pathogen developed its major epidemics in Panama causing the total collapse of 'Gros Michel', the banana on which the export trades were then based (Ploetz, 1994; Ploetz, 2015a). There the disease gained its notoriety and its common name of Panama disease (Ordoñez et al., 2015; Ploetz, 2005).

Fortunately, productive and resistant Cavendish cultivars found in the United Fruit Company collection in Honduras and botanical gardens in the United Kingdom replaced 'Gros Michel' (Ordoñez et al., 2015; Stover, 1962). Unfortunately, the history is repeating itself and in the 1990s Cavendish plantations failed to establish due to a new race of FOC known as tropical race 4 (TR4) (Ploetz, 2005). Since then, the disease have been found in Southeast Asia, Africa, Western Asia, Australia, China, Indonesia, Jordan, Lebanon, Malaysia, Mozambique, Oman, Pakistan, the Philippines and Taiwan (**Fig. 2**) (Butler, 2013; Freshplaza, 2015; Garcia et al., 2013; Molina et al., 2010; Ordoñez et al., 2015; ; Ploetz et al., 2015) Currently, Panama disease is the most widely disseminated banana pathogen (Ploetz, 2015b) and the most destructive in the history of agriculture (Perez-Vicente, 2004).

The anamorphic stage of this soilborne pathogen is responsible for causing the disease. Morphologically, the filamentous fungus is characterized by white to purple colonies with abundant aerial mycelium and growth rates of 4 to 7 mm per day when grown in potato dextrose agar (PDA) (Ploetz, 2000; 2006). Pale violet to dark red color pigments are also produced in PDA (Leslie and Sumerell, 2006; Perez-Vicente et al., 2003). For reproduction, survival and dispersal, the fungus produces macroconidias, microconidias, chlamydospores and sclerotia (Leslie and

Sumerell, 2006; Ploetz, 2000; 2006). Macroconidias are produced in tan to orange sporodochia which may vary from 27 to 55 µm long and 3.3 to 5.5 µm wide. Macroconidia of FOC are usually four to eight celled and sickle- shaped with a foot-shaped basal cells. Microconidias, formed in false-heads in short monophialides, are 5 to 16 µm in length and 2.4 to 3.5 µm wide, are one to two celled and oval to kidney shaped (Leslie and Sumerell, 2006; Ploetz, 2000 and 2006). Other characteristic morphological traits of FOC are blue sclerotia and globose chlamydospores which are 7 to 11 µm in diameter and formed in pairs or singly in hyphae or conidia (Leslie and Sumerell, 2006; Ploetz, 2000 and 2006).

To date, 4 races of FOC have been described and among these, only three are recognized to infect bananas (Ploetz, 2005 and 2000; Perez-Vicente, 2004). Cultivars that are differentially susceptible or resistant to the different isolates of FOC have been used to determine race structure. For example, 'Gros Michel' (and closely related cultivars within the subgroup) is susceptible to race 1; but resistant to race 2 isolates; whereas 'Bluggoe', and closely related cultivars, are susceptible to race 2 and resistant to race 1 isolates. Other important cultivars, in distinct, subgroups susceptible to race 1 include 'Manzano', 'Pisang Awak', 'Prata' and 'Maqueño' (Ploetz, 2000, 2005 and 2006). *M. textilis*, or abaca as it is commonly known, is an important species of banana used for its fiber is also known to be susceptible to race 1 of FOC (Ploetz, 2000; 2005; 2006; Perez-Vicente, 2004). Aside from Bluggoe subgroup cultivars, some of the man-made synthetic tetraploids derived from international breeding programs have also shown susceptibility to race 2 isolates (Ploetz, 2000 and 2006). 'Cavendish' subgroup cultivars have been resistant to both race 1 and 2 isolates, but are susceptible to race 4 isolates.

The two race 4 strains are divided into subtropical race 4 (STR4) and tropical race 4 (TR4). STR4 affects Cavendish, race 1 and race 2 susceptible cultivars strictly in the subtropics where

colder temperatures stresses the plants and predisposes them to the disease (Ploetz, 2015a; Ploetz et al., 2015). TR4 affects the same cultivars as STR4, but in the absence of any abiotic stressors (Ploetz, 2015a; Ploetz et al., 2015). Many of the popular cultivars around the world, including the plantain subgroup, are also affected by the aggressive TR4 race (Ploetz, 2000; 2005). Race 3 was described by Waite (1963) in *Heliconia*, but due to distant association between *Musa* and *Heliconia*, this race may be considered a distinct *forma specialis* (Perez-Vicente, 2004).

Since 1950s race designations have been used to facilitate comparisons among different populations of FOC (Stover, 1962). However, race designations do not represent the full variability and genetic diversity of the pathogen (Ploetz, 2006). Thus, other methods for unveiling genetic diversity were developed (Ordoñez et al., 2015). A technique where Nitrate non-utilizing (*nit*) mutants are used to test for vegetative compatibility was developed by Puhalla (1985) and improved by Correll et al. (1987). Today, vegetative compatibility group analyses divide FOC into 24 unique VCGs (Bentley et al., 1998; Kistler et al., 1998; Ploetz, 2005). Furthermore, results obtained from genetic studies carried out using electrophoretic karyotyping, RFLPs, AFLPs, RAPDs, and phylogenies with multiple genes divided FOC into at least eight different lineages (Bentley et al., 1994; Boehm et al., 1994; Fourie et al., 2009; Groenwald et al., 2006; Koenig et al., 1997; O'Donnell et al., 1998; Pegg et al., 1995). According to O'Donnell et al. (1998), FOC lineages show an outstanding dichotomy, known to types or clades. More recently, Ordoñez et al. (2015) used genome-wide Diversity Array Technology sequence (or DarTseq) markers to divided the 24 FOC strains (representing all known VCGs) into two major clades. In summary, all phylogenetic studies indicate that FOC is a clonal polyphyletic fungus with various strains with convergent evolution (Bentley et al., 1998; Fourie et al., 2011; O'Donnell et al., 1998, Ploetz,

2006) and that it has co-evolved with its host (bananas) in South East Asia (O'Donnell et al., 1998; Ploetz and Pegg, 2000).

The first symptoms of FOC infection in the plant are observed at the root hair tips, which are the first site of infection (Perez-Vicente, 2004; Ploetz, 2000 and 2006). First typical external symptoms can be observed two to five months after initial infection when, the appearance of pale green streaks and a reddish brown discoloration of the vascular vessels occur under the leaf petiole epidermis (Perez-Vicente, 2004). FOC produces two types of external symptoms: (a) yellow leaf syndrome and (b) green leaf syndrome (Perez-Vicente, 2004). The yellow leaf syndrome (**Fig. 3A and 3B**) is the classic symptom of Panama disease and is commonly confused with potassium deficiency (Perez-Vicente et al., 2014). Damage to the chloroplasts by Fusaric acid, a phytotoxin has been identified as the cause of the intense leaf chlorosis (Dong et al., 2012 and 2014). The green leaf syndrome is associated with leaves that remain green, but bend and collapse at the petiole base (Perez-Vicente et al., 2014). Afterward, a yellowing and a longitudinally splitting of the base of the older leaves (i.e., pseudostem) can occur around four months after initial infection (Ploetz, 2006).

In some cases, if the plant is actively growing, the splitting of the pseudostem (**Fig. 3C**) at the soil level can be observed (Perez-Vicente, 2004). Early on during infection, the most common internal symptoms are a reddish-brown discoloration of the xylem vessels in the roots, (**Fig. 3D**) which progresses to the rhizome and finally proceeds up through the pseudostem and into the leaf petioles and laminas (Ploetz, 2006). In the advanced stages of the disease, younger leaves wilt and collapse until the entire canopy consists of dead or dying leaves (Ploetz, 2006). Most of the symptoms mentioned above do not develop on young rhizomes or adventitious suckers (<5 months). Often, the symptoms produced by FOC and by the bacteria, *Ralstonia solanacearum*

("Moko" disease) can be confused. However, Moko can be effectively distinguished by the presence of internal discoloration and symptoms in the fruits (Perez-Vicente, 2004).

Unlike most soilborne pathogens, postharvest diseases and rusts - without urediospores – that generally are characterized by the production of a monocyclic disease, FOC is a soilborne pathogen with a polycyclic disease cycle (Ploetz, 2015b). Rhizomes and adventitious suckers are commonly used as vegetative seed pieces for banana propagation. Because rhizomes are usually symptomless when infected, they are the most common means by which the pathogen is spread (Ploetz, 2000; Stover, 1962). Other means by which the pathogen can be disseminated are by movement of contaminated soil in running water, footwear, clothes, farm implements and machinery (Ploetz, 2015b; Stover, 1962). In a recent study, Meldrum et al. (2013) isolated conidias from the exoskeleton of the banana weevil, *Cosmopolites sordidus*, and suggested the insect as a potential vector of the pathogen. Macroconidia and chlamydospores, produced in the advanced stages of the disease, are the principal survival structures of the pathogen allowing it to survive up to 30 years in the absence of the host (Ploetz, 2000 and 2006). However, this long survival trait might not be strictly related to resistant spore persistence, rather to the pathogen's capacity to survive as a saprophyte in alternate hosts (e.g., weed roots) (Hennessy et al., 2013; Ploetz, 2015b). Hennessy et al. (2013) reported isolating FOC from asymptomatic roots of several weed species, *Chloris inflata*, *Euphorbia heterophylla*, *Cyanthilium cinereum* and *Tridax procumbens*. *C. cinereum* is the only weed species with broad distribution in tropical countries (Hennessy et al., 2013).

Heterokaryosis is the condition in which, as a result of fertilization or anastomosis, cells of fungal hyphae contain two or more nuclei that are genetically distinct (Agrios, 2005). Heterokaryon formation between different individual is an important component of many fungal

life cycles and may serve as the initial step in the parasexual cycle (Leslie, 1993). Parasexuality, mainly occurs in fungi that lack a sexual stage such as FOC. Parasexualism is the process by which genetic recombination can occur within fungal heterokaryon and throughout this process genetic variability may equal or surpass that brought about by sexual reproduction (Agrios, 2005). When two isolates fuse their hyphae and are capable of producing a stable heterokaryon, it is said that they are vegetative compatible. Vegetative compatibility is mediated by multiple vegetative incompatibility loci (*vic*, or *het*, loci) (Leslie, 1993). Also, vegetative compatibility, in most cases, is a homogenic trait. Homogenic means that two fungal isolates are vegetative compatible only if the alleles at each of the corresponding *vic* loci are identical (Leslie, 1993). Therefore, since vegetative compatibility requires alleles at *vic* loci to be identical, members of a vegetative compatibility group are usually clonally derived and genetically related (Perez-Vicente, 2004).

Developed by Cove (1976) and later improved by Puhalla (1985) and Correll et al. (1987), a straight-forward technique for determining VCGs is using nitrate non-utilizing (*nit*) mutants. Since then, vegetative compatibility has proven to be a useful mechanism for studying the genetic diversity of FOC and to date more than twenty VCGs have been reported (Perez-Vicente, 2004; Ploetz, 2006). Some of the reported VCGs have a worldwide distribution while others have a much more limited area of dispersal (Ploetz, 2005). Furthermore, some VCGs have been recovered from a wide range of cultivars and genetic backgrounds, others come from specific banana subgroups and still others have been found in single cultivars (Perez-Vicente, 2004). Isolates in the 01213 VCG are responsible for the current epidemic of TR4 in Cavendish bananas (Ploetz, 2005).

Microorganisms, such as nonpathogenic strains of *F. oxysporum* and *Pseudomonas fluorescens*, have been evaluated as FOC biocontrol agents. However, most of these studies were

carried out under lab or greenhouse conditions and were not reliable in the field (Ploetz, 2015b). Root dips (Nel et al., 2007) and injections of fungicides into plants (Herbert and Max, 1990) have been tested as chemical measures, but studies were carried out *in vitro* or results varied between locations. Soil fumigation with methyl bromide (Herbert and Max, 1990) reduced the disease incidence for 26 months after treatment, but eventually areas were re-invaded. As a sanitary measure, tools, farm equipment and shoes can be disinfected with polymeric biquanidine hydrochloride, sodium hypochloride (bleach), poly dimethyl ammonium chloride or quaternary ammonium compound (Nel et al., 2007). Physical management techniques like rice hull burning on infested soils (Molina et al., 2010) and soil solarization (Hermanto et al., 2012) have been tested. Of the two, solarization was most effective since symptoms were delayed up to 6 months following 10 months of treatment. Soils in which a virulent pathogen does not cause typical levels of disease on a susceptible host are known as disease suppressive soils and have been described for FOC/bananas (Ploetz, 2015b). According to Stover (1990), the suppressive capacity might be biologically based. However, the requirements to convert a disease conducive soil to a disease suppressive soil are not well understood (Ploetz, 2015b).

Host resistance is the only consistently effective measure for managing the disease (Ploetz, 2015b). Conventional breeding programs have been working in the development of resistant cultivars since 1922 (Ortiz, 2013). However, the long generation times from planting to seed production, the need for final products to be parthenocarpic and sterile and the low fertility of cultivars are few of the limiting factors (Ortiz, 2013). Some bred hybrids developed by the Fundación Hondureña de Investigación Agrícola (FHIA) have shown tolerance to TR4 (Rowe and Rosales, 2000). Despite their tolerance to TR4, few agronomic, post-harvest and organoleptic standards are met (Ploetz, 2015b). Giant Cavendish Tissue Culture Variants (GCTV series)

tolerant to FOC have been developed using mutation breeding by the Taiwan Banana Research Institute (TBRI). Although deficient in other horticultural traits, they are the best tolerant alternatives for the exported Cavendish clones (Ploetz, 2015b). Lastly, a wide range of genes have been identified for resistance based on genetic transformation, but only short-term results from greenhouse evaluations have been reported (Ploetz, 2015b).

Generally, there are no cultural, biological or chemical tools to effective manage FOC. In pathogen free regions, exclusion and quarantines are essential since FOC cannot be eradicated once the soil is infected and pathogen becomes established (Ploetz, 2015b). In new infected areas, early detection is crucial to avoid widespread dissemination (Ploetz, 2015b). With the idea that early detection could prevent rampant spread, an intergenic region sequence (IGS) PCR-based diagnostic for TR4 was developed (Dita et al., 2010). Another effective measure to avoid dissemination, is to use disease-free certified tissue culture-derived plantlets either as the source of clean material planting material or to initiate disease free nurseries (Lule et al., 2013).

3. MATERIALS AND METHODS

3.1 Survey and isolation

The recovery of isolates was carried out in an island-wide survey which included the municipalities of Isabela, Aguada, Mayaguez and Gurabo. Banana plants showing typical symptoms of FOC infection were chosen. Symptomatic tissues were collected from internal portions of the pseudostems, labeled appropriately, placed in polypropylene bags and transported back to the laboratory for processing.

Once in the laboratory, smaller pieces approximately 2 cm^2 were surface sterilized as follows: ethyl alcohol (70%) and commercial chlorine (10%) for one minute each and rinsed with sterile distilled water.

Once surface sterilized, four pseudostem tissue pieces per isolate were transferred to Petri dishes with 2% water agar (WA) (**Appendix A**) (Fisher BioReagents, Fisher Scientific, Waltham, MA) and incubated in an Imperial III incubator (Barnstead International Inc., Dubuque, IA) at 25°C with alternating 12-hour periods of light/dark for 2 to 4 days.

After incubation, and from the water agar plates, a single germinating microconidia, macroconidia or a hyphal tip was identified with the aid of a stereoscope, excised and transferred to potato dextrose agar (PDA) (**Appendix A**) (BD Difco Microbiology, Houston, TX) with a 7 cm diameter sterile Whatman filter paper disk (GE Healthcare Bio-Sciences, Pittsburgh, PA) on the surface. The PDA Petri dishes were then incubated, as mentioned above, for eight days.

Once colonies had covered the surface of the filter paper, the paper was peeled off the medium and allowed to air-dry in an empty sterile Petri dish in a laminar flow hood. Once dry, and using sterile techniques, the filter paper was cut into 5mm^2 pieces, placed into a sterile 2 ml

sterile screw-thread borosilicate glass sample vials (Kimble Chase, Rockwood, TN) and stored at 4ºC following the same methodology outlined by Correll et al. (1986).

3.2 Morphological characterization

All *Fusarium* spp. isolates were characterized using morphological and cultural characteristics described in Leslie and Sumerell (2006). For cultural characterizations, a square 5 mm^2 piece of filter paper (previously colonized by the fungus and which had been store at 4°C) was placed in the center of a Petri dish containing PDA and incubated at 25°C with alternating 12-hour periods of light/dark for 7 days. After 7 days, colony pigmentation and color were recorded. Colony growth rates were recorded for each isolate from a monosporic culture. For this, a single germinating macro or microconidia was transferred to the center of a Petri dish containing PDA. Cultures were incubated at 25°C in complete darkness and colony diameter was measured (in mm) after 72 hours of growth.

For micro and macroconidia shape (length and width measurements) four 5 mm^2 filter paper pieces colonized by *Fusarium* spp. were transferred to Petri dishes with carnation leaf agar (CLA) (**Appendix A**). CLA plates were incubated at 25°C with alternating 12-hour periods of light/dark for 14 days. After the 14-day incubation, each CLA plate was examined with an Olympus BX 40 compound microscope (Olympus, Melville, NY) *in situ* under the 10X or 20X objectives. This was carried out to establish presence/absence of micro, macroconidia and chlamydospores. If present, then the shape of both types of conidia, the manner in which conidia are formed (i.e., heads or chains), whether conidia are formed on conidiogenous cells or phialides, and the presence or absence of chlamydospores was established.

Standard glass microscope slides were prepared in water for detailed morphological examinations of micro and macroconidias and (i.e., size, shape, apical and basal cell) from each fungal isolate. For both types of conidia, size (width and length) was measured on 25 spores per isolate using the compound microscope and the software *Pax it!* (Pax it, IL). The analysis of variance (ANOVA) was carried out using the software InfoStat (**Appendix B**).

3.3 Pathogenicity tests

Pathogenicity tests were performed following the methods described by Ploetz and Shepard (1989) and Dita et al. (2010) with some minor modifications which are described herein. Healthy plants for the pathogenicity tests were micro-propagated at the USDA-ARS Tropical Agriculture Research Station (TARS) tissue culture laboratory facilities. The Gros Michel accessions '2-R-2, 500' (race 1 susceptible), Bluggoe accession 'Dole' (race 2 susceptible) and the Cavendish accessions '5-A' (race 4 susceptible) were chosen for their ability to differentiate FOC races and because isolates recovered in the island survey originated from some of the same cultivars/subgroups.

Once sufficient plants had been multiplied in the laboratories and once they had differentiated roots, they were taken to the greenhouses for transplanting. For the first set of plants used in the pathogenicity tests, rooted plantlets were transplanted into 10 cm plastic sterile pots filled with sterile silica sand and fertilized once every two weeks with an all-purpose (20-20-20) Nutri-Leaf (Miller Chemical and Fertilizer Corp., Hanover, PA) water soluble fertilizer. A second set of plants used in a replicate pathogenicity test were also grown in sterile sand until inoculation, but were transplanted immediately following inoculation to 10 cm plastic pots with Sunshine Growing Mix #1 (Sungro Horticulture, Agawam, MA) growing medium. This was due to poor

plant grown and performance in the sterile sand medium and in an effort to produce ideal growing conditions to plants post-inoculation.

Plantlets were acclimated for four weeks under a shade cloth covered greenhouse under ambient (~24-28°C) greenhouse conditions. Shade cloth was used to reduced sunlight (30%) and temperatures while plants were acclimating.

The spore suspension used in the inoculations was prepared from individual isolates which were grown on 7-day old cultures on Petri dishes containing PDA. A sterile glass rod and 15 ml of sterile water were used to dislodge spores from the surface of each culture by scraping without removing the medium. The 15 ml of sterile distilled water was then transferred to a 40 ml plastic screw cap centrifuge tube and pelletized using a 5810R laboratory centrifuge (Eppendorf, Hauppauge, NY) for 10 minutes at 4,000 rpm. The supernatant was carefully discarded and pellet (i.e., spores) re-suspended by adding 15 ml sterile water. The spore concentration of each isolate was determined by using a hemocytometer (LW Scientific, Lawrenceville, GA) and then standardized to 10^6 microconidia ml^{-1} by addition of different volumes of water.

Inoculations were performed with each of the *Fusarium* spp. isolates recovered during the survey. Roots of each plant was immersed in the 10^6 conidia/ml sterile water spore suspension for five minutes and then transplanted back into the sterile sand containing pot. To ensure infection, plantlet roots were mechanically damaged by manually hand squeezing and damaging the root system before inoculation (**Fig. 4**). Extra precautions were incorporated to not cross-contaminate plants with different isolates (e.g., changing latex gloves between isolates). Each isolate was inoculated onto all three differential accessions and each group of three inoculated plants were subsequently placed in a standard 1020 greenhouse tray (Hummert International, Earth City, MO) with no holes in the bottom to prevent cross contamination. Non-inoculated control plants were

plants that corresponded to the cultivar/accessions from which the original isolate was recovered from. For example, if an isolate was originally recovered from a Bluggoe cultivar/accession, then the non-inoculated control would be a 'Dole' plant. Non-inoculated control plants were treated the same as inoculated plants, except these plant's roots were dipped in sterile water.

Following inoculations and during their incubation period, inoculated plantlets were maintained in a separate greenhouse from where healthy plants were being kept. Forty days after inoculation, plantlet disease severity was measured using a severity scale (**Table 1**). A 1 to 10 severity scale was used based on internal pseudostem discoloration and symptoms (Ploetz and Shepard, 1989). Pathogenicity tests for all isolates were conducted twice.

3.4 VCG determination

Vegetative Compatibility Groups (VCGs) were determine following the methods described by Correll et al., (1987) and Leslie and Summerell (2006). Four known VCGs (i.e., testers), provided by Dr. Randy Ploetz of the University of Florida, were used in this study: VCG 0124, VCG 0120, VCG 0125 and VCG 01210. Nitrate non-utilizing mutants (*nit*) mutants were generated by placing a piece (5 mm^2) of filter paper containing the fungal isolate in the center of a Petri dish containing PDA amended with 1.5% chlorate (Sigma Aldrich, St. Louis, MO) or (PDC) (**Appendix A**). Plates were incubated at 25°C with alternating 12-hour periods of light/darkness for four to six days, until the appearance of fast growing sectors from the initially restricted fungal colony. Sectors were then transferred to minimal media (MM) (**Appendix A**) and, if they grew expansive thin colonies with no aerial mycelium, they were considered good a *nit* mutants. The type of *nit* mutants where also determined by use of phenotyping media.

In complementation tests, mycelial pairings were made on MM by placing the *nit*M tester in the center of a Petri dish surrounded by up to five *nit* mutants from original isolates. Pairings were incubated at 25°C in complete darkness for 7 to14 days and then scored for complementation and/or heterokaryon formation. Dense aerial growth (i.e., heterokaryon) was observed for isolates where the *nit* mutants were complimentary to the *nit*M testers and where they anastomosed. For those *F. oxysporum* isolates that did not form a heterokaryon with none of the *nit*M testers used, multiple *nit* mutants were generated and classified as *nit*1, *nit*3 or *nit*M based on their growth in the four phenotyping media (i.e. Nitrate, Nitrite, Ammonium and Hypoxanthine). Those *nit* mutants classified as *nit1* and *nit*M were used for further investigation and were tested for heterokaryon self-incompatibility (HSI) by setting up a complementation test between the *nit1* and *nit*M originated from the same isolate. HSI was determined by the lack of heterokaryon formation.

3.5 Molecular Characterization

For DNA extraction of unknown *Fusarium* spp. from Puerto Rico, monosporic isolates were grown on PDA medium for 5 days at 25°C with alternating 12-hour periods of light/darkness. VCG testers were also grown on PDA and grown under the same conditions for DNA extraction. Approximately, three square pieces (1 cm^2) per isolate were then transferred from the PDA cultures to 125 ml Erlenmeyer flasks containing 50 ml of potato dextrose broth (PDB) (MP Biomedicals, OH) (**Appendix A**). Flasks were incubated on a Labline 56400 orbital shaker (Labline instruments, Melrose Park, ILL) at 150 to 200 rpm at room temperature (~24°C) for five days. Mycelium was harvested/separated from PDB by pouring the liquid media, containing fungal mycelium, through a sterile filter paper on a Buchner funnel connected to a vacuum pump. Approximately 100 mg of fresh mycelium was collected and transferred to a 2 ml microcentrifuge tube (Eppendorf, Hauppauge, NY) containing 2 tungsten beads.

The samples were then disrupted using the TissueLyser II homogenizer (Qiagen, Valencia, CA) using a setting of 30.0 1/s for 1 minute. Once the samples were disrupted, total genomic DNA was isolated using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA) according to manufacturer's instructions. Undiluted DNA was quantified and assessed for purity using a NanoPhotometer spectrophotometer (Implen, Westlake Village, CA).

For DNA amplifications, to address taxon determination and race identify (race-specific primers), three distinct primer sets were used (**Table 2**). Primer pair EF-1/EF-2 (O'Donnell et al., 1998) was used to amplify a portion of the translation elongation factor 1α (TEF-1α) gene. This region was used to molecularly characterize *Fusarium* spp. isolates to the species by comparing sequences generated for the Puerto Rican isolate collection unknowns to published sequences of known isolates in the GenBank database. The primer pairs PBL/PBR (Liu et al., 2006) and FocTR4-F/FocTR4-R (Dita et al., 2010) were used as molecular diagnostic methods to differentiate between race 1 and 4 isolates of FOC, respectively.

PCR reaction mixture for all primer sets are summarized in (**Table 3**). The amplification reaction for primer pair EF-1/EF-2 was carried out in a T100 Thermal Cycler (Bio Rad, Hercules, CA) using the following program: 95°C for 2 minutes (1 cycle), 95°C for 1 minutes, 62°C for 1 minutes, 72°C for 1 minute (35 cycles), 72°C for 5 minutes final extension (1 cycle) and a 4°C hold. For the primer pairs PBL/PBR and FocTR4-F/FocTR4-R, thermocycling conditions were the same described above, except different annealing temperatures (55°C and 68°C, respectively) were used. The annealing temperature for the Dita et al (2010) primers had to be optimized using gradient PCR in an attempt to increase the annealing temperature and eliminate non-specific amplification.

For the primer sets EF-1/EF-2 and PBL/PBR, the desired fragments were visualized through electrophoresis in a 1.5% OmniPur agarose gel (EMD Millipore, Billerica, MA) with 1X TBE buffer (Promega, Madison, WI) and stained with ethidium bromide. For the primer set FocTR4-F/FocTR4-R, the desired fragment was visualized in a 4% MetaPhor high resolution agarose gel (Lonza, Rockland, ME). Four microliters of 100 bp DNA ladder (Promega, Madison, WI) were used for size reference in all gels. The electrophoresis was carried out at 110 volt and 400 milliamps for 60 minutes. Agarose gels were visualized and photographed using the Epi Chemi II Darkroom (UVP, Upland, CA) trans-illuminator.

DNA amplifications, using the EF-1/EF-2 primer set, were purified using QIAquick PCR Purification Kit (Qiagen, Valencia, CA) following manufacturer's instructions. After purification, DNA amplification products were shipped to a commercial company (Macrogen Corp.) for sequencing. The sequences obtained were edited with the software Sequencher 5.3 (Gene Codes Corporation, Ann Abor, MI). Once edited, the nucleotide sequences were analyzed using the BLAST tool of the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov). The BLAST sequences with the highest homology to the unknown isolates were used as references. The phylogenetic tree was developed using MEGA 6.06 according to the default parameters established by the software. The model and statistical method employed were Kimura 2 parameter model (K2) and Maximum Likelihood, respectively. *F. graminearum* was used as an outgroup.

4. RESULTS

4.1 Survey and Isolation

A total of 28 *Fusarium* spp. isolates were recovered from symptomatic banana (*Musa* spp.) plants from four municipalities. Most of the isolates were recovered from two municipalities (Isabela and Mayaguez). The isolates from Isabela all came from affected plants grown as part of the USDA-ARS Tropical Agriculture Research Station's germplasm *Musa* spp. collection. Isolates originated from plants in several *Musa* spp. taxonomic subgroups. Many of the isolates were from symptomatic plants that where in Gros Michel, Silk, Bluggoe or closely related subgroups (**Table 4**).

4.2 Morphological characterization

Based on the macro and microscopic morphological and phenotypic characteristics observed on CLA and PDA, the isolates appeared to be in two distinct groups (**Table 7**). Microconidias were present in all isolates and all were produced on false heads. For a group of six isolates (5, 6, 7, 10, 16 and 28) false heads were produced on long mono or polyphialides, whereas in the rest of isolates the conidia were born on short monophialides (**Fig. 6**). Chlamydospores were present in all isolates except for 5 and 7. When grown on PDA, six isolates (not the same six as above – 2, 4, 5, 10, 13 and 18) produced no pigmentation on the medium. Color varied from red to purple for the rest of the isolates that produced pigmentation (**Fig. 7**). Most of the isolates produced a white, pinkish to peach colony with abundant aerial mycelium when grown on PDA, with the exception of isolates 5, 10, 16 and 22 that produce an orange colony with little or none aerial mycelium (**Table 7**). Macroconidias were produce in tan to orange sporodochia for all isolates (data not shown).

ANOVA showed significant differences for the length and width of both microconidias and macroconidias, although no clear trend was observed between isolates in the two distinct groups previously identified based on the conidiogenous cells and on media phenotypes (**Table 8**). The possible exception was the trend seen in the length of conidia for the isolates 6, 7 and 28 which presented long statistically significantly different conidia. These three isolates (6, 7, and 28) were part of the group of six isolates that separated based on the conidiogenous cells. Statistical differences were also observed for average growth rate, however no separation into groups was observed. Based on the macro and microscopic morphological and phenotypic characteristics, six of the isolates collected were tentatively identified as *F. sacchari* and 22 as FOC.

4.3 Pathogenicity

Forty days after the inoculation, all isolates (including those identified morphologically and genetically as *F. sacchari* – see results section below) appeared to be pathogenic on differential banana accessions based on external symptoms and internal vascular discoloration. The symptoms produced in the pathogenicity assays by the *Fusarium* spp. isolates recovered from Puerto Rico were consistent with those generally described previously for Fusarium wilt (i.e., intense yellowing of older leaves and reddish to brown vascular discoloration). Surprisingly, no major differences in symptoms between the isolates identified as *F. sacchari* and those identified as FOC were observed. Pathogenicity tests were not entirely consistent across replicates and in some cases results did not match perfectly with what had been expected. When an isolate was recovered from a field plant in the Gros Michel subgroup it was expected to be a race 1 isolate and cause symptoms on plants in that same subgroup during pathogenicity tests. This was not always the case, for example isolate 8 recovered from a Bluggoe subgroup plant in the first pathogenicity replication

caused severe vascular discoloration on the Gros Michel differential and very little discoloration on the Bluggoe differential (**Table 5**).

All non-inoculated controls remained symptomless during evaluation period. Based on the ratings for disease severity on the three differentials accessions utilized (i.e., 'Gros Michel', 'Dole' and '5-A'), race structure could be determined for the FOC isolates (**Fig. 5**). Although pathogenicity tests were not consistent in the two separate replicates, the mean disease severity (averaging across replicates) for each isolate showed a definitive trend and a clearer picture of race structure (**Table 6**).

4.4 VCG determination

Four known VCGs were used in this study as testers: VCG 0120, VCG 0124, VCG 0125 and VCG 01210. Nitrate non-utilizing mutants (*nit*) sectors and mutants were generated readily on PDA amended with 1.5% chlorate. The type of *nit* generated (i.e., *nit* 1, 3 or M) was determine based on their growth on one four different types of phenotyping media (i.e., nitrate, nitrite, ammonium and hypoxanthine) (**Fig. 8; Table 9)**. Sixteen of the FOC isolates formed a stable heterokaryon with the *nit*M tester for the known VCG 0124 (**Table 10**). Isolate 25 formed a weak heterokaryon with the *nit*M tester for the known VCG 0120 in only one of the two replicate pairings. For isolates that did not readily form a heterokaryon with any of the *nit*M testers used in either pairing replication, multiple *nit* mutants were generated and classified as *nit*1, *nit*3 or *nit*M based on their growth on the four phenotyping media. Mutants classified as *nit1* and *nit*M were used for further investigation and were tested for heterokaryon self-incompatibility (HSI) by setting up complementation tests between *nit1* and *nit*M mutants originating from the same isolate. HSI was determined by the lack of heterokaryon formation, but none of the isolates tested were found to be HSI (i.e., they all formed a heterokaryon and were self-compatible).

4.5 Molecular characterization

The DNeasy Plant Mini Kit worked well and good quantity and quality DNA was successfully extracted from all 28 *Fusarium* spp. isolates recovered from affected banana plants in Puerto Rico as well as from the VCG tester isolates.

The partial translation elongation factor 1α (TEF-1α) gene was amplified with the primer pair EF-1/EF-2. The successful amplification of a DNA fragment, of approximately 648 bp, was confirmed in a 1.5% agarose gel using 5 µl of the PCR product (not shown). Two species, *F. sacchari* and *F. oxysporum* were identified based on sequence similarity to sequences available in GenBank. DNA sequence homology was 97% or higher for all matches (**Table 11**). A phylogenetic tree was constructed with the Kimura 2 parameter model (K2) and the statistical method Maximum Likelihood. The phylogenetic tree constructed separated the isolates into three strongly supported clusters or clades (bootstrap values of 96, 97 and 99) corresponding to the *F. oxysporum*, *F. sacchari* species and *F. graminearum* as the outgroup (**Fig. 9**).

Amplification of DNA samples with race 1 specific primer pair PBL/PBR (Liu et al., 2006) produced a fragment of ~355 bp as originally reported. The fragment was amplified in all of the Puerto Rican FOC isolates as well as DNA extracted from testers in VCGs 0124 and 0125. No amplification was observed for DNA from VCG 0120, VCG 01210, *F. sacchari* or the water control. A single *F. sacchari* DNA sample was included as a negative control, as primer pair was not expected to amplify DNA of this species. Based on this amplification pattern, the primer pair was unable to clearly differentiate between race 1 and 2 isolates identified by pathogenicity tests (**Fig. 10**).

The primer pair FocTR4-F/FocTR4-R (Dita et al., 2010), which is supposed to be specific for the FOC TR4 strain in VCG 01213, was used as a molecular diagnostic tool to ensure no Puerto Rican isolates of FOC belonged to this virulent race. Surprisingly, all isolates recovered from Puerto Rico and that were identify as *F. oxysporum* amplified several fragments. After attempting to optimize the PCR conditions and trying to eliminate the non-specific amplification products by raising annealing temperature to 68C, a clear amplification product still was produced with this primer combination. However, the resulting fragment was clearly larger (~500 bp) than the expected for FOC TR4 diagnostic fragment (~463 bp) (**Fig. 10**).

5. DISCUSSION

5.1 Survey and Isolation

Fusarium spp. isolates were recovered from the North, East and Western regions of Puerto Rico. However, the majority of the isolates were collected from symptomatic plants in the Northern part of the island, specifically from the municipality of Isabela, PR. This was due to accessibility since this municipality is where the USDA-ARS TARS banana germplasm collection is located and where the disease symptoms were first noticed. In contrast to Brandes (1919), where typical cases of banana wilt were observed and reported in Puerto Rico only on the cultivar 'Gros Michel', the collection of isolates in the current study comes from host accessions and banana sub-groups where Fusarium wilt susceptibility has not been well characterized or well understood. For example, *Fusarium* spp. isolates (later confirmed to be FOC isolates) were recovered from 'TMB 1378' a synthetic hybrid, 'Cardaba' (Saba sub-group) and 'Tigua' (Iholena sub-group) all of which are host accessions and sub-groups for which race susceptibility is not well characterized or understood.

5.2 Morphological characterization

The *F. oxysporum* species complex is comprised of a suite of anamorphic, filamentous, morphologically similar, pathogenic and non-pathogenic strains that affect a wide variety of plants, animals and humans (O'Donnell et al., 1998). *F. oxysporum* is characterized by white to purple colonies with abundant aerial mycelium, but colony morphology on PDA varies widely (Leslie and Sumerell, 2006; Ploetz, 2000; 2006). Growth rates vary from 4 to 7 mm per day when grown on PDA at 25ºC (Ploetz, 2000; 2006). Pale violet to dark red color pigments are also produced PDA in some instances, with some isolates producing no pigmentation of any kind (Leslie and

Sumerell, 2006; Perez-Vicente et al., 2003). For reproduction, survival and dispersal the fungus produces macroconidias, microconidias, chlamydospores and sclerotia (Leslie and Sumerell, 2006; Ploetz, 2000; 2006). Microconidias, formed in false-heads in short monophialides, are 5.0 to 16.0 µm in length and 2.4 to 3.5 µm wide, are one to two celled and oval to kidney shaped (Leslie and Sumerell, 2006; Ploetz, 2000 and 2006). In most cases macroconidias are produced in tan to orange sporodochia which may vary from 27.0 to 55.0 µm long and 3.3 to 5.5 µm wide. Macroconidia of FOC are usually four to eight celled and sickle-shaped with a foot-shaped basal cells. Other characteristic morphological traits of FOC are blue sclerotia and globose chlamydospores which are 7.0 to 11.0 µm in diameter and formed in pairs or singly in hyphae or conidia (Leslie and Sumerell, 2006; Ploetz, 2000 and 2006).

Considering FOC morphological traits, 22 of the isolates could be classified as *F. oxysporum* species. For isolates 1, 3, 7, 18 and 22 (**Table 8**) a significant difference was observed in the length of macroconidias when compared with the rest of the isolates. Different morphotypes of *F. oxysporum* have been identified in previous studies. Hafizi et al (2013) identified two morphotypes of *F. oxysporum* associated with crown disease of oil palm in Indonesia. One of the most noteworthy morphological differences between Morphotype I and Morphotype II was the mean length of macroconidias 42.2 ± 4.5 and 34.9 ± 2.1, respectively.

F. sacchari is characterized by a pale mycelium that turns purple over time with the formation of a violet pigmentation when grown on PDA (Leslie and Sumerell, 2006). For reproduction, survival and dispersal the fungus produces macroconidias, microconidias, sclerotia, but chlamydospores are usually absent (Leslie and Sumerell, 2006). In rare cases macroconidias are produced in orange sporodochia that can be found on carnation leaf pieces (Leslie and Sumerell, 2006). Macroconidias vary from 19.0 to 46.2 µm long and 2.7 to 3.3 µm wide (Siti

Nordahliawate et al., 2008). Macroconidia of *F. sacchari* are usually three septate, slightly falcate, thin walled and poorly developed basal cells (Leslie and Sumerell, 2006). Microconidias, are formed in false-heads in either monophialides or polyphialides, are 5.4 to 15.0 µm in length and 1.9 to 4.1 µm wide (Siti Nordahliawate et al., 2008) and sin some cases elongated mesoconidia can be present (Leslie and Sumerell, 2006).

Six of the isolates recovered from symptomatic banana plants were identified morphologically as *F. sacchari*. *F. sacchari* is classified within the section Liseola, whereas *F. oxysporum* is classified within the section Elegans. *Fusarium* species within the section Liseola fulfill certain morphological requirements, among these, the absence of chlamydospores (Waalwijk et al., 1996). Surprisingly, four of the isolates identified morphologically and molecularly as *F. sacchari* in the current study produced chlamydospores. Studies based on the sequence analysis of 28S ribosomal RNA gene indicated that these two sections are closely related (Baayen et al., 1997). Furthermore, according to Baayen (1997) the distinction between the section Liseola without chlamydospores and section Elegans with chlamydospores is still questionable.

5.3 Pathogenicity

Pathogenicity assays were conducted twice, however disease severity results were not consistent across the two replications and some clear differences were noticed.

In the first replicate, all isolates including those identified morphologically and molecularly as *F. sacchari* were pathogenic. Disease severity rating was consistently higher for all isolates and many of the plants used in the pathogenicity assays in the Cavendish subgroup (Accession '5-A' - race 4 susceptible) appeared to be susceptibility.

In contrast, during the second replicate of the pathogenicity test none of the *F. sacchari* isolates caused symptoms and not all FOC isolates caused disease. Overall, disease severity was notably lower overall and fewer 'Cavendish' accessions showed symptoms or vascular discoloration (i.e., severity) of rhizomes as compared to the first pathogenicity assay. From the original 28 isolates in the collection, all 28 appeared to be pathogenic on banana plants in the first inoculation, whereas only 13 caused symptoms on plants the second assay.

F. sacchari has been described as a plant pathogenic fungus causing sugarcane wilt (Viswanathan et al., 2011). Viswanathan et al., also suggest that *F. saccharii* is associated with the sugarcane leaf disease known as pokkah boeng. Li-sha et al (2011) were the first to report *F. sacchari* in bananas and, along with *F. concentricum*, were the most common species recovered from the fruit. However, no pathogenicity assays were carried out by Li-sha et al (2011) with any of the *Fusarium* species described. Therefore, the current research is the first to report *F. sacchari* as a potential plant pathogen infecting and affecting banana plants. Unfortunately, due to the lack of consistency between replications in the pathogenicity tests, *F. sacchari* pathogenicity on bananas remains questionable. Additional pathogenicity tests with *F. sachari* might resolve observed discrepancies.

Another important difference observed between pathogenicity test replicates was the decrease in number of isolates that caused disease and overall severity seen in the second pathogenicity replication. The differences observed might have been related to disease predisposing factors in the host. It is commonly known that *Fusarium* species can survive as saprophytes or opportunistic plant pathogens and only become virulent once host is weakened by stress. Stress factors may include biological stresses (e.g., insect, nematode) or abiotic (e.g., water stress, temperature) or even physiological stresses such as plant nutritional status (e.g., too

much/too little fertilizer). More specifically for plant nutrition, the type of fertilizer, whether it is granular, slow release or water soluble, the source/form of nitrogen present and its concentration are all very important in determining optimal plant health. According to Ploetz (2015b), the nitrogen source and its form potentially can have an effect on Fusarium wilt disease severity adding that nitrate (NO_3) fertilizers generally decrease disease severity and ammonium (NH_4) fertilizers may increase it.

However, according to Zhang et al (2013) in *in vitro* and hydroponics experiments, results, suggested that high concentrations of ammoniacal nitrogen (NH_4) inhibited penetration of FOC through secondary roots. The all-purpose (20-20-20) Nutri-Leaf water soluble fertilizer was used during the first pathogenicity test replicate and was applied according to the manufacturer's instructions (once every two weeks) from plant transplant until destructive rating of rhizome discoloration. Nutri-Leaf fertilizer is composed of three nitrogen sources including ammoniacal (4%), nitrate (6%) and Urea (10%). During the acclimatization process, plants in the first pathogenicity test were often water stressed (too little or too much water). This was primarily because it was difficult to keep plantlets properly hydrated due to the poor water holding capacity of the sand. In an effort to keep plants sufficiently watered, an 8 cm plastic saucer was used under the base of each pot. When this technique was implemented, plants and plant roots were often waterlogged. The combination of frequent water soluble fertilizer applications with a high urea source of nitrogen and the water stress caused to the plants in the sterile sand medium might have predisposed them to pathogenicity.

These observations could have been responsible for the increased disease severity ratings seen in the first replication of the pathogenicity tests. Post-inoculation, plants in the second pathogenicity replicate were grown in a peat moss-based growing medium. Organic mediums are

thought to affect susceptibly and pathogenicity of bananas in previous FOC pathogenicity tests (Randy Ploetz, *personal communication*). In addition, a proprietary formulation of fertilizer is used in the medium so the exact source of nitrogen is unknown and an effect due to sources/form of nitrogen cannot be determined.

Another possible explanation for the susceptibility observed during the first pathogenicity test replicate in the 'Cavendish' accession might be due to the fact that most of the isolates formed robust heterokaryons with VCG 0124 tester (see VCG discussion below). Although FOC isolates in VCG 01213 are responsible of the current epidemic in 'Cavendish' plantations (i.e., TR4), several reports exists showing that isolates VCG 0124 have also been recovered from symptomatic 'Cavendish' plants. Thangavelu and Mustaffa (2010) not only demonstrated that isolates in this particular VCG were capable of infecting FOC race 1 susceptible cultivars, but also was able to show infection of 'Grand Naine', a cultivar thought be only susceptible to race 4 isolates. Susceptibility of certain 'Cavendish' cultivars to non-TR4 strains of FOC appears to be somewhat linked to disease predisposing factors. Factors that influence FOC susceptibility of Cavendish plants are not well understood and further studies with single-spore isolates of the different races are required to present a clearer picture.

Results showed that race designation of the isolates, based on disease susceptibility (incidence and severity) of differential cultivars, was not as consistent as expected. According to Ploetz and Correll (1988), FOC race designation via pathogenicity assays are time consuming, may be affected by minor changes in growing conditions and by the cultivar or accessions evaluated in the study. Moreover, isolates within a particular VCG may belong to the same race or belong to more than one race (Ploetz and Correll, 1988). In the case of the VCG 0124, Ploetz and Correll (1988) concluded in their study that this VCG along with 0123, 0125, 0129 and 01210

contained isolates of unknown race or possibly two different races. Conversely, Thangavelu and Mustaffa (2010) suggested that the isolates they worked with within VCG 0124 strictly belonged to race 1. The discrepancy between these two studies suggests that pathogenicity assays for race designation have limitations and should be accompanied by other diagnostic techniques such as genetic/molecular markers. Race-specific PCR primers, when available, have been both useful and controversial at the same time.

5.4 VCG determination

To the date, over 20 VCGs of FOC are known (Ploetz and Pegg, 1997). Some of these known VCGs have been recovered from a variety of cultivars and genomes, while others have been found on single cultivars (Somrith et al., 2011). In addition, there are VCGs that have a localized or limited distribution, whereas others have a cosmopolitan distribution. The known VCGs 0120, 0124, 0125 and 01210 were used in the present study. These particular known VCGs were chosen because they have a cosmopolitan distribution and because they were more likely to be found in Puerto Rico as they have been previously reported in the Americas and the Caribbean. 16 of our isolates were classified within the VCG 0124 since they formed a robust heterokaryon with *nit*M tester for this particular VCG. This is the first report of the VCG 0124 affecting banana plants in Puerto Rico. According to our results, one of the isolates formed a weak heterokaryon with the *nit*M tester for the known VCG 0120 in one of the two replicates. This result could be related to a misinterpretation or human error. The remaining 11 isolates did not formed a heterokaryon with any of the testers used. These isolates were tested for HSI, but none of them resulted to be HSI. Two things might be happening, they could belong to other known VCGs not considered in this study or they could be part of a new unknown VCG.

5.5 Molecular characterization

To the date, no one technique has proven to be practical for managing FOC as chemical, biological or cultural measures have been ineffective. A successful disease management program would incorporate clean planting material (e.g., tissue culture plantlets) and would implement an early detection (monitoring) and eradication (quarantine) program to avoid broad dispersion. Quick and accurate disease diagnosis is imperative when dealing with FOC outbreaks and with this in mind several molecular diagnostic methods based on race-specific primers have been developed. Liu et al (2006) developed, through a Random Amplified Polymorphic DNA (RAPD) technique, a race 1 specific primer set.

However, its specificity was not as accurate as predicted since this primer set amplified DNA of race 2 isolates as well as isolates from several VCGs (0124 and 0125) in the current study. Although it is unclear which race (or races) belong in VCG 0125 (Ploetz and Correll, 1988), isolates have mostly been recovered from 'Lady Finger' (Pome Subgroup - AAB), a race 1 susceptible cultivar. VCG 01210 did not amplify with the Liu et al (2006) primers either, even though isolates in this VCG are associated with race 1. Many isolates in this VCG have been recovered from the cultivar 'Apple' (Silk subgroup – AAB) which is race 1 susceptible. As expected, the race 1 specific primer set did not amplify DNA for the *F. sacchari* isolates or control DNA of the TR4 strain in VCG 01213. So, some specificity exists with Liu et al (2006) primer set to the species level and to some FOC genetic backgrounds.

Since the initial appearance and subsequent outbreaks of race 4 FOC strains (STR4 and TR4) affecting Cavendish banana production, there has been a great effort to develop race-specific primers for prompt diagnosis. Lin et al (2009) designed a race 4 specific primer set using a

Sequence Characterized Amplified Region (SCAR) methodology. However, Dita et al (2010) suggested that a primer set they developed was more specific and allowed for distinction of the different strains (STR4 and TR4) of FOC race 4 affecting Cavendish. DNA markers developed by Dita et al (2010) were elaborated using a single nucleotide polymorphism (SNP) present in the intergenic spacer (IGS) region of the nuclear ribosomal operon. When Dita et al (2010) primers were used in the current study at the annealing temperature established by the authors, non-specific amplification was observed. A gradient PCR reaction was implemented in order to optimize annealing temperature (increasing the published annealing temperatures) and eliminating background amplification/noise. In spite of increasing annealing temperature up to 68°C (from 60°C), all isolates identified as FOC appeared to amplify the diagnostic fragment.

However after closer inspection and separation in a high resolution agarose gel, it was determined that the fragment was considerably larger (~ 500 bp) than the expected TR4 diagnostic fragment (463 bp). These results were associated with false positive, because there was a discernable difference in the fragment size. Many isolates amplified with the race 1 specific primers by Liu et al. (2006) and not with positive control DNA from TR4, and because all FOC isolates clearly formed heterokaryons with VCG 0124. According to Sumerell (*personal communication*), false positives have been previously recorded in Australia when using Dita et al (2010) primer set as diagnostic method. Although this was not done in the current study, another way to determine that the amplification observed is due to the non-specificity of the primers would be to sequence the fragment amplified for the Puerto Rican FOC isolates and determine if the sequence belongs to the TR4 strain IGS region.

These results suggest that molecular diagnostic methods, such as the ones used in this study, should be paired with other techniques such as VCG complementation assays in order to

add confidence to the diagnosis. Results showed poor primer specificity and therefore the use of virulence genes might be a more effective strategy in future development of race-specific diagnostic primer sets.

6. CONCLUSION

The causal agent of Panama disease or Fusarium wilt, *Fusarium oxysporum* f. sp. *cubense*, is present in Puerto Rico, since we were able to recovered it from symptomatic banana plants. Our research provide an insight of the genetic diversity and race structure of the FOC population present in the island never reported before. Based on our representative sample, Puerto Rico's FOC population is limited to one VCG (0124) and to race 1 and 2 isolates. This is the first report of VCG (0124) affecting banana cultivars in the island. The races or strains found in Puerto Rico affect banana cultivars that are grown, sold and consumed locally (e.g., Manzano, Gros Michel). However, these cultivars are not produced on any commercial scale and therefore FOC races 1 and 2 do not have an important economic impact. Both plantains and 'Cavendish' bananas are sizeable industries in Puerto Rico and neither of these genetic backgrounds are susceptible to the races described in this study. Although only shown in one of the replicates of the pathogenicity tests, it is conceivable that under stressed conditions 'Cavendish' cultivars (maybe other cultivars as well) may be susceptible to FOC isolates in the described VCG (0124). It is also important to be mindful that these FOC strains are found in Puerto Rican banana farmer's fields and that any time soil is moved there is a possibility of disseminating the fungus as well.

FOC TR4 was not found in the current study and it is not thought to be on the island for now. In order to prevent the entry of TR4, or for that matter any other important pest, precautions must be taken including establishing regulations and quarantine measures on the movement of plant propagative material. Educational workshops to inform the public should be considered as a training tool and as a preventative measure. TR4 has not been reported in the Americas and keeping it out of Puerto Rico, the Caribbean region and the American continent is of utmost importance.

The current study was the first of its kind in Puerto Rico and will serve as a starting point for further studies, however it did have some limitations. For further studies, would be good to include a larger collection of isolates from more geographically diverse regions in order to have a more representative sample of the FOC population present on the island. Also, since several of the isolates did not form heterokaryon with any of the testers, including more VCG testers could help classify all isolates to VCG. Following through with the sequencing of the non-specific fragment amplified with the race-specific TR4 primers could have added strength to the evidence against TR4 being found in PR. Lastly, more genes like RPB2 and Beta-tubulin can be included in order to have a more reliable molecular characterization.

7. FIGURES AND TABLES

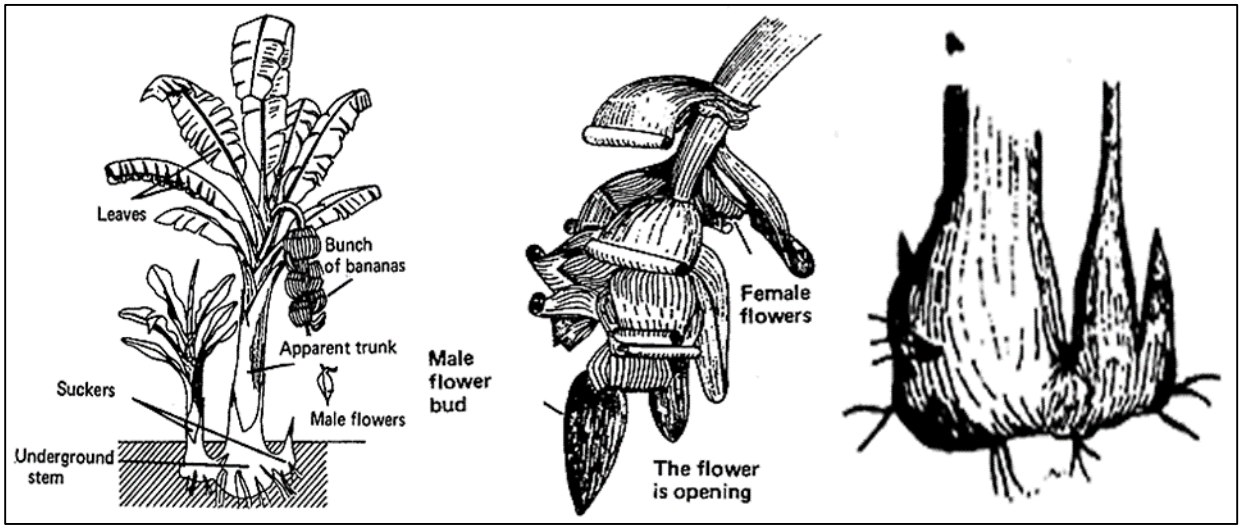

Figure 1. Parts of a banana plant. (A) Pseudostem, which is comprised of fused petioles and leaves which also form the canopy; (B) male and female portion of the inflorescence; and (C) rhizomes with a lateral sucker, both used to vegetatively propagate the plant.

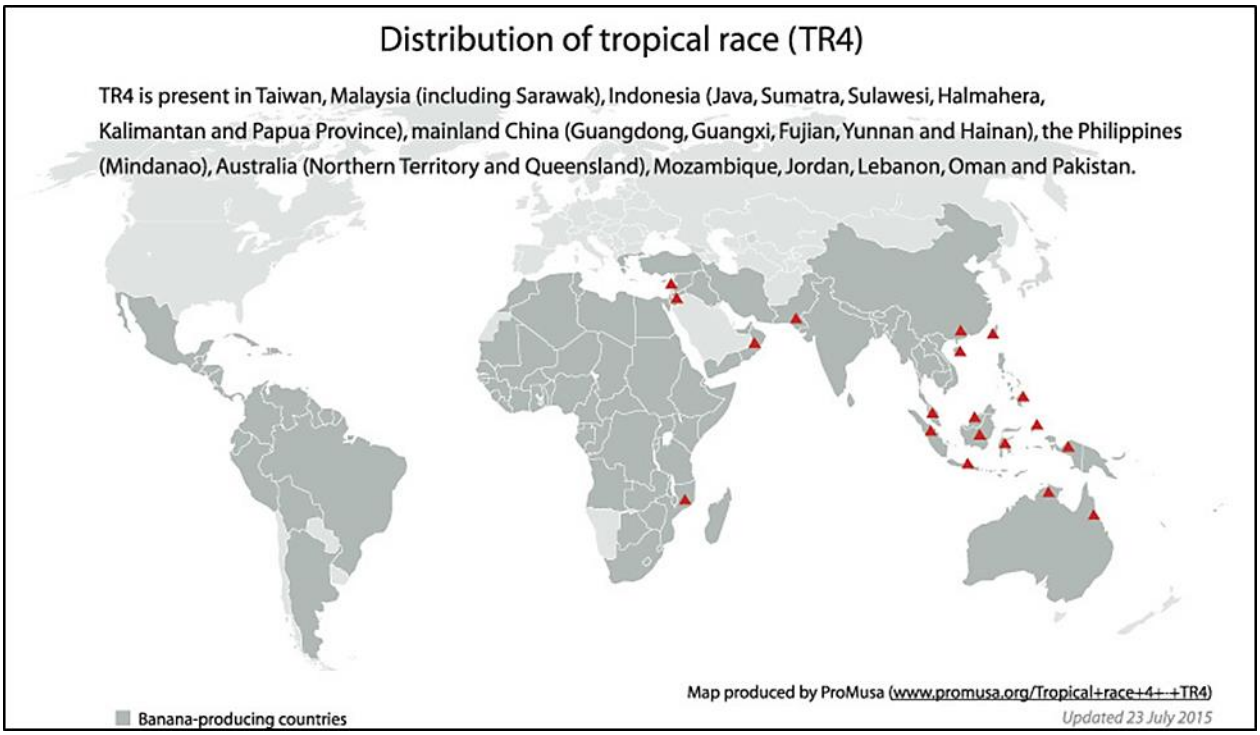

Figure 2. Banana producing regions where the aggressive form of *Fusarium oxysporum* f. sp. *cubense*, tropical race 4 (TR4), has been reported affecting Cavendish banana plantations. (http://www.promusa.org/preview2578).

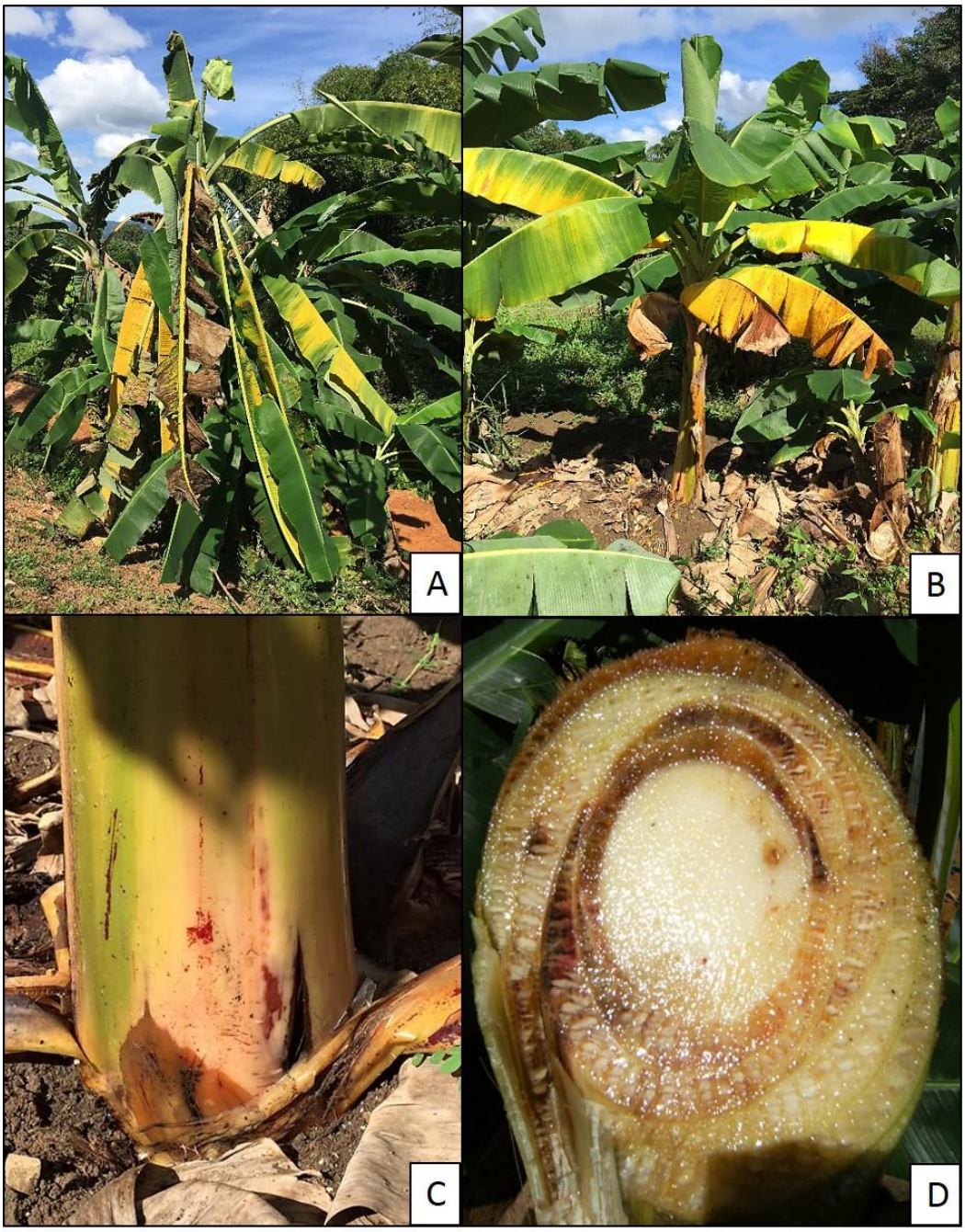

Figure 3. Typical symptoms of Panama disease. (A) Silk or "Manzano" (AAB) and with chlorotic and wilted leaves; (B) Bluggoe or "Mafafo de Adjuntas" (ABB) showing intense yellowing of older leaves; (C) Longitudinal splitting of the pseudostem at the soil level; and (D) Reddish to brown discoloration of vascular tissue.

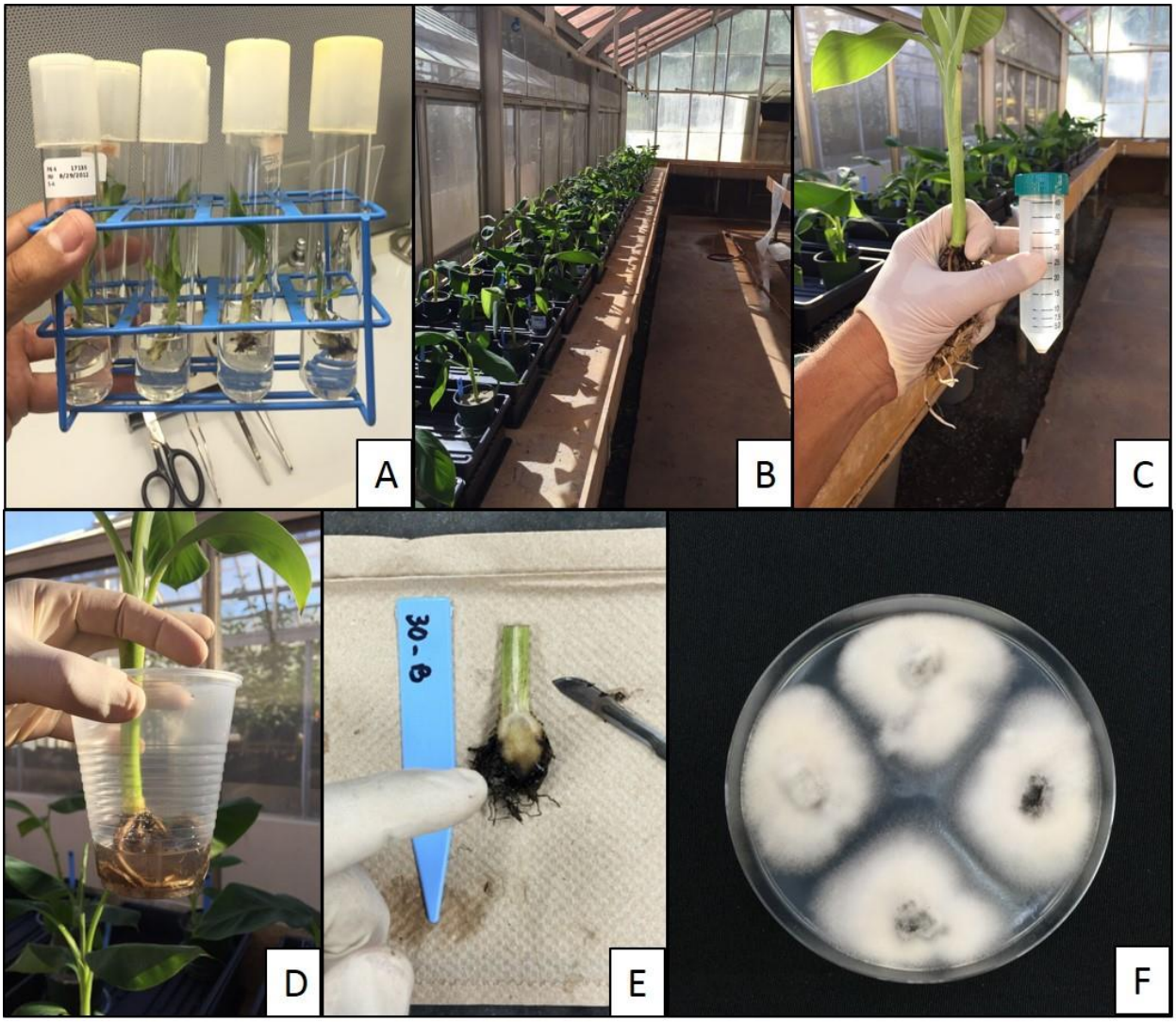

Figure 4. Methodology used to determine pathogenicity of *Fusarium* spp. isolates recovered from banana plants in Puerto Rico. (A) Plantlets were propagated by tissue culture [‘2-R-2, 500’ (race 1 differential), ‘Dole’ (race 2 differential), ‘5-A’ (race 4 differential)]; (B) prior to inoculation plantlets were acclimatized for a month under greenhouse conditions; (C and D) inoculations were carried out with a conidial spore suspension (10^6 spores/mL) for five minutes; (E) 40 days post inoculation disease severity was scored based on internal pseudostem discoloration; and (F) pathogen was recovered on Komada’s medium.

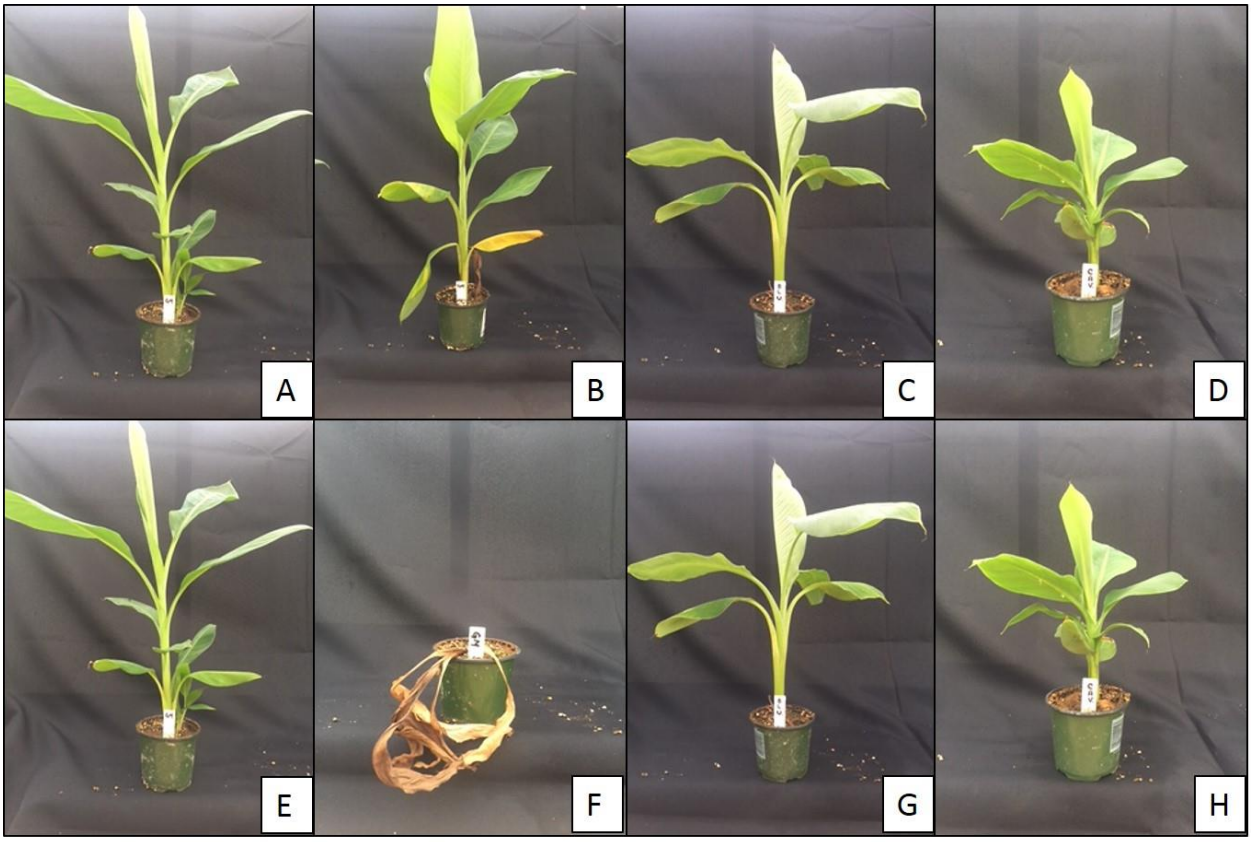

Figure 5. Disease progression and differential banana cultivar response to a race 1 isolate of *Fusarium oxysporum* f. sp. *cubense* (FOC) recovered from banana plants in Puerto Rico. Series (A-D)/20 days after inoculation: (A) Non-inoculated '2-R-2,500' control plant showing no external symptoms; (B) inoculated '2-R-2,500' showing intense yellowing of older leaves; (C) inoculated 'Dole,' showing no external symptoms; (D) inoculated '5-A' showing no external symptoms. Series (E-H)/40 days after inoculation: (E) non-inoculated '2-R-2,500' control plant showing no external symptoms; (F) inoculated '2-R-2,500' dead; (G) inoculated 'Dole,' showing no external symptoms and (H) inoculated '5-A' showing no external symptoms.

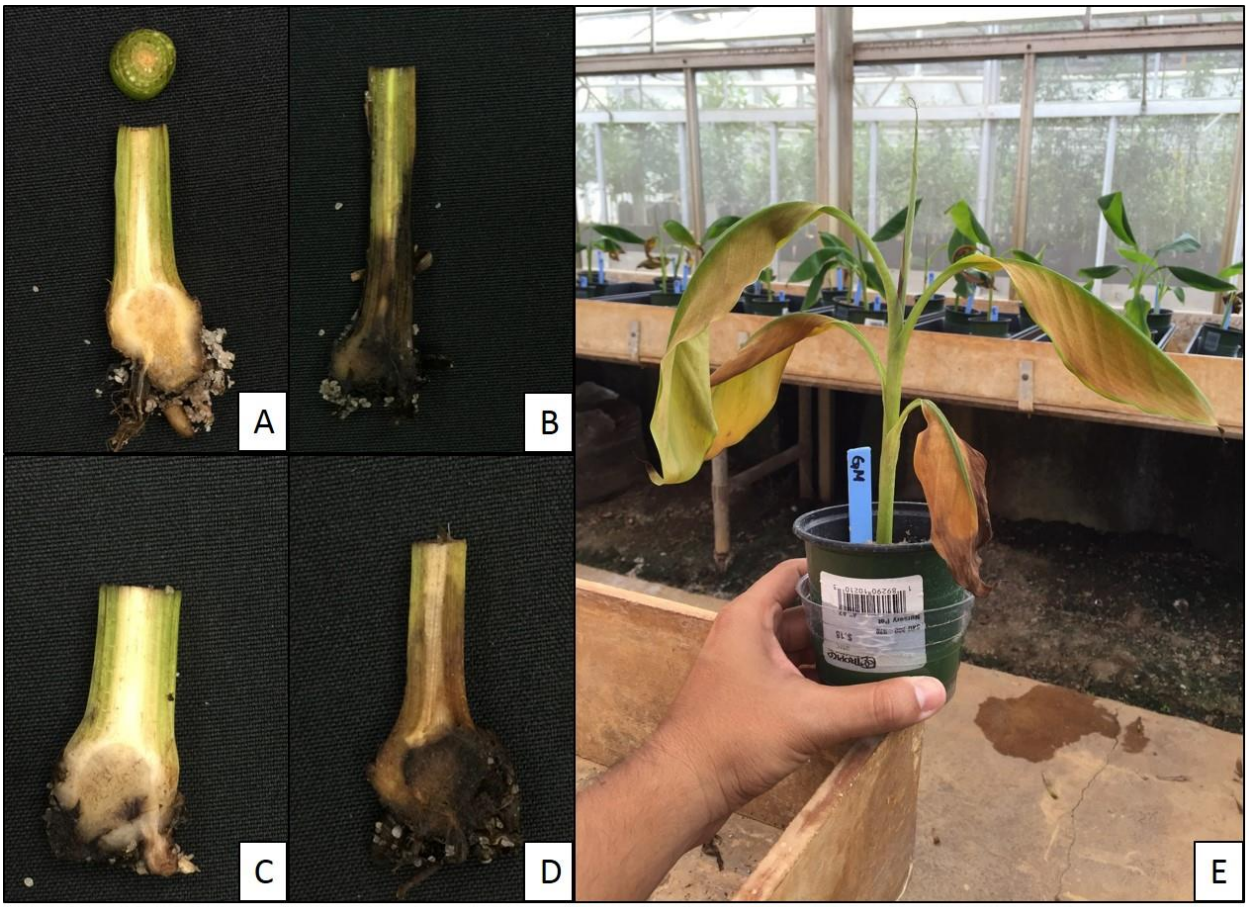

Figure 6. Differential banana cultivar response to a race 2 isolate of *Fusarium oxysporum* f. sp. *cubense* (FOC) recovered from banana plants in Puerto Rico. (A) non-inoculated 'Dole' control plant showing no vascular discoloration; (B) inoculated 'Dole' showing a rating of 8 (50-74% pseudostem discoloration); (C) inoculated '2-R-2,500' showing slight internal rhizome discoloration; (D) inoculated '5-A' (Cavendish) showing a rating of 7 (25-49% rhizome discoloration); (E) Inoculated tissue culture plantlet showing characteristic symptoms of Panama disease including wilting and intense yellowing of older leaves.

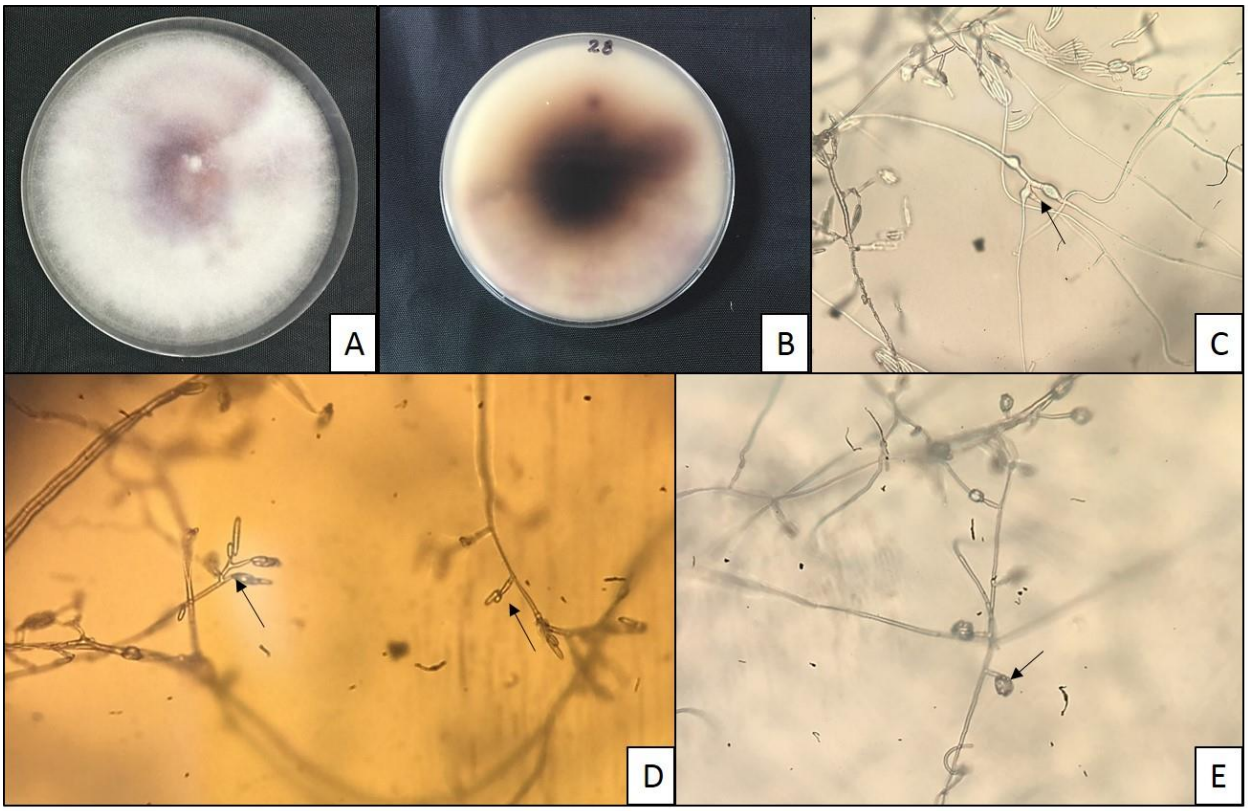

Figure 7. Macroscopic and microscopic morphological traits used in the identification of *Fusarium* spp. isolates recovered from banana plants in Puerto Rico. (A) Colony color and (B) pigmentation produced after 7 days in complete darkness on potato dextrose agar (PDA); (C) chlamydospores produced after 14 days on carnation leaf agar (CLA); (D) polyphialides (left) and monophialides (right) of *F. sacchari*; and (E) monophialide of *F.oxysporum*.

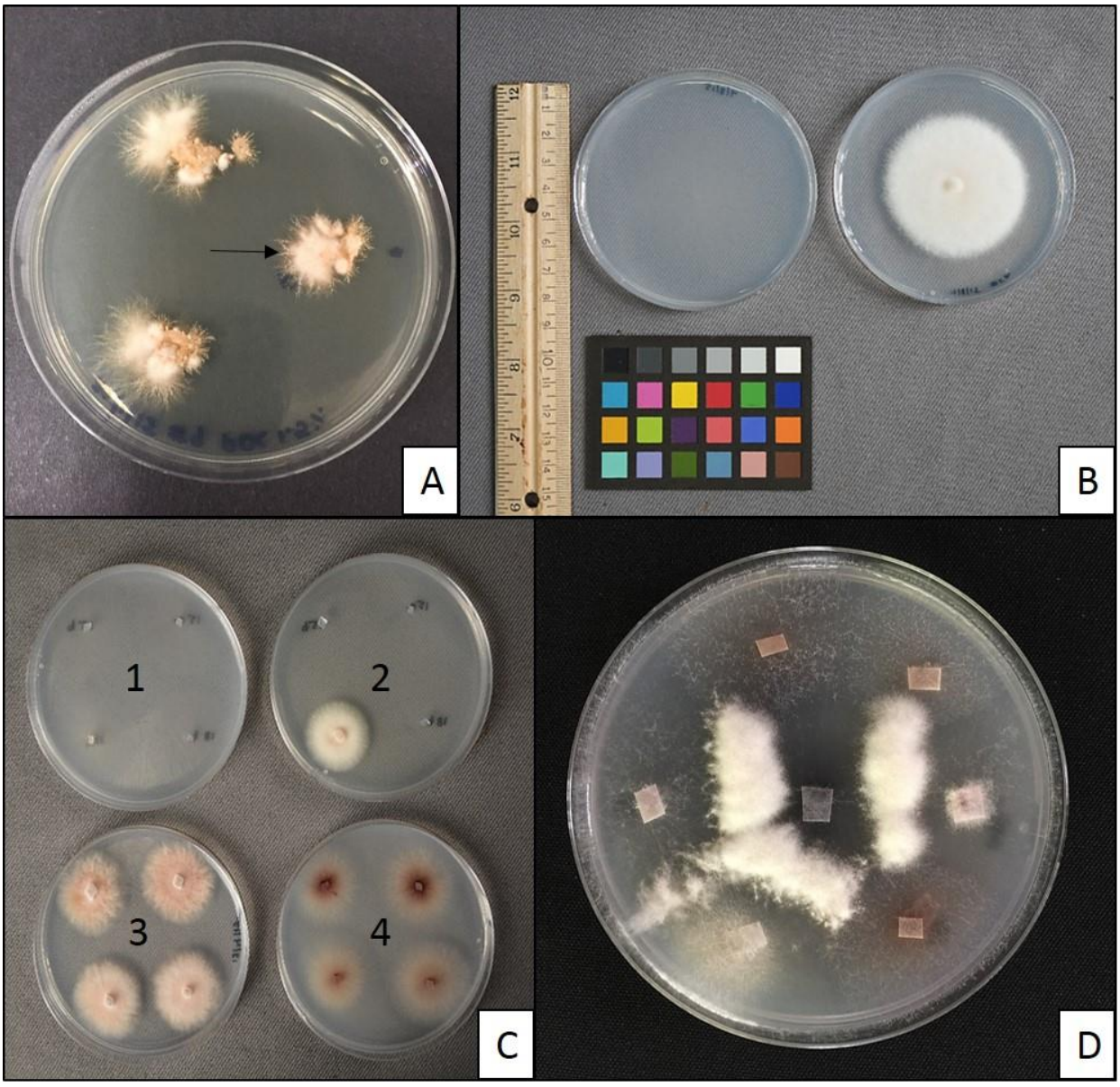

Figure 8. Vegetative compatibility group (VCG) determination procedure for *Fusarium* spp. isolates recovered from banana plants in Puerto Rico. (A) Sectors from wild type isolates generated on potato dextrose agar (PDA) amended with 1.5% chlorate (note arrow); (B) phenotypic differences between a *nit* mutant and a wild-type sector on minimal media (MM); (C) four phenotyping media containing (1) nitrate, (2) nitrite, (3) hypoxanthine and (4) ammonium; (D) heterokaryon formation between tester (center) and unknown isolates in the same VCG.

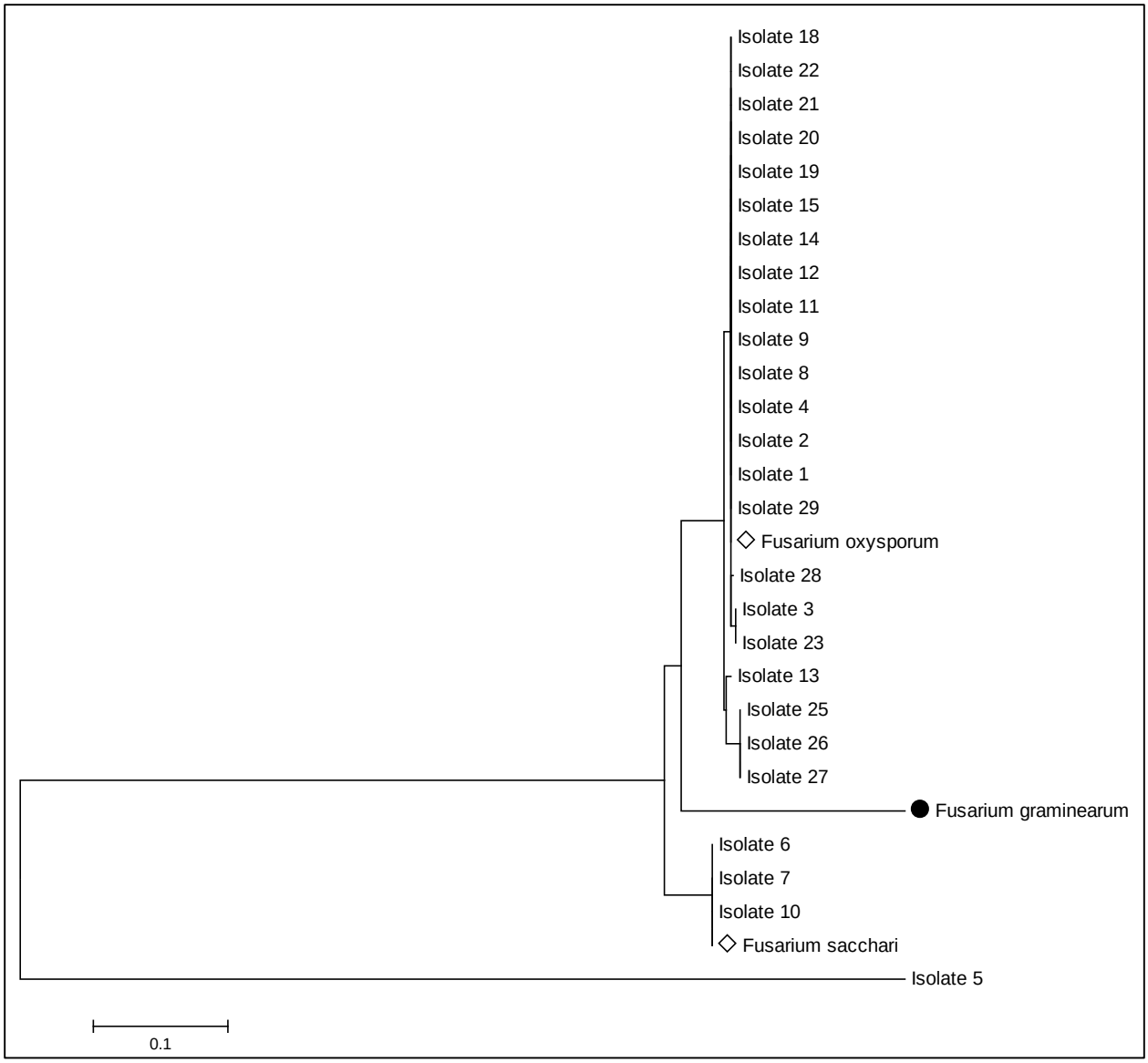

Figure 9. Phylogenetic tree for *Fusarium* spp. isolates recovered from banana plants in Puerto Rico built based on the sequences of the partial translation elongation factor 1α (TEF-1α) gene (•) Outgroup: *F. inflexum*. (◊) Sequences from GenBank with the highest homology.

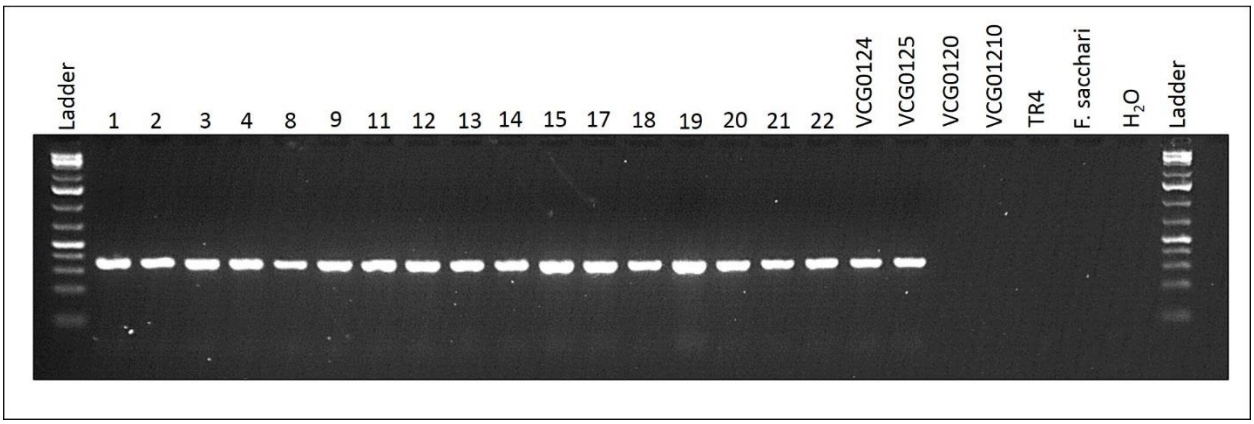

Figure 10. Results from PCR amplification with Liu et al. (2006) *Fusarium oxysporum* f. sp. *cubense* (FOC) race 1 specific primers in a 1.5% agarose gel. Ladder – 100 bp ladder; Lanes 2 - 18 – FOC isolates recovered from symptomatic *Musa* spp. in Puerto Rico (*numbers above lanes correspond to the number of the isolate in the tables*); VCG 0124 tester isolate; VCG 0125 tester isolate; VCG 0120 tester isolate; VCG 01210 tester isolate; TR4 control (DNA); *F. sacchari* – negative control; and H_2O – negative control. Expected fragment for FOC race 1 specific primers was 355 bp.

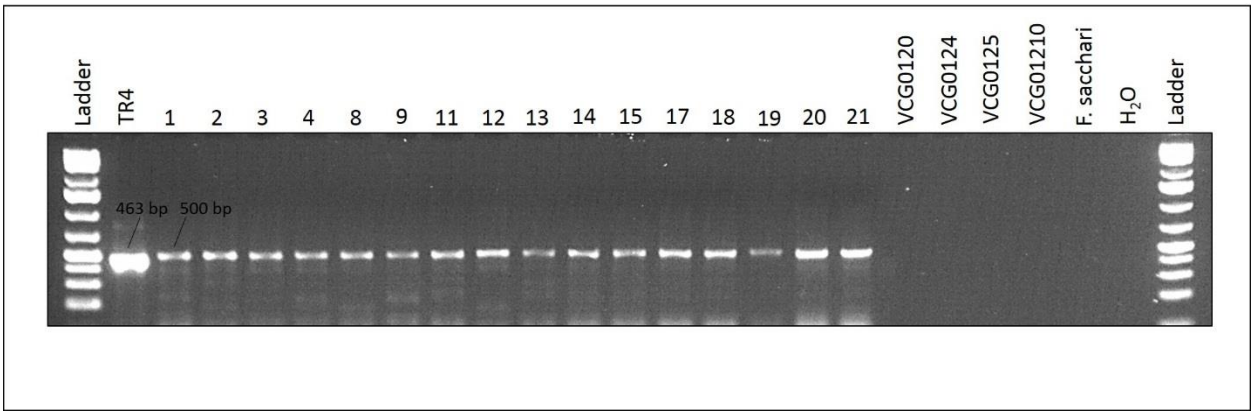

Figure 11. Results from PCR amplification with Dita et al. (2010) *Fusarium oxysporum* f. sp. *cubense* (FOC) TR4 specific primers in a 1.5% agarose gel. Ladder – 100 bp ladder; TR4 - Control (DNA); Lanes 3-18 – FOC isolates recovered from symptomatic *Musa* spp. in Puerto Rico (*numbers above lanes correspond to the number of the isolate in the tables*); VCG 0120 - tester isolate; VCG 0124 - tester isolate; VCG 0125 - tester isolate; VCG 01210 - tester isolate; *F. sacchari* – negative control; and H_2O – negative control. Expected fragment for FOC TR4 specific primers was 463 bp.

Table 1. Disease severity scale used in rating *Fusarium* spp. isolates pathogenicity on banana (*Musa* spp.).[a]

Class	Description
1	No rhizome discoloration
2	Slight internal rhizome discoloration restricted < 10%; no pseudostem discoloration
3	10-25% rhizome discoloration; no pseudostem discoloration
4	< 25% rhizome discoloration, pseudostem slightly discolored limited single sheath
5	< 10% pseudostem discolored
6	10-24% pseudostem discolored
7	25-49% pseudostem discolored
8	50-74% pseudostem discolored
9	< 75% pseudostem discolored
10	Dead

[a] Disease severity scale adapted from Ploetz and Shepard, 1989

Table 2. Forward and reverse primer sequences used in PCR amplification of the partial translation elongation factor 1α (TEF-1α) gene in *Fusarium* spp. and *F. oxysporum* f. sp. *cubense* race specific primers sets.

Primer	Sequence	Size (bp)
O'Donnell et al., 1998 (TEF-1α)	EF-1: 5'ATGGGTAAGGA(A/G)GACAAGAC EF-2: 5'GGA(G/A)GTACCAGT(G/C)ATCATGTT	**648**
Liu et al., 2006 (FOC race 1 specific)	PBL: 5'AGTCGCCCTTAACATACAGATATA PBR: 5′AGTCGCCCTTCGCATATGAGATGC	**355**
Dita et al., 2010 (FOC race TR4 specific)	FocTR4-F: 5'CACGTTTAAGGTGCCATGAGAG FocTR4-R: 5'CGCACGCCAGGACTGCCTCGTGA	**463**

Table 3. Reagents, concentrations and volumes used in single PCR reactions for the *Fusarium* spp. partial translation elongation factor 1α (TEF-1α) gene and *F. oxysporum* f. sp. *cubense* race specific primers sets.

Reagent	**Concentration**	**TEF-1α**[a]	**FOC Race 1**[b]	**FOC RaceTR4**[c]
5X PCR Buffer[d]		5.0	5.0	5.0
$MgCL_2$	(25mM)	2.5	2.5	2.5
dNTPs	(10 µm)	1.0	1.0	1.0
Primer F	(2.5 pmoles/µL)	1.0	1.0	1.0
Primer R	(2.5 pmoles/µL)	1.0	1.0	1.0
Taq Polymerase	(5U/µL)	0.25	0.25	0.25
ddH_20	-	12.25	12.25	12.25
Template	(5-10 ng/µL)	2.0	2.0	2.0

[a] O'Donnell et al., 1998 (TEF-1α)

[b] Liu et al., 2006 (FOC race 1 specific)

[c] Dita et al., 2010 (FOC race TR4 specific)

[d] All reagents were part of a PCR package from Promega including the GoTaq® Flexi DNA Polymerase (Promegea, Madison, WI)

Table 4. Summary information for host, sub-group, genome, date and location for *Fusarium* spp. fungal isolates recovered from symptomatic banana (*Musa* spp.) plants in Puerto Rico.

Isolate	Host accession	Sub-group	Genome	Date	Township
1	2-R-2,500	Gross Michel	AAA	08/13/08	Isabela
2	Golden Pillow	Silk	AAB	12/18/08	Isabela
3	Manzano	Silk	AAB	03/26/09	Isabela
4	2-R-2,500	Gross Michel	AAA	03/26/09	Isabela
5	Dwarf Orinoco	Bluggoe	ABB	05/08/09	Isabela
6	Dwarf Orinoco	Bluggoe	ABB	05/08/09	Isabela
7	Dwarf Chamaluco	Bluggoe	ABB	05/08/09	Isabela
8	Dwarf Chamaluco	Bluggoe	ABB	05/08/09	Isabela
9	Mafafo Dominicano	Bluggoe	ABB	05/08/09	Isabela
10	Cardaba	Saba	ABB	05/08/09	Isabela
11	Ney Poovan	Ney Poovan	AB	05/08/09	Isabela
12	TMB 1378	Synthetic hybrid	AABB	05/08/09	Isabela
13	Dwarf Orinoco	Bluggoe	ABB	06/28/10	Isabela
14	Mafafo Dominicano	Bluggoe	ABB	06/28/10	Isabela
15	TMB 1378	Synthetic hybrid	AABB	06/28/10	Isabela
16	Cacambou	Bluggoe	ABB	06/28/10	Isabela
17	Mossmun	Bluggoe	ABB	06/28/10	Isabela
18	Tigua	Iholena	AAB	09/28/10	Isabela
19	Mafafo de Adjuntas	Bluggoe	ABB	09/28/10	Isabela
20	Mossmum	Bluggoe	ABB	09/28/10	Isabela
21	Bluggoe	Bluggoe	ABB	10/26/10	Mayaguez
22	Bluggoe	Bluggoe	ABB	05/05/12	Aguada
23	Gross Michel	Gross Michel	AAA	12/23/14	Mayaguez
24	Gross Michel	Gross Michel	AAA	12/23/14	Mayaguez
25	Gross Michel	Gross Michel	AAA	12/23/14	Mayaguez
26	Bluggoe	Bluggoe	ABB	12/23/14	Mayaguez
27	Mafafo	Bluggoe	ABB	02/01/15	Gurabo
28	Mafafo	Bluggoe	ABB	11/26/15	Gurabo

Table 5. Diseases severity of banana (*Musa* spp.) plantlets inoculated with *Fusarium* spp. isolates recovered from symptomatic banana plants in Puerto Rico (results shown for two separate inoculations).

Isolate	Disease severity[a] (R1)				Disease severity (R2)			
	G.M.[b]	Bluggoe	Caven.	Control[c]	G.M.	Bluggoe	Caven.	Control
1	2^{2}	2^{2}	10^{1}	1	4	2	2	1
2	7^{2}	4^{2}	10^{2}	1	10^{1}	1	1	1
3	10^{2}	1^{2}	10^{1}	1	10^{1}	1	1	1
4	10^{2}	10^{12}	9^{2}	1	1	2	1	1
5	1^{2}	10^{2}	5^{2}	1	1	1	1	1
6	10^{2}	1^{2}	1^{2}	1	1	1	1	1
7	3^{2}	1^{2}	2^{2}	1	1	1	1	1
8	9^{2}	1^{2}	10^{2}	1	1	10^{1}	1	1
9	2^{2}	3^{2}	1^{2}	1	1	1	1	1
10	10^{2}	1^{2}	9^{2}	1	1	1	1	1
11	1^{2}	10^{2}	9^{2}	1	3	10^{1}	2	1
12	2^{2}	9^{2}	7^{2}	1	1	4	1	1
13	1^{2}	10^{12}	4^{2}	1	1	1	1	1
14	2^{2}	1^{2}	10^{2}	1	1	1	1	1
15	1^{2}	1^{2}	1^{2}	1	4	1	1	1
16	1^{2}	1^{2}	10^{2}	1	1	1	1	1
17	1^{2}	10^{2}	9^{2}	1	1	1	1	1
18	1^{2}	10^{12}	9^{2}	1	4	1	1	1
19	2^{2}	10^{12}	10^{12}	1	1	1	1	1
20	1^{2}	10^{12}	2^{2}	1	1	1	1	1
21	2^{2}	1^{2}	1^{2}	1	2	1	1	1
22	2^{2}	10^{12}	1^{2}	1	1	10^{1}	3	1
23	3^{2}	1^{2}	10^{2}	1	1	1	1	1
24	10^{2}	1^{2}	10^{2}	1	3	1	1	1
25	10^{12}	10^{2}	2^{2}	1	4	3	3	1
26	10^{2}	10^{12}	2^{2}	1	1	1	1	1
27	4^{2}	9^{2}	9^{2}	1	1	1	1	1
28	3^{2}	10^{12}	9^{2}	1	1	1	1	1

[b] Accessions/cultivars used in pathogenicity tests included 'Gros Michel' (G.M.), 'Dole' (Bluggoe) and '5-A' (Cavendish)

[1] Plant dead before 40 days after inoculation

[2] *Fusarium* spp. recovered on Komada's medium

Table 6. Mean diseases severity for banana (*Musa* spp.) plantlets inoculated with *Fusarium* spp. isolates recovered from symptomatic banana plants in Puerto Rico.

Isolate	Mean disease severity			
	G.M.	Bluggoe	Caven.	Control
1	3	2	6	1
2	8.5	2.5	5.5	1
3	10	1	5.5	1
4	5.5	6	3	1
5	1	5.5	3	1
6	5.5	1	1	1
7	2	1	1.5	1
8	5	5.5	5.5	1
9	1.5	2	1	1
10	5.5	1	5	1
11	2	10	5.5	1
12	1.5	6.5	4	1
13	1	5.5	2.5	1
14	1.5	1	5.5	1
15	2.5	1	1	1
16	1	1	5.5	1
17	1	5.5	5	1
18	2.5	5.5	5	1
19	1.5	5.5	5.5	1
20	1	5.5	1.5	1
21	2	1	1	1
22	1.5	10	2	1
23	2	1	5.5	1
24	6.5	1	5.5	1
25	7	6.5	2.5	1
26	5.5	6.5	1.5	1
27	2.5	5	5	1
28	2	5.5	5	1

[b] Accessions/cultivars used in pathogenicity tests included 'Gros Michel' (G.M.), 'Dole' (Bluggoe) and '5-A' (Cavendish)

[1] Plant dead before 40 days after inoculation

[2] *Fusarium* spp. recovered on Komada's medium

Table 7. Microscopic and macroscopic morphological and phenotypic traits used for identification of *Fusarium* spp. isolates recovered from symptomatic banana (*Musa* spp.) plants in Puerto Rico.

Isolate	CLA Characteristics[a]				PDA Characteristics[b]	
	Microconidia[c]		Phialides	Chlam.		
	+/-	Heads/Chains			Pigment	Color
1	+	Heads	Short mono	+	Purple	Pinkish
2	+	Heads	Short mono	+	No pigment	Pinkish
3	+	Heads	Short mono	+	Purple	White
4	+	Heads	Short mono	+	No pigment	Pinkish
5	+	Heads	Mono/Poly	-	No pigment	Orange
6	+	Heads	Mono/Poly	+	Purple	Pinkish
7	+	Heads	Mono/Poly	-	Red	Pinkish
8	+	Heads	Short mono	+	Purple	Pinkish
9	+	Heads	Short mono	+	Purple	Pinkish
10	+	Heads	Mono/Poly	+	No pigment	Orange
11	+	Heads	Short mono	+	Purple	Pinkish
12	+	Heads	Short mono	+	Purple	Pinkish
13	+	Heads	Short mono	+	No pigment	Pinkish
14	+	Heads	Short mono	+	Purple	Peach
15	+	Heads	Short mono	+	Purple	Pinkish
16	+	Heads	Mono/Poly	+	Red	Orange
17	+	Heads	Short mono	+	Purple	Peach
18	+	Heads	Short mono	+	No pigment	Peach
19	+	Heads	Short mono	+	Purple	Peach
20	+	Heads	Short mono	+	Purple	White
21	+	Heads	Short mono	+	Purple	Pinkish
22	+	Heads	Short mono	+	Red	Orange
23	+	Heads	Short mono	+	Purple	Peach
24	+	Heads	Short mono	+	Purple	White
25	+	Heads	Short mono	+	Red	Pinkish
26	+	Heads	Short mono	+	Purple	Pinkish
27	+	Heads	Short mono	+	Purple	Pinkish
28	+	Heads	Mono/Poly	+	Purple	Peach

[a] Determined on CLA after 14 days at 25ºC in alternating 12-hour periods of light/darkness
[b] Determined on PDA after 7 days at 25ºC in complete darkness
[c] (+) Presence or (–) absence

Table 8. Shape, length, width and growth rate for micro and macroconidia for *Fusarium* spp. isolates recovered from symptomatic banana (*Musa* spp.) plants in Puerto Rico.

Isolate	Microconidia			Macroconidia			
	Shape	Length (µm)	Width (µm)	Shape	Length (µm)	Width (µm)	Growth (mm)[b]
1	Oval to kidney	8.61 hijk	1.85 a	Slightly curved	45.41 n	3.05 hi	23.00 a
2	Oval to kidney	7.73 defgh	1.95 abcde	Slightly curved	24.01 abc	2.36 a	30.00 fgh
3	Oval to kidney	8.30 ghijk	1.86 ab	Slightly curved	55.28 o	2.80 defg	28.00 cdefgh
4	Oval to kidney	7.16 bcde	1.92 abcd	Slightly curved	23.12 ab	2.54 abc	31.00 h
5	Oval	8.08 efghij	1.84 a	Falcate	28.62 efg	2.72 cdef	30.00 fgh
6	Oval	10.38 l	2.17 fghij	Falcate	26.22 cde	2.44 ab	27.50 cdefg
7	Oval	11.76 m	2.34 jk	Falcate	42.09 m	3.07 hij	25.00 abc
8	Oval to kidney	7.50 cdefg	1.88 abc	Slightly curved	32.44 ij	2.82 defg	29.50 efgh
9	Oval to kidney	6.98 abcd	2.01 abcdefg	Slightly curved	37.52 kl	2.97 ghi	26.50 bcde
10	Oval	8.14 fghij	1.99 abcdef	Falcate	21.02 a	2.62 bcd	31.00 h
11	Oval to kidney	8.12 fghij	2.06 cdefghi	Slightly curved	29.20 efgh	2.69 cde	25.50 abcd
12	Oval to kidney	7.10 bcd	2.00 abcdef	Slightly curved	25.04 bcd	3.06 hij	30.50 gh
13	Oval to kidney	7.28 bcdef	2.12 efghi	Slightly curved	31.97 hij	2.76 defg	30.50 gh
14	Oval to kidney	6.85 abcd	2.06 cdefghi	Slightly curved	30.66 ghi	2.90 fgh	29.50 efgh
15	Oval to kidney	8.12 fghij	2.05 cdefg	Slightly curved	38.01 l	3.26 jk	23.00 a
16	Oval	8.06 efghi	2.06 cdefgh	Falcate	24.19 bc	2.74 cdef	30.00 fgh
17	Oval to kidney	7.47 cdefg	2.09 defghi	Slightly curved	25.37 bcd	2.84 efg	30.00 fgh
18	Oval to kidney	8.68 ijk	2.04 bcdefg	Slightly curved	45.51 n	2.97 ghi	23.50 ab
19	Oval to kidney	8.39 ghijk	2.08 defghi	Slightly curved	36.33 kl	2.90 efgh	23.00 a
20	Oval to kidney	6.37 ab	2.18 ghijk	Slightly curved	26.56 cde	2.74 cdef	29.50 efgh
21	Oval to kidney	6.08 a	2.12 efghi	Slightly curved	37.19 kl	3.63 l	27.00 cdef
22	Oval to kidney	8.99 jk	2.11 efghi	Slightly curved	48.46 n	3.12 ijk	25.50 abcd
23	Oval to kidney	9.15 k	1.84 a	Slightly curved	24.66 bcd	3.72 l	28.50 defgh
24	Oval to kidney	8.04 efghi	2.24 ijk	Slightly curved	27.29 def	2.77 defg	27.50 cdefg
25	Oval to kidney	6.80 abc	2.23 hijk	Slightly curved	29.25 efgh	3.07 hij	26.50 bcde
26	Oval to kidney	7.70 cdefgh	1.91 abcd	Slightly curved	34.84 jk	4.26 m	26.50 bcde
27	Oval to kidney	10.43 l	2.36 k	Slightly curved	36.52 kl	3.65 l	29.00 efgh
28	Oval	12.89 n	2.24 ijk	Falcate	29.81 fghi	3.30 k	29.00 efgh

[a] Means with the same letter within a column are not significantly different ($p > 0.05$); Mean based on 25 measurements

[b] Radial growth determined by placing a single macroconidium on PDA at 25ºC in complete darkness and measuring after 72 h

Table 9. Phenotyping media and isolate reaction used to determine type of *nit* mutant for *Fusarium* spp. isolates recovered from symptomatic banana (*Musa* spp.) plants in Puerto Rico.

Isolate	Phenotyping Media				Type of *nit*
	Nitrate	Nitrite	Hypoxanthine	Ammonium	
1[a]	**-, -, -**	**+, +, +**	**+, +, -**	**+, +, +**	1, 1, M
2	**-**	**+**	**+**	**+**	1
3	**-, -**	**+, +**	**+, -**	**+, +**	1, M
4	**-**	**+**	**+**	**+**	1
5	**-**	**+**	**+**	**+**	1
6	**-**	**+**	**+**	**+**	1
7	**-**	**+**	**+**	**+**	1
8	**-**	**-**	**+**	**+**	3
9	**-**	**+**	**+**	**+**	1
10	**-**	**+**	**+**	**+**	1
11	**-**	**+**	**+**	**+**	1
12	**-**	**+**	**+**	**+**	1
13	**-, -**	**-, -**	**+, +**	**+, +**	3, 3
14	**-**	**+**	**+**	**+**	1
15	**-**	**-**	**+**	**+**	3
16	**-**	**-**	**+**	**+**	3
17	**-, -**	**+, +**	**+, +**	**+, +**	1, 1
18	**-, -**	**+, +**	**+, -**	**+, +**	1, M
19	**-, -**	**+, -**	**+, +**	**+, +**	1, 3
20	**-**	**+**	**+**	**+**	1
21	**-**	**+**	**+**	**+**	1
22	**-**	**-**	**+**	**+**	3
23	**-, -**	**-, +**	**+, -**	**+, +**	3, M
24	**-, -, -**	**+, -, -**	**+, +, +**	**+, +, +**	1, 3, 3
25	**-**	**-**	**+**	**+**	3
26	**-**	**+**	**+**	**+**	1
27	**-, -**	**+, +**	**+, -**	**+, +**	1, M
28	**-**	**-**	**+**	**+**	3

[a] In some cases multiple *nit* mutant (e.g., 1, 3 and/or M) had to be generated for a particular isolate to include in complementation assays.

Table 10. Vegetative compatibility group (VCG) test results from complementation assays for *Fusarium* spp. isolates recovered from symptomatic banana (*Musa* spp.) plants in Puerto Rico (results shown for two replicates).

Isolate	VCG tester - NitM (n = 1)[a]				VCG tester - NitM (n = 2)				HSI[b]
	0120	0124	0125	01210	0120	0124	0125	01210	
1[c]	-	-	-	-	-	-	-	-	**no**
2	-	+	-	-	-	+	-	-	
3	-	+	-	-	-	+	-	-	
4	-	+	-	-	-	+	-	-	
5	-	-	-	-	-	-	-	-	
6	-	-	-	-	-	-	-	-	
7	-	-	-	-	-	-	-	-	
8	-	+	-	-	-	+	-	-	
9	-	+	-	-	-	+	-	-	
10	-	-	-	-	-	-	-	-	
11	-	+	-	-	-	+	-	-	
12	-	+	-	-	-	+	-	-	
13[c]	-	-	-	-	-	-	-	-	**no**
14	-	+	-	-	-	+	-	-	
15	-	+	-	-	-	+	-	-	
16	-	-	-	-	-	-	-	-	
17	-	+	-	-	-	+	-	-	
18[c]	-	-	-	-	-	-	-	-	**no**
19	-	+	-	-	-	+	-	-	
20	-	+	-	-	-	+	-	-	
21	-	+	-	-	-	+	-	-	
22	-	+	-	-	-	+	-	-	
23[c]	-	-	-	-	-	-	-	-	**no**
24[c]	-	-	-	-	-	-	-	-	**no**
25[d]	+	-	-	-	-	-	-	-	
26	-	+	-	-	-	+	-	-	
27	-	+	-	-	-	+	-	-	
28	-	-	-	-	-	-	-	-	

[a] (+) = Heterokaryon formation; (-) = No heterokaryon formation

[b] Heterokaryon self-incompatible (HSI)

[c] These isolates did not form heterokaryon with any of the testers used and were evaluated for heterokaryon self-incompatibility

[d] Formed heterokaryon only during one of the two complementation assays/pairings

Table 11. Translation elongation factor 1α (TEF 1-α) sequences of *Fusarium* spp. from isolates in this study and GenBank and used as reference during the construction of the phylogenetic tree.

Isolate	Q. coverage (%)	Identity (%)	Accession #	Species
1	93	98	KP964900.1	*F. oxysporum*
2	92	99	KP964900.1	*F. oxysporum*
3	91	98	KP964900.1	*F. oxysporum*
4	92	99	KP964900.1	*F. oxysporum*
5	90	99	DQ465939.1	*F. sacchari*
6	88	99	DQ465939.1	*F. sacchari*
7	91	98	DQ465939.1	*F. sacchari*
8	92	98	KP964900.1	*F. oxysporum*
9	87	99	KP964900.1	*F. oxysporum*
10	85	99	DQ465939.1	*F. sacchari*
11	93	99	KP964900.1	*F. oxysporum*
12	92	98	KP964900.1	*F. oxysporum*
13	86	99	KP964900.1	*F. oxysporum*
14	87	99	KP964900.1	*F. oxysporum*
15	86	98	KP964900.1	*F. oxysporum*
16[a]	-	-	-	-
17	90	98	KP964900.1	*F. oxysporum*
18	89	99	KP964900.1	*F. oxysporum*
19	89	99	KP964900.1	*F. oxysporum*
20	89	99	KP964900.1	*F. oxysporum*
21	92	97	KP964900.1	*F. oxysporum*
22	89	99	KP964900.1	*F. oxysporum*
23	91	99	KR072627.1	*F. oxysporum*
24	87	99	KR072627.1	*F. oxysporum*
25	90	99	JQ965436.1	*F. oxysporum*
26	86	97	KP964900.1	*F. oxysporum*
27	88	98	KP964900.1	*F. oxysporum*
28[b]	-	-	-	-
Outgroup			AF008479.1	*F. inflexum*

[a] DNA extracted from these isolates did not amplify.
[b] PCR amplification has not been sent for sequencing

Table 12. Determination of species, race and vegetative compatibility group (VCG) for *Fusarium* spp. isolates recovered from symptomatic banana (*Musa* spp.) plants in Puerto Rico.

Isolate	Host accession	Sub-group	Species	Race	VCG
1	2-R-2,500	Gross Michel	*F. oxysporum*	Race 1	-
2	Golden Pillow	Silk	*F. oxysporum*	Race 1	0124
3	Manzano	Silk	*F. oxysporum*	Race 1	0124
4	2-R-2,500	Gross Michel	*F. oxysporum*	Race 1	0124
5	Dwarf Orinoco	Bluggoe	*F. sacchari*	-	-
6	Dwarf Orinoco	Bluggoe	*F. sacchari*	-	-
7	Dwarf Chamaluco	Bluggoe	*F. sacchari*	-	-
8	Dwarf Chamaluco	Bluggoe	*F. oxysporum*	Race 2	0124
9	Mafafo Dominicano	Bluggoe	*F. oxysporum*	Race 2	0124
10	Cardaba	Saba	*F. sacchari*	-	-
11	Ney Poovan	Ney Poovan	*F. oxysporum*	Race 1	0124
12	TMB 1378	Synthetic hybrid	*F. oxysporum*	Race 1	0124
13	Dwarf Orinoco	Bluggoe	*F. oxysporum*	Race 2	-
14	Mafafo Dominicano	Bluggoe	*F. oxysporum*	Race 2	0124
15	TMB 1378	Synthetic hybrid	*F. oxysporum*	Race 2	0124
16	Cacambou	Bluggoe	*F. sacchari*	-	-
17	Mossmun	Bluggoe	*F. oxysporum*	Race 1	0124
18	Tigua	Iholena	*F. oxysporum*	Race 1	-
19	Mafafo de Adjuntas	Bluggoe	*F. oxysporum*	Race 2	0124
20	Mossmum	Bluggoe	*F. oxysporum*	Race 1	0124
21	Bluggoe	Bluggoe	*F. oxysporum*	Race 2	0124
22	Bluggoe	Bluggoe	*F. oxysporum*	Race 2	0124
23	Gross Michel	Gross Michel	*F. oxysporum*	Race 1	-
24	Gross Michel	Gross Michel	*F. oxysporum*	Race 1	-
25	Gross Michel	Gross Michel	*F. oxysporum*	Race 1	0120[d]
26	Bluggoe	Bluggoe	*F. oxysporum*	Race 2	0124
27	Mafafo	Bluggoe	*F. oxysporum*	Race 2	0124
28	Mafafo	Bluggoe	*F. sacchari*	-	-

[a] Species determined from pathogenicity tests, morphological assays and by sequence comparisons.
[b] Race determined from pathogenicity tests and by race specific primers/PCR amplifications.
[c] VCG determined during complementation assays.
[d] Paired in only one of the two complementation tests.

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APPENDIX

APPENDIX A – MEDIA RECIPE

Media	Recipe (1L H_2O)[a]
Water agar (2%)	20g Agar
Potato Dextrose Agar (PDA)	39g PDA
PDC (PDA+$KCLO_3$) (1.5%)	39g PDA+15g $KCLO_3$
Basal media	30g Sucrose, 1g KH_2PO_4, 0.50g $MgSO_4.7H_2O$, 0.50g KCL, 0.01g $FeSO_4.7H_2O$, 20g Agar, 0.2mL Trace element solution: (5g/95mL H_2O) Citric acid, (5g/95mL H_2O) $ZnSO_4.7H_2O$, (1g/95mL H_2O) $Fe(NH_4)_2(SO_4)_2.6H_2O$, (0.25g/95mL H_2O) $CuSO_4.5H_2O$, (0.05g/95mL H_2O) $MnSO_4.H_2O$, (0.05g/95mL H_2O) H_3BO_4, (0.05g/95mL H_2O) $NaMoO_4.2H_2O$
Phenotyping	Minimal media: Basal media + 1g Nitrate ($NaNO_3$), Ammonium: Basal media + 0.5g Ammonium tartrate, Hypoxanthine: Basal media + 0.1g Hypoxanthine, Nitrite: Basal media + 0.25g Nitrite ($NaNO_2$)
Komada's medium[b]	**Part 1**: 1g $Na_2B_4O_7.10H_2O$, 1g KH_2PO_4, 0.50g KCL, 0.50g $MgSO_4.7H_2O$, 0.01g Fe-Na-EDTA, 20g D - Galactose, 2g L – Asparagine, 15g Agar, **Part 2**: 1g PCNB, 0.5g Oxgall (Bile bovine), 0.3g Streptomycin sulfate.

[a]Autoclave conditions: 15 minutes, 15 psi and 141 ºC.

[b]Autoclave ingredients in **Part 1,** cool to 50 – 55 ºC and then add ingredients in **Part 2**. Acidify the medium to 3.8 – 4.0 pH with 10% Phosphoric acid

APPENDIX B – STATISTICAL ANALYSIS

Analysis of variance - Macroconidias

Length

Variable	N	R^2	Adj R^2	CV
Length	700	0.71	0.70	16.82

Analysis of variance table (Partial SS)

S.V.	SS	df	MS	F	p-value
Model.	49803.96	27	1844.59	60.82	<0.0001
Isolate	49803.96	27	1844.59	60.82	<0.0001
Error	20381.16	672	30.33		
Total	70185.12	699			

Test:Fisher LSD Alpha:=0.05 LSD:=3.05848

Error: 30.3291 df: 672

Isolate	Means	n	S.E.														
10	21.02	25	1.10	A													
4	23.12	25	1.10	A	B												
2	24.01	25	1.10	A	B	C											
17	24.19	25	1.10		B	C											
25	24.66	25	1.10		B	C	D										
12	25.04	25	1.10		B	C	D										
18	25.37	25	1.10		B	C	D										
6	26.22	25	1.10			C	D	E									
21	26.56	25	1.10			C	D	E									
26	27.29	25	1.10				D	E	F								
5	28.62	25	1.10					E	F	G							
11	29.20	25	1.10					E	F	G	H						
27	29.25	25	1.10					E	F	G	H						
30	29.81	25	1.10						F	G	H	I					
14	30.66	25	1.10							G	H	I					
13	31.97	25	1.10								H	I	J				
8	32.44	25	1.10									I	J				
28	34.84	25	1.10										J	K			
20	36.33	25	1.10											K	L		
29	36.52	25	1.10											K	L		
22	37.19	25	1.10											K	L		
9	37.52	25	1.10											K	L		
15	38.01	25	1.10												L		
7	42.09	25	1.10													M	
1	45.41	25	1.10														N
19	45.51	25	1.10														N
23	48.46	25	1.10														N
3	55.28	25	1.10														O

Means with a common letter are not significantly different (p > 0.05)

Width

Variable	N	R^2	Adj R^2	CV
Width	700	0.56	0.54	12.52

Analysis of variance table (Partial SS)

S.V.	SS	df	MS	F	p-value
Model.	117.87	27	4.37	31.12	<0.0001
Isolate	117.87	27	4.37	31.12	<0.0001
Error	94.28	672	0.14		
Total	212.15	699			

Test:Fisher LSD Alpha:=0.05 LSD:=0.20801

Error: 0.1403 df: 672

Isolate	Means	n	S.E.													
2	2.36	25	0.07	A												
6	2.44	25	0.07	A	B											
4	2.54	25	0.07	A	B	C										
10	2.62	25	0.07		B	C	D									
11	2.69	25	0.07			C	D	E								
5	2.72	25	0.07			C	D	E	F							
17	2.74	25	0.07			C	D	E	F							
21	2.74	25	0.07			C	D	E	F							
13	2.76	25	0.07				D	E	F	G						
26	2.77	25	0.07				D	E	F	G						
3	2.80	25	0.07				D	E	F	G						
8	2.82	25	0.07				D	E	F	G						
18	2.84	25	0.07					E	F	G						
20	2.90	25	0.07					E	F	G	H					
14	2.90	25	0.07						F	G	H					
9	2.97	25	0.07							G	H	I				
19	2.97	25	0.07							G	H	I				
1	3.05	25	0.07								H	I				
12	3.06	25	0.07								H	I	J			
27	3.07	25	0.07								H	I	J			
7	3.07	25	0.07								H	I	J			
23	3.12	25	0.07									I	J	K		
15	3.26	25	0.07										J	K		
30	3.30	25	0.07											K		
22	3.63	25	0.07												L	
29	3.65	25	0.07												L	
25	3.72	25	0.07												L	
28	4.26	25	0.07													M

Means with a common letter are not significantly different (p > 0.05)

Analysis of variance - Microconidia

Length

Variable	N	R^2	Adj R^2	CV
Length	700	0.46	0.44	20.22

Analysis of variance table (Partial SS)

S.V.	SS	df	MS	F	p-value
Model.	1604.13	27	59.41	21.32	<0.0001
Isolate	1604.13	27	59.41	21.32	<0.0001
Error	1872.64	672	2.79		
Total	3476.78	699			

Test:Fisher LSD Alpha:=0.05 LSD:=0.92708

Error: 2.7867 df: 672

Isolate	Means	n	S.E.													
22	6.08	25	0.33	A												
21	6.37	25	0.33	A	B											
27	6.80	25	0.33	A	B	C										
14	6.85	25	0.33	A	B	C	D									
9	6.98	25	0.33	A	B	C	D									
12	7.10	25	0.33		B	C	D									
4	7.16	25	0.33		B	C	D	E								
13	7.28	25	0.33		B	C	D	E	F							
18	7.47	25	0.33			C	D	E	F	G						
8	7.50	25	0.33			C	D	E	F	G						
28	7.70	25	0.33			C	D	E	F	G	H					
2	7.73	25	0.33				D	E	F	G	H					
26	8.04	25	0.33					E	F	G	H	I				
17	8.06	25	0.33					E	F	G	H	I				
5	8.08	25	0.33					E	F	G	H	I	J			
15	8.12	25	0.33						F	G	H	I	J			
11	8.12	25	0.33						F	G	H	I	J			
10	8.14	25	0.33						F	G	H	I	J			
3	8.30	25	0.33							G	H	I	J	K		
20	8.39	25	0.33							G	H	I	J	K		
1	8.61	25	0.33								H	I	J	K		
19	8.68	25	0.33									I	J	K		
23	8.99	25	0.33										J	K		
25	9.15	25	0.33											K		
6	10.38	25	0.33												L	
29	10.43	25	0.33												L	
7	11.76	25	0.33													M
30	12.89	25	0.33													N

Means with a common letter are not significantly different (p > 0.05)

Width

Variable	N	R^2	Adj R^2	CV
Width	700	0.16	0.13	16.04

Analysis of variance table (Partial SS)

S.V.	SS	df	MS	F	p-value
Model.	14.26	27	0.53	4.85	<0.0001
Isolate	14.26	27	0.53	4.85	<0.0001
Error	73.18	672	0.11		
Total	87.44	699			

Test:Fisher LSD Alpha:=0.05 LSD:=0.18327

Error: 0.1089 df: 672

Isolate	Means	n	S.E.										
5	1.84	25	0.07	A									
25	1.84	25	0.07	A									
1	1.85	25	0.07	A									
3	1.86	25	0.07	A	B								
8	1.88	25	0.07	A	B	C							
28	1.91	25	0.07	A	B	C	D						
4	1.92	25	0.07	A	B	C	D						
2	1.95	25	0.07	A	B	C	D	E					
10	1.99	25	0.07	A	B	C	D	E	F				
12	2.00	25	0.07	A	B	C	D	E	F				
9	2.01	25	0.07	A	B	C	D	E	F	G			
19	2.04	25	0.07		B	C	D	E	F	G			
15	2.05	25	0.07			C	D	E	F	G			
17	2.06	25	0.07			C	D	E	F	G	H		
14	2.06	25	0.07			C	D	E	F	G	H	I	
11	2.06	25	0.07			C	D	E	F	G	H	I	
20	2.08	25	0.07				D	E	F	G	H	I	
18	2.09	25	0.07				D	E	F	G	H	I	
23	2.11	25	0.07					E	F	G	H	I	
22	2.12	25	0.07					E	F	G	H	I	
13	2.12	25	0.07					E	F	G	H	I	
6	2.17	25	0.07						F	G	H	I	J
21	2.18	25	0.07							G	H	I	J
27	2.23	25	0.07								H	I	J
26	2.24	25	0.07									I	J
30	2.24	25	0.07									I	J
7	2.34	25	0.07										J
29	2.36	25	0.07										

Means with a common letter are not significantly different (p > 0.05)

Analysis of variance – Growth rate

Variable	N	R^2	Adj R^2	CV	
Growth rate	56	0.82	0.65	5.98	

Analysis of variance table (Partial SS)

S.V.	SS	df	MS	F	p-value
Model.	356.43	27	13.20	4.80	<0.0001
Isolate	356.43	27	13.20	4.80	<0.0001
Error	77.00	28	2.75		
Total	433.43	55			

Test:Fisher LSD Alpha:=0.05 LSD:=3.39690

Error: 2.7500 df: 28

Isolate	Means	n	S.E.								
1	23.00	2	1.17	A							
15	23.00	2	1.17	A							
20	23.00	2	1.17	A							
19	23.50	2	1.17	A	B						
7	25.00	2	1.17	A	B	C					
11	25.50	2	1.17	A	B	C	D				
23	25.50	2	1.17	A	B	C	D				
9	26.50	2	1.17		B	C	D	E			
28	26.50	2	1.17		B	C	D	E			
27	26.50	2	1.17		B	C	D	E			
22	27.00	2	1.17			C	D	E	F		
26	27.50	2	1.17			C	D	E	F	G	
6	27.50	2	1.17			C	D	E	F	G	
3	28.00	2	1.17			C	D	E	F	G	H
25	28.50	2	1.17				D	E	F	G	H
30	29.00	2	1.17					E	F	G	H
29	29.00	2	1.17					E	F	G	H
21	29.50	2	1.17					E	F	G	H
8	29.50	2	1.17					E	F	G	H
14	29.50	2	1.17					E	F	G	H
18	30.00	2	1.17						F	G	H
5	30.00	2	1.17						F	G	H
2	30.00	2	1.17						F	G	H
17	30.00	2	1.17						F	G	H
13	30.50	2	1.17							G	H
12	30.50	2	1.17							G	H
4	31.00	2	1.17								H
10	31.00	2	1.17								H

Means with a common letter are not significantly different (p > 0.05)