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Clonality and limited population diversity of *Fusarium circinatum* in Colombia

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Abstract

Fusarium circinatum is an important fungal pathogen of *Pinus* species utilized in commercial forestry worldwide. In Colombia, it was first found on nursery plants and later in plantations associated with basal cankers on young trees. In this study, we explored the population diversity of the pathogen in Colombia by analyzing 136 isolates collected from diseased nursery plants (2005–2007) and plantation trees (2017 and 2020). These were sourced from different geographical regions and *Pinus* species. Genotyping was performed using 10 microsatellite markers, while mating types were identified with PCRs targeting the MAT1 locus. Using microsatellites, a total of 33 multilocus haplotypes were detected. Genetic diversity indices showed low levels of diversity in both the overall collection and in specific collection groupings. The data also suggested that a small number of isolates had unique origins in the country ($p < .05$), and relatively low levels of population differentiation were detected between the nursery and plantation collections. All the isolates were scored as having the MAT1-1 mating type, and no evidence for the random association among microsatellite alleles ($p = .0001$) was found. Overall, these data suggest that *F. circinatum* was introduced into Colombia a small number of times, likely on seed for nursery production. Furthermore, the data also indicate that the pathogen has spread from nurseries to the plantations via asexual reproduction and on asymptomatic plants. This has resulted in a highly clonal *F. circinatum* population in Colombia that has resulted from accidental introductions of the pathogen into a production nursery.

KEYWORDS
clonality, mating type, microsatellites, pine pitch canker, population structure, reproductive mode, South America

1 | INTRODUCTION

Pitch canker is a serious disease of *Pinus* species and is responsible for major economic losses to forestry industries worldwide (Wingfield et al., 2008). Its causal agent is the filamentous fungus *Fusarium circinatum* (Dwinell et al., 1985; Hepting & Roth, 1946). On established trees, symptoms of infection typically include wilted yellow to reddish-brown needles, shoot or branch dieback, and the

occurrence of large resinous cankers on stems and branches (Gordon et al., 2001; Storer et al., 1995; Wingfield et al., 2008). The pathogen can also reduce seed germination, while infected seedlings may display pre- and post-emergence damping-off, as well as root and root collar disease (Barnard & Blakeslee, 1980; Gordon et al., 2001, 2015; Storer et al., 1998; Viljoen et al., 1994; Wingfield et al., 2001). Plant mortality is usually higher in nursery seedlings than in established trees in plantations or natural stands, although both are almost always associated with significant economic losses (Wingfield et al., 2008).

The centre of origin of *F. circinatum* is thought to be Mexico/Central America (Correll et al., 1992; Guerra-Santos, 1999; Santos & Tovar, 1991; Wikler, 1999; Wikler & Gordon, 2000). From there it was introduced to other parts of the world, including various countries in North and South America, Europe, Asia, and Africa (Drenkhan et al., 2020). The results of population genetic studies have shown that the pathogen's introduction into new *Pinus* production regions generally follows a similar scenario (Berbegal et al., 2013; Britz et al., 2005; Correll et al., 1992; Fru et al., 2017, 2019; Gordon et al., 2006; Gordon & Okamoto, 1996; Iturritxa et al., 2011; Santana et al., 2016; Steenkamp et al., 2014; Viljoen et al., 1997; Wikler, 1999; Wikler & Gordon, 2000). After initially gaining entry into a new area (usually via the import of infected planting stock), *F. circinatum*-associated disease usually goes unnoticed, which in the case of seedlings is exacerbated by the fact that infected plants often remain asymptomatic (Storer et al., 1998; Swett et al., 2016, 2018). Once planted in the field, these infected, but asymptomatic, plants typically succumb to root and root collar disease within the first 1–2 years due to stresses associated with planting and establishment (Crous, 2005; Wingfield et al., 2008). These diseased plants might then contribute to the inoculum resulting in above-ground infections, yielding the pitch canker symptoms typically seen in established stands or plantations (Coutinho et al., 2007; Fru et al., 2023; Gordon et al., 2015; Mitchell et al., 2011; Wingfield et al., 2001, 2008).

As for most plant pathogens that have become established in specific regions, effective disease management strategies against *F. circinatum* are largely limited to breeding for resistant planting stock (Martín-García et al., 2019; Mitchell et al., 2011). Because the durability of such resistance is largely dependent on the pathogen's population dynamics (Martín-García et al., 2019), numerous studies have investigated the population genetics of *F. circinatum* (Berbegal et al., 2013; Correll et al., 1992; Fru et al., 2023). These have shown that the diversity of *F. circinatum* in non-native regions is mostly linked to the genetic make-up of the inoculum associated with the pathogen's introduction and not subsequent cycles of sexual reproduction. This is because, despite being heterothallic and capable of sexual reproduction in the laboratory (Britz et al., 1998, 1999; Leslie, 1995; Leslie & Klein, 1996; Leslie & Summerell, 2008), introduced populations of *F. circinatum* are usually dominated by a single mating type and/or the general lack of female-fertile individuals (Berbegal et al., 2013; Fru et al., 2017, 2019, 2023; Iturritxa et al., 2011; Santana et al., 2016; Steenkamp et al., 2014). As a result, introduced populations are largely clonal with limited structure and low genetic diversity, although mutation might impact this pattern to some extent (Berbegal et al., 2013; Fru et al., 2023; Gordon et al., 2021).

In Colombia, *F. circinatum* was first detected in 2005 on diseased *P. patula*, *P. maximinoi*, and high elevation (HE) *P. tecunumanii* plants in Valle del Cauca where symptoms included wilting, shoot dieback as well as root and root collar lesions (Rodas Pelaez, 2013; Steenkamp et al., 2012). Later, in 2006 the pathogen was isolated from the diseased tissue of an 11-year-old *P. patula* tree, where symptoms included shoot and tip dieback, as well as resinous trunk and branch cankers (Rodas Pelaez, 2013; Steenkamp et al., 2012). In 2007, pitch canker infections were reported on plantation-grown *P. patula* in Valle del Cauca and from *P. kesiya* plantation trees in Antioquia (Rodas Pelaez, 2013; Steenkamp et al., 2012). Based on the results of pathogenicity tests with representative isolates from the various disease outbreaks, Steenkamp et al. (2012) highlighted the value of developing and deploying resistant planting stock to mitigate *F. circinatum*-associated losses. However, despite eradication attempts, the pathogen remains a significant risk to *Pinus*-based forestry in Colombia, where the disease is commonly encountered in young plantations. Yet nothing is known regarding the population biology of the pathogen or evolutionary potential to overcome the genetic resistance (McDonald & Linde, 2002) in *Pinus* planting stock.

Against the background of a newly emerging and serious disease problem, the aim of this study was to investigate the evolutionary potential of *F. circinatum* in Colombia. This was done by analyzing the genetic diversity, population structure, and mode of reproduction of the pathogen in the country. To achieve this objective, a comprehensive set of molecular and population genetic analyses were employed. These methods broadly included DNA barcoding and statistical modelling, which allowed us to gain insights into the pathogen's adaptability and spread in varying environmental conditions and across a time scale.

2 | MATERIALS AND METHODS

2.1 | Isolates

A total of 136 *F. circinatum* isolates (Table S1) were used in this study. These were collected from the diseased *Pinus* tissues in Colombia (Figure 1) following the procedure outlined by Steenkamp et al. (2012). Pure cultures were prepared by transferring a single hyphal tip for each isolate to malt extract agar (MEA; 20 g/L; Merck, Wadeville, South Africa) followed by incubation in the dark at 25°C for a week (Leslie & Summerell, 2008). Isolate identity as *F. circinatum* was confirmed using the diagnostic method involving the ribosomal RNA operon's intergenic spacer (Fru et al., 2017; Schweigkofler et al., 2004), as well as DNA sequence comparisons of gene encoding translation elongation factor 1α against the *Fusarium*-ID database (Geiser et al., 2004; Steenkamp et al., 2014). All isolates utilised

FIGURE 1 Disease symptoms associated with *Fusarium circinatum* of *Pinus* plants in the nursery (**a**) and plantation (**b**–**d**) environment in Colombia. In nurseries, the pathogen causes root and collar infections resulting in seedling death (**a**). In plantations, resinous cankers develop on the root collars of young plants (**b**, **c**), with trees often displaying typical flagging symptoms (**d**).

in this study are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

Twenty of the 136 isolates included in this study were collected in 2005–2007 and were utilised in the study of Steenkamp et al. (2012). Of these, 17 isolates were from diseased *P. patula* and *P. tecunumanii* (HE) nursery plants and were all collected in 2005 in Valle del Cauca. In addition, two isolates were from diseased *P. patula* trees, sampled in 2006 in Risaralda and Valle del Cauca, and one isolate was from diseased *P. kesiya*, sampled in 2007 in Antioquia (Figure 2). The remaining 116 isolates were collected in 2017 and 2020 from two farms, one in Caldas (75°44′42″ W, 5°24′15″ N and 75°44′41″ W, 5°25′08″ N) and the other in Risaralda (75°44′10″ W, 5°23′34″ N). Of these, 37 were from diseased *P. tecunumanii* (HE) trees in Caldas, and 79 isolates were from *P. patula* trees in Risaralda.

For population genetic analyses, isolates were assigned to groups (i.e., Nursery and Plantation 1) according to their corresponding collection metadata (i.e., sampling location). Thus, in addition to the total collection, analyses were performed on collections that consisted of isolate groupings separated by their corresponding metadata. Based on sampling location, the isolates from the nursery in Valle del Cauca, and the established plantation trees in Caldas and Risaralda were grouped into the Nursery ($n=17$), Plantation 1 ($n=79$), and Plantation 2 ($n=37$) groupings, respectively. Three isolates were removed from this grouping due to not falling into any of the relevant categories ($n=133$). Based on the sampling date, the isolates were assigned to either the 2005–2007 group ($n=20$) or the 2017 and 2020 group ($n=116$).

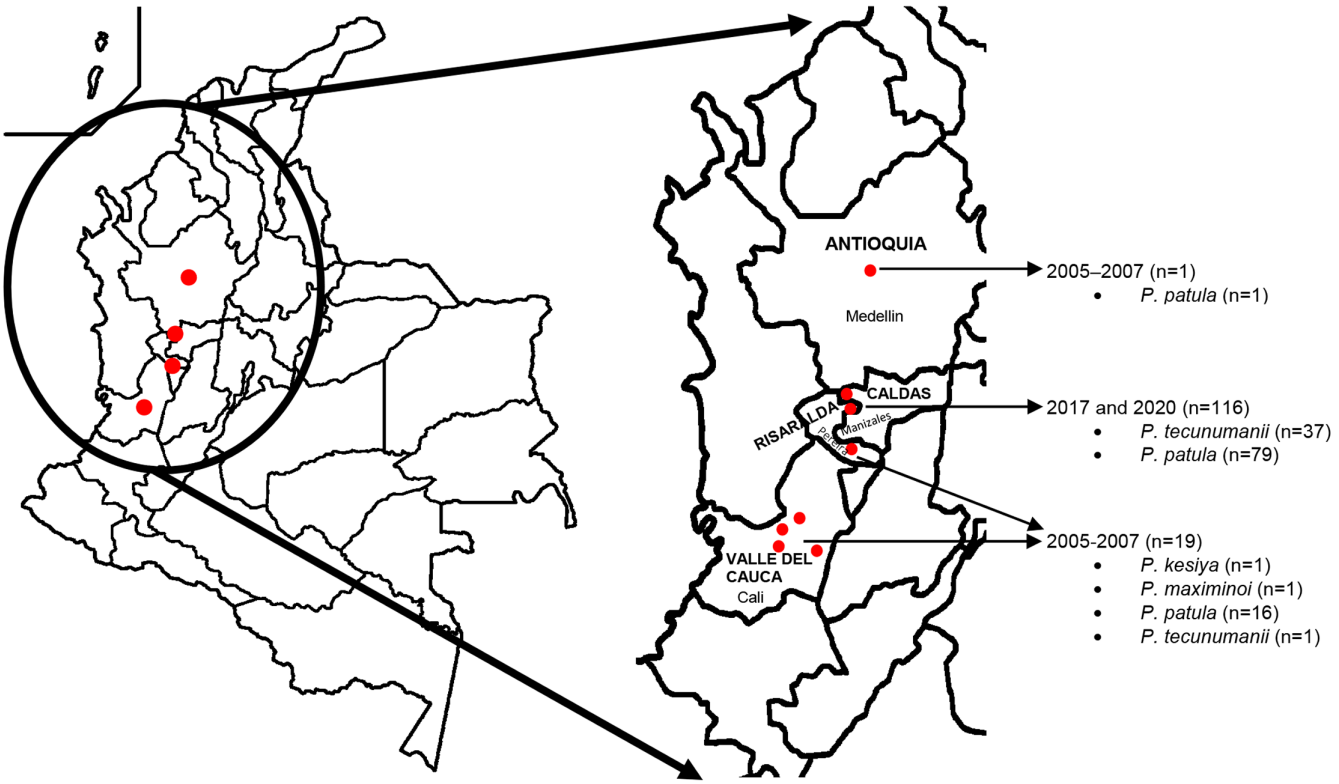

FIGURE 2 Map of Colombia showing the sampling information of the *F. circinatum* isolates collected and used in this study. The figure displays the number of isolates obtained from each collection date, *Pinus* host species, and geographical region in Colombia.

2.2 | DNA extraction and microsatellite analysis

Mycelium was scraped from the surface of cultures on MEA with a sterilized scalpel and transferred to a sterile Eppendorf tube. Genomic DNA was extracted from the mycelium using a modified sodium dodecyl sulphate (SDS) and Phenol-chloroform based extraction method (Aljanabi & Martinez, 1997; Duong et al., 2013). The extracted DNA was quantified with a NanoDrop® ND-1000 UV–Vis Spectrophotometer (Thermo Fisher Scientific; Waltham, Massachusetts). Using these DNAs and fluorescently labelled primers (Table S2), 10 microsatellite loci were then amplified for all the isolates as described previously (Fru et al., 2017; Santana et al., 2009). The amplicons were multiplexed into 96-well panels and separated by size with an ABI Prism 3500xl Genetic Analyser (Applied Biosystems, Waltham, MA, USA). For allele sizing, amplicon peaks were compared against those of the GeneScan™LIZ-500 molecular size standard (Applied Biosystems) using ABI PRISM® GeneMapper v 6.0 software (Applied Biosystems). All alleles and their sizes were manually confirmed by visual inspection of the electropherograms obtained at each microsatellite locus for each isolate.

2.3 | Genetic diversity

The allele sizes at each locus were used to generate a data matrix from which the multilocus haplotype (MLH) for each isolate was inferred. This was done by generating a 10-numbered sequence from the allele sizes of the respective loci (i.e., 12,345,678,910, where 1 is the allele of the first locus and 2 is the allele of the second locus, etc.) for each isolate. Standard genetic diversity metrics were determined as previously described (Fru et al., 2017, 2019; Santana et al., 2016) using R (v4.3.1) (RCoreTeam, 2022) and Rstudio (v4.2.2) (Posit Team, 2023), along with the *poppr* (v2.9.4) (Kamvar et al., 2014, 2015) and *adegent* (v2.1.7) packages (Jombart, 2008; Jombart & Ahmed, 2011), unless otherwise stated. All genetic diversity analyses were performed on the total collection, as well as the metadata associated collections, and the respective grouping assignments.

The allele frequency per locus was determined for all loci. Where relevant, parameters were calculated both with and without a clone correction, which was used to account for possible bias associated with sampling of the same genetic individual more than once (Chen et al., 1994). Gene diversity (H) was determined with the equation $H = 1 - \sum x_k^2$, where x is the frequency of the kth allele (Nei, 1973). For each primer pair, the Polymorphic Information Content (PIC) (Botstein et al., 1980) was calculated for the total collection using the equation $1 - \left(\sum_{i=1}^{n} pi^2\right) = \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} 2_{pi^2 pj^2}$ with the package *polysat* (v1.7–7) (Clark & Jasieniuk, 2011), which was also used to check for the occurrence of null alleles in the dataset. Genotypic diversity (G) was calculated using the equation $G = \frac{1}{\sum p_i^2}$, where p_i is the observed frequency of the ith genotype in the population (Stoddart & Taylor, 1988). The maximum estimator of genotypic diversity was determined with the equation $\hat{G} = \frac{G}{n} \times 100$ (Chen et al., 1994). Population diversity, as reflected by the Shannon

diversity index (SI), was calculated using the equation $SI = -\sum \rho_i \ln \rho_i$, where ρ_i represents the frequency of the ith genotype in the population (Sheldon, 1969). To allow for comparison between populations, the normalized $SI(H_s)$ was calculated with $H_s = SI / \ln N$, where N is the number of individuals in the population. Genotypic evenness or the within-population distribution of MLH abundance was evaluated using the Evenness index (E_5) with the equation $E_5 = (G - 1) / (e^{Hs} - 1)$ (Grünwald et al., 2003).

2.4 | Population structure

To explore patterns of clustering among *F. circinatum* individuals, either de novo or with the use of prior meta-data associated collection assignments, Principal Coordinate Analysis (PCoA) analysis was performed on the MLH data. This was done using GenAlEx, with the options of ploidy and marker choice being set to "Haploid-SSR". The use of the Covariance-Standardized option to conduct a multivariate analysis of the MLH data-derived Tri-Distance Matrix was to allow the ordination of isolates according to the set of uncorrelated axes summarizing the total variability in the data (Peakall & Smouse, 2006).

To investigate population structure, the microsatellite data and the respective groupings were subjected to unsupervised model-based Bayesian clustering with STRUCTURE (v2.3.4) (Falush et al., 2003, 2007; Hubisz et al., 2009; Pritchard et al., 2000, 2003). This allowed the estimation of the optimal *K* (number of ancestral or genetically distinct source populations) and the assignment of isolates to the distinct *K*s (Falush et al., 2003; Hubisz et al., 2009; Pritchard et al., 2000). The optimal *K* was determined using 20 independent iterations or runs for each possible *K*-value from 1 to 5. In all cases, analyses utilised a burn-in of 100,000 and 1,000,000 Markov Chain Monte Carlo post-burn-in repetitions, as well as the admixture model with separate alpha values for each ancestral population to accommodate for differences in cluster sizes (Wang, 2017), and independent allele frequencies with no prior information about population demography (Falush et al., 2003; Hubisz et al., 2009; Pritchard et al., 2000). From the data for all of the runs, the optimal *K*-value was determined by computing the Evanno best *K* or Delta *K* (ΔK) and the log-likelihood value Prob($K=k$) (Evanno et al., 2005). The summary of the STRUCTURE iterations and optimal *K* analysis results were then graphically interpreted using the best *K* and DISTRUCT for many *K*s Cluster Markov Packager Across *K* (CLUMPAK) (Kopelman et al., 2015) web server features.

Population clustering was also investigated using the model-free multivariate statistical tool Discriminate Analysis of the Principal Coordinates (DAPC) (Agapow & Burt, 2001). This was because STRUCTURE assumes that markers are unlinked and populations are panmictic (Pritchard et al., 2000), which is often not the case for *F. circinatum* (Fru et al., 2017, 2023; Iturritxa et al., 2011; Santana et al., 2016). de novo DAPC was performed using the *poppr* and *adegenet* packages in Rstudio. During the analysis, DAPC transformed the MLH data with principal component analysis (PCA) to obtain a set of uncorrelated variables or Principal Components (PCs) that captured most of the genetic variation in the dataset. DAPC then performed a discriminant analysis on these PCs to find linear combinations of variables (i.e., the discriminant functions or Linear Discriminants, LDs) that best discriminate between groups of individuals, with the optimal clustering solution (i.e., *K*-value) being associated with the lowest Bayesian Information Criterion. The number of PCs retained was determined with a cross-validation analysis in *poppr*. Here, the MLH dataset was separated into training and testing subsets and subjected to 1000 replicate analyses at each PC between 1 and 20. The optimal number of PCs for retaining were those which achieved the highest mean successful assignment of the group membership of individuals in the testing set (i.e., average accuracy) and lowest root mean squared error (average prediction error). Clusters were graphically represented by plotting isolates according to the first and second LDs, using DAPC's "scatter" function.

2.5 | Inference of population differentiation and genetic relationships

Genetic differentiation and partitioning within and between the various de novo clusters and predefined groupings within collections were explored using Analysis of Molecular Variance (AMOVA) (Excoffier et al., 1992) as implemented in GenAlEx 6.503 (Peakall & Smouse, 2006). These analyses utilized the haploid Tri-Distance Matrix inferred from the MLH data, the "Standard Permute" options with 9999 permutations and 9999 pairwise population permutations, and the null hypothesis of no population differentiation between groups. The latter is based on PhiPT (ΦPT), an analogue of Sewall Wright's to F_{ST}, which is used to exploit both the frequency and evolutionary distances between alleles, especially when mutations contributed substantially to allelic differences observed (Holsinger & Weir, 2009). The PhiPT metric scales from 0 to 1, with 0 indicating no genetic differentiation (populations are genetically identical) and 1 indicating complete genetic differentiation (populations are genetically distinct and have no shared variance).

To infer ancestry-descendance relationships among isolates, their MLH data were subjected to distance analysis that accounts for the stepwise mutational model of microsatellites (Bruvo et al., 2004). These data were then used to construct a minimum spanning network (MSN) reflecting the relationships among MLHs (Bandelt et al., 1999). The MSN analysis was conducted with *poppr* using data for all 10 loci across all isolates (Table S2).

2.6 | Mating type and reproductive mode

Reproductive mode was explored using two approaches. The first approach involved scoring the mating type of all isolates using diagnostic PCRs targeting the alpha box and high mobility group (HMG) motifs encoded by MAT1-1 and MAT1-2 isolates, respectively (Steenkamp et al., 2000). These PCRs utilized the extracted

genomic DNA (see above) as a template, as well as previously described reaction and cycling conditions (Steenkamp et al., 2000, 2014). The possibility of sexual reproduction was then inferred from the mating-type frequencies and their distributions across isolate collections (Milgroom, 1996; Santana et al., 2016; Steenkamp et al., 2014).

The second approach utilized the MLH data and tested for the non-random association and recombination of alleles (Otto & Lenormand, 2002) within the respective isolate groupings. For this purpose, the *poppr* package was used to calculate the index of association (I_A) and $\bar{r}_d$, values with clone correction. The I_A and $\bar{r}_d$ indices provide estimates of multilocus linkage disequilibrium and the frequency and distribution of recombination events among individuals, respectively, thereby allowing quantification of the association between alleles at different loci in individuals in the population (Agapow & Burt, 2001; Brown et al., 1980; Smith et al., 1993). For each dataset, the null hypothesis that alleles in the population are randomly associated or recombining and that the population(s) freely undergo(es) recombination was tested with 10,000 randomizations of the microsatellite data. The values for I_A range and can be interpreted as follows $I_A = 0$ (random association), $I_A > 0$ (non-random association), $I_A < 0$ (excess random association). The $\bar{r}_d$ values can be interpreted similarly to that of the I_A values: $\bar{r}_d = 0$ (random recombination), $\bar{r}_d > 0$ (limited recombination), $\bar{r}_d < 0$ (excess recombination events) (Smith et al., 1993).

3 | RESULTS

3.1 | Genetic diversity

A total of 48 alleles across the 10 microsatellite loci were identified among the 136 isolates of *F. circinatum* examined (Table 1, Table S3). The greatest number of alleles was detected at the FCM-4 locus with 12 alleles, followed by the loci FCM-2 (9) and FCM-7 (8). The remaining microsatellite markers (FCM-3, FCM-6, FCM-16, FCM-20, FCM-23, FCM-24, and FCM-25) had noticeably fewer alleles each (i.e., 2, 5, 2, 3, 2, 3, and 2 alleles, respectively). The PIC values for the total collection ranged from 0.0291 at FCM-25 and FCM-23 to 0.582 at FCM-4, while no null alleles were observed at any of the loci. When considering the entire collection of isolates, none of the loci were monomorphic. This same trend was seen when considering the 2017 and 2020, and Plantation 1 groupings. These collections contained 36 and 25 private alleles within their groupings, respectively. In contrast, the 2005–2007, Nursery, and Plantation 2 groupings were monomorphic at 8, 8, and 4 of the loci, respectively, each with a limited number of private alleles (i.e., 0, 0, and 9, respectively). The entire collection had a gene diversity value of $H = 0.201$. Plantation 2 had the highest value of $H = 0.275$, followed by the 2017 and 2020 grouping of $H = 0.226$ (Table 1, Table S3). The H values for the 2005–2007, Nursery, and Plantation 1 groupings were lower in comparison,

TABLE 1 Genetic diversity statistics for each of the isolate collections and their respective groupings based on the microsatellite data for the 10 loci used in this study.

Isolate collection definition	Isolate groupings	No. of isolates	No. of alleles	No. of private alleles	No. of MLHs[a]	H[b]	G[c]	$\hat{G}$[d]	SI[e]	H_S[f]	E_5[g]
Sampling year	2005–2007	20	12	0	3	0.020 (0.133)	1.23	6.15%	0.394	0.132	0.469
	2017 and 2020	116	48	36	33	0.226 (0.323)	8.72	7.52%	2.746	0.578	0.529
Sampling location	Nursery	17	12	0	3	0.024 (0.133)	1.27	7.47%	0.444	0.157	0.489
	Plantation 1	79	39	25	27	0.180 (0.303)	8.20	10.38%	2.605	0.596	0.574
	Plantation 2	37	22	9	7	0.275 (0.338)	4.11	11.11%	1.646	0.456	0.743
Total	All	136	48	0	33	0.201 (0.323)	6.24	4.59%	2.55	0.519	0.442

[a]MLH, Multilocus haplotype.
[b]H, Nei's Gene diversity (1978). The value in parenthesis is for clone corrected dataset (Nei, 1973).
[c]G, The Genotypic diversity (Stoddart & Taylor, 1988) of the *F. circinatum* collections groupings.
[d]$\hat{G}$, The Maximum estimator of genotypic diversity (Chen et al., 1994) in the population expressed as a percentage (%).
[e]SI, The Shannon-Weiner Diversity Index (Sheldon, 1969).
[f]H, The Normalized Shannon Diversity Index.
[g]$E5$, The Evenness index (Grünwald et al., 2003).

and were 0.020, 0.024, and 0.180, respectively. A similar trend was observed in the clone corrected data, except that the *H* values were somewhat higher (e.g., $H=0.323$ for the total collection, compared to an *H*-value of 0.201).

A total of 33 MLHs were detected among the 136 isolates (Table 1, Figure 3). Most isolates (35%) were represented by MLH1, while 18 MLHs were represented by single isolates once. When considering individual isolate groupings, the 2005–2007 and Nursery groupings contained only 3 MLHs (i.e., MLH1, MLH2, and MLH3); by contrast, the 2017 and 2020 groupings contained 33 MLHs. Of the 33 MLHs detected, only MLH1 appeared in all the various isolate groupings examined, while MLH2 and MLH3 were also found in the found in 2017 and 2020, and Plantation 1 groupings. Normalized genetic diversity indices obtained for the entire isolate collection were $\hat{G}=4.59\%$ and $H_s=0.519$ (Table 1, Table S3). However, higher $\hat{G}$-values were obtained when considering the various isolate groupings, with $\hat{G}$-values ranging from 6.15% to 11.11% for the 2005–2007 and Plantation 2 groupings, respectively. When considering population diversity, however, H_s-values of as low as 0.123 and 0.157 were obtained for the 2005–2007 and Nursery groupings, while the remaining groupings were characterized by values exceeding 0.456. Additionally, the highest level of genotypic evenness was observed for the Plantation 2 grouping as the E_5-value (i.e, 0.743) indicated MLH abundance

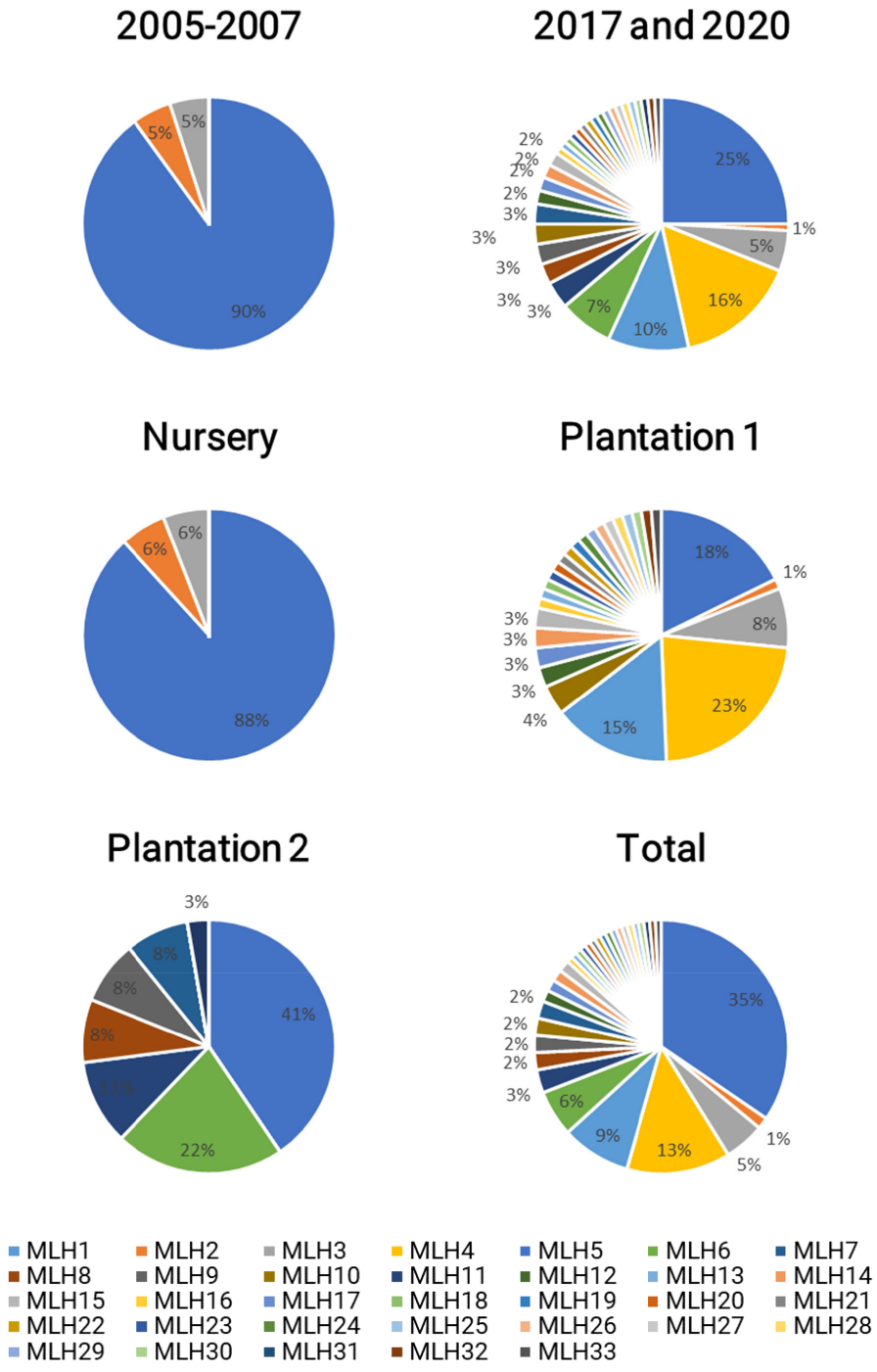

FIGURE 3 The distribution and frequency of the multilocus haplotypes (MLHs) found in the different collections of *Fusarium circinatum* in Colombia. MLHs are indicated according to colour in the carious pie graphs. The size of each section is representative of the percentage of individuals found in each MLH.

closer to equal than for the other groupings with E_5-values ranging from 0.442 to 0.574.

3.2 | Population structure

PCoA-based multivariate analysis of MLH-derived Euclidian distances indicated that 98.98% of the total diversity could be summarized using a set of three uncorrelated axes (Figure S1). Ordination of isolates along the first two of these axes revealed three potential clusters of closely related *F. circinatum* individuals. One included 9 isolates originating from Plantation 2 (*P. tecunumanii*), while the second included two isolates from Plantation 1 (*P. patula*). The third and largest cluster (i.e., Cluster 1), included the remaining isolates, of which many of the 2017 and 2020, Plantation 1, and Plantation 2 isolates ordinated around those from the 2005–2007 and the Nursery collection (Figure S2). The collection based on sampling location showed that the largest cluster consisted of individuals found in both plantations and consequently, either host. The latter and largest cluster was accordingly designated as Cluster 1, while the 2-isolate cluster was named Cluster 2, and the 9-isolate cluster was named Cluster 3. These clusters did not reflect the sampling location, sampling date, or *Pinus* hosts from which isolates were obtained and are considered a reflection of the de novo population structure.

STRUCTURE-based Bayesian modelling of the MLH data groupings yielded an optimal *K*-value of 3, suggesting that a proportion of each individual's genome was derived from three ancestral or source populations (Figure S1). Assignment of individuals to these three ancestral backgrounds allowed the recovery of the three groups identified with PCoA. Inspection of the ancestry proportions predicted for the respective isolates showed that each of the Clusters 1, 2, and 3 were derived mainly from only one of the three suggested ancestral *K*s. This same trend was seen when looking at the collections based on sampling date and location (Figure S3).

The findings for the de novo population structure were confirmed using model-free analysis with DAPC which utilized the MLH data for predicting groups among the isolates (Figure 4). DAPC also suggested a value of 3 for *K*. Additionally, PCA-based transformation of the data and discriminant analysis of the retained PCs suggested that 10 PCs and 2 LDs had the highest average accuracy (1.00) and lowest average prediction error (0.00). This model conserved 0.885 or 88.5% of the variance. Projection of the isolates onto the first two of these LDs revealed clusters that matched those identified using Bayesian modelling and PCoA.

3.3 | Inference of population differentiation and genetic relationships

The observed de novo genetic patterns within the set of 136 *F. circinatum* examined were supported using AMOVA of isolate collections resembling Clusters 1, 2 and 3 (Table 2). As much as 82% of the total MLH variation could be attributed to variance between the Clusters. Furthermore, ΦPT-values ranging from 0.810 to 0.929 were obtained for a pairwise comparison between Clusters 1, 2 and 3 (Table S4), suggesting high genetic differentiation with limited shared molecular variance. These results were all highly significant ($p \leq .05$). When exploring the collections based on sampling date and location, the largest proportion of the total variation could be attributed to the variation within collections rather than between

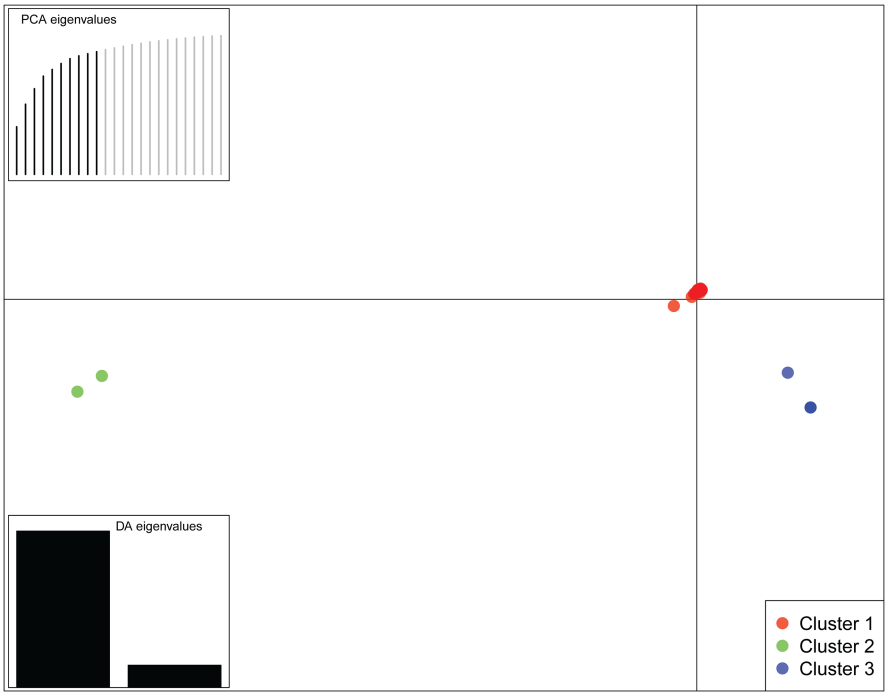

FIGURE 4 Model-free Discriminant Analysis of Principal Components (DAPC) clustering analyses of *Fusarium circinatum* isolates from Colombia. Plotting of isolates along the first two Linear Discriminants (LDs) using 10 Principal Components (PCs) retained by DAPC produced three clusters of isolates. The Median and Confidence Intervals for Random Chance at 2.5%, 50%, and 97.5% were 0.304, 0.309, and 0.473, respectively. The proportion of conserved variance was 0.885.

TABLE 2 Analysis of Molecular Variance (AMOVA) results for each of the predefined isolate collections and their respective groupings in the microsatellite dataset.

Isolate collection definition	Source of the variation	Degrees of freedom[a]	Sum of squares[b]	Mean square deviations	Estimate of variance component[c]	% of the molecular variance[d]	PhiPT (ΦPT) value[e]	p[f]
Sampling year	Between groupings	1	3.846	3.846	0.084	8	0.079	.0047
	Within groupings	134	131.728	0.983	0.983	92		
	Total	135	135.574		1.067			
Sampling location	Between groupings	2	13.304	6.652	0.155	14	0.142	.0001
	Within groupings	130	121.651	0.936	0.936	86		
	Total	132	134.955		1.091			
de novo-based clusters	Between groupings	2	56.908	19.797	2.720	82	0.821	.0001
	Within groupings	133	78.666	0.577	0.591	18		
	Total	135	135.574		3.312			

[a]The number of independent categories or groups (−1), used for estimating variance components and determining statistical significance.
[b]Variability or deviation from the overall mean that is attributed to different components of genetic variation.
[c]Quantitative measure of the genetic variation contributed to different hierarchical levels.
[d]The proportion of the total genetic variation that is attributed to different levels of hierarchical population structure.
[e]The PhiPT or Fixation index is a measure of population differentiation that quantifies the proportion of genetic variation attributed to differences among populations relative to the total genetic variation.
[f]Statistical significance of the AMOVA results.

collections. The "between grouping" molecular variance never exceeded 15% in either collection (Table 2). This limited level of genetic differentiation between the isolate collections was also evident from the low ΦPT-values recovered (Table S4). For the collection based on sampling year, the calculated ΦPT-value was 0.079 ($p = .0057$), while the collections based on sampling location had pairwise ΦPT-values ranging from 0.100 to 0.166 ($p \leq .0043$). These values are suggestive of significantly low to moderate differentiation among and between the groups examined.

Analyzing the distance-based relationships between MLHs revealed a MSN framework comprising of three primary components (Figure 5). Here the MLHs predefined to Cluster 1 could not be confidently linked with those from Clusters 2 and Cluster 3, which further supports the strong molecular differentiation among the three clusters (Table 2). Additionally, many of the singleton MLHs were closely related, often differing from one another at only a single allele. Nevertheless, the MSNs relating to sampling date and location clearly illustrated the co-occurrence of isolates with the same MLH in different isolate groupings. For example, MLHs 1, 2, and 3 were originally identified in 2005–2007 and from the Nursery, and were shared by collections made between 2017 and 2020 in plantations (and the broader Cluster 1) (Figure S4). Other than MLHs from the nursery, MLHs found in the plantations were not shared between plantations (i.e., these MLHs were found exclusively in either Plantation 1 or 2). However, these MLH collections showed little differentiation and often only differed by a single allele from another.

3.4 | Mating type and reproductive mode

All the isolates examined in this study were of the MAT1-1 mating type and no evidence of random mating or recombination of alleles was found when the MLH data sets were analysed (Table 3). For the entire data set, as well as the sampling date and location collections, I_A and $\bar{r}_d$ values were calculated, with the only exception being the isolates from the Nursery and 2005–2007 grouping that contained inordinately few genotypes to be informative. In the case of the total collection, statistically significant ($p < .05$) I_A and $\bar{r}_d$ values were obtained of 2.711 and 0.310, respectively. These values are indicative of a non-random association of alleles and a lack of recombination in the dataset, respectively. This trend also followed suit in the various isolate groupings, which in all cases statically significant I_A and $\bar{r}_d$ values were obtained. This allowed for the rejection of the null hypothesis that the alleles were randomly associated or recombined.

4 | DISCUSSION

The findings presented here showed that the occurrence of *F. circinatum* on *Pinus* in Colombia is highly clonal with limited genetic diversity. This is similar to reports for introductions of the

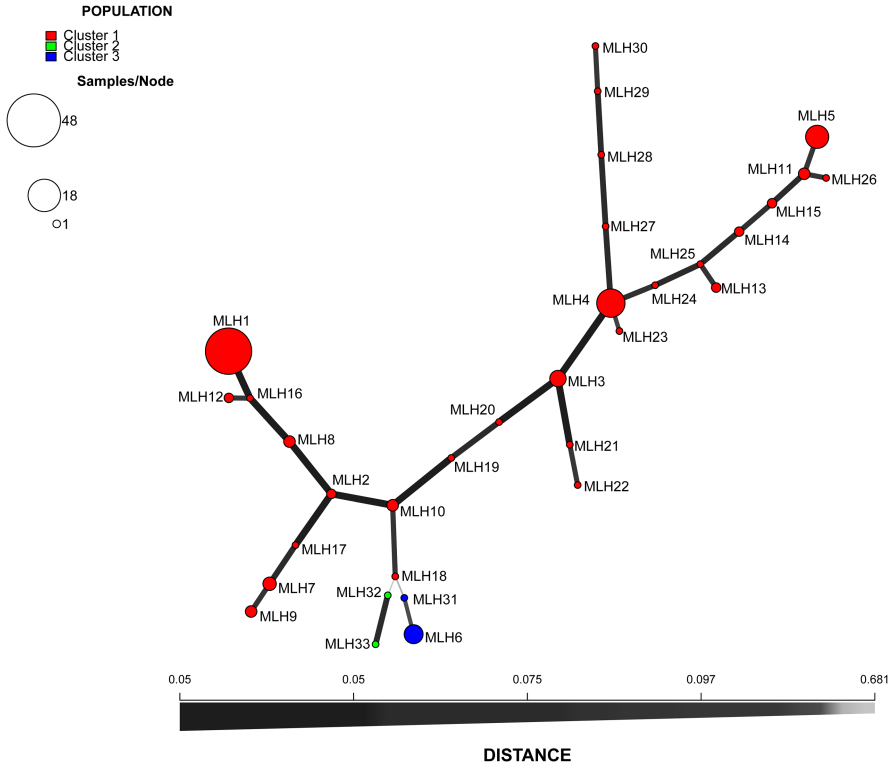

FIGURE 5 Minimum-spanning network of the isolates from the total collection, using the previously identified clusters to group MLHs. These are Cluster 1, Cluster 2, and Cluster 3. The collections are represented by different coloured circles. The size of each of the circles or nodes is proportional to the number of multilocus haplotypes or samples in each node. The link between nodes represents a unique mutational event between haplotypes.

TABLE 3 Index of association values (I_A), RbarD ($\bar{r}_d$) values, and the frequency and distribution of the mating type(s) across each of the predefined isolate collections and respective groupings.

					Mating type[e]	
Isolate collection definition[a]	Isolate groupings	I_A[b]	$\bar{r}_d$[c]	p[d]	MAT1-1	MAT1-2
Sampling year	2005–2007	–	–	–	20	0
	2017 and 2020	2.712	0.310	.0001	116	0
Sampling location	Nursery	–	–	–	17	0
	Plantation 1	2.895	0.331	.0001	79	0
	Plantation 2	2.604	0.555	.0003	37	0
Total	All	2.711	0.310	.0001	136	0

[a]Isolates are pre-defined to the collections based on the sampling date or location of isolation within the total isolate collection.
[b]I_A, Index of association measure of multilocus linkage disequilibrium (Smith et al., 1993). Values are for the clone-corrected dataset.
[c]$\bar{r}_d$, Measure of multilocus linkage disequilibrium independent of sample size (Smith et al., 1993). Values are for the clone-corrected dataset.
[d]The *p*-value for the null hypothesis that the alleles are randomly associated in the population.
[e]The number of individuals in each of the isolate collections containing either the MAT1-1 or MAT1-2 mating type.

pathogen in other parts of the world, where it is typically clonal and dominated by one or only a few MLHs (Berbegal et al., 2013; Correll et al., 1992; Fariña-Flores et al., 2023; Fru et al., 2017, 2023; Gordon & Okamoto, 1996; Iturritxa et al., 2011; Santana et al., 2016; Steenkamp et al., 2014). Having identified only three genetic clusters, with all isolates carrying the MAT1-1 mating type, the view that this *F. circinatum* population represents a limited introduction with a lack of potential for sexual reproduction is validated. In addition, evidence was found for the pathogen having been first introduced into the nursery environment and subsequently moved with asymptomatic seedlings during plantation establishment.

The genetic diversity indices observed were comparable to introductions of this pathogen studied worldwide. The ranges for the normalized genotypic ($\hat{G}$) and clone-corrected gene diversity (H) were 6.15–11.11% and 0.133–0.338, respectively. This is somewhat lower and indicative of a higher degree of clonality than has been reported for introduced populations in California (Gordon & Okamoto, 1996), South Africa (Britz et al., 2005; Fru et al., 2017, 2019, 2023; Santana et al., 2016), and Spain (Fariña-Flores et al., 2023). However, a comparison of the indices from the initial introduction (2005–2007) to a more present-day representation of the population in the field (2017 and 2020) suggests that gene diversity is slowly increasing

over time, and consequently, genotypic diversity too. Therefore, the pathogen is likely reproducing asexually, with clonal diversity and population biology largely being maintained.

The inoculum responsible for the disease affecting *P. patula* and *P. tecunumanii* in Colombian plantations could be traced back to the nursery environment. This is based on the isolates from the nursery sharing MLHs with those from trees in plantations. In addition, the isolate groups representing those from the nursery and plantations had low genetic differentiation between them. Thus, asymptomatic but *F. circinatum*-infected nursery plants were likely used to establish new plantations, where stresses associated with planting triggered disease expression. Such a scenario is similar to the situation found for the pathogen in South Africa (Fru et al., 2023; Mitchell et al., 2011), whereby, evidence supports the link between nursery and field infections (Santana et al., 2016). Interestingly, the fact that some of the MLHs among the nursery-collected isolates were prevalent in those collected in plantations many years later, further suggests that these genotypes are capable of surviving for decades across a range of environments.

The change in genetic diversity over time is indicative that mutation plays an important role in generating diversity within the Colombian population of *F. circinatum*. Although the initial introduction(s) into the country likely provided the bulk of the genotypes observed, no direct or indirect evidence for sexual recombination was found. This is despite meiotic recombination typically suppling the genetic variation for natural selection to act upon (McDonald & Linde, 2002). While lower frequency genotypes found in the population differed by only one or a few consecutive microsatellite allele changes from a more dominant MLH. This is similar to recent reports of mutation giving rise to closely related MLHs in South African populations of *F. circinatum* (Fru et al., 2017, 2023). In other words, a substantial proportion of the change in genetic diversity detected between the initial introduction (2005–2007) and the recent field collection (2017 and 2020) could be attributed to mutation and subsequent random genetic drift over time. However, the possibility that sampling or large differences in collection sizes or dates may have skewed this observation is plausible, especially when considering the smaller number of samples collected during the 2005–2007 period compared to the 2017 and 2020 collections. Our future research will seek to shed light on how sampling and mutation might impact the conclusions of microsatellite-based population studies of *F. circinatum*.

The origin of *F. circinatum* in Colombia and other South American countries remains unknown. One speculation is that the pathogen may have accidentally entered South America via routes involving sources in or near the proposed centre origin of the fungus in Mexico/Central America. This scenario might explain the close relationship between Chilean and Mexican MLHs (Berbegal et al., 2013), as well as the presence of both mating types in *F. circinatum* populations from Brazil and Chile (Jacobs et al., 2007; Pfenning et al., 2014). Alternatively, the pathogen could have been introduced into Colombia or any of the other South American countries via sources elsewhere in the world. For example, the microsatellite-based study by Berbegal et al. (2013) proposed that the populations of *F. circinatum* from Uruguay and various European countries may have originated from the USA. Whatever the case may be, the movement of the fungus across South American landscapes represents a significant concern as the main *Pinus*-growing countries have all reported the presence of *F. circinatum* within their borders (Alonso & Bettucci, 2009; Jacobs et al., 2007; Pfenning et al., 2014; Wingfield et al., 2002).

As in many parts of the world, *F. circinatum* remains an important threat to *Pinus*-based forestry in Colombia. Although the reproductive mode and mechanisms of dispersal of the pathogen typically limit its evolutionary potential (Fru et al., 2017; McDonald & Linde, 2002), *F. circinatum* evidently has all the attributes necessary to overcome the defences of its plant host given the appropriate conditions. Together with the growing evidence of mutation contributing to its genetic diversity, there is an increasing risk that *F. circinatum* might overcome the inherent disease resistance/tolerance of planting and breeding stock (McDonald & Linde, 2002) in Colombia. Long-term efforts to reduce the negative impacts of the pathogen should, therefore, rely on traditional disease management strategies aimed at preventing pathogen establishment and spread (inoculum load), combined with modern tree breeding techniques to ensure durable resistance (Mitchell et al., 2011; Wingfield et al., 2001, 2008; Yin et al., 2021).

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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