UNIVERSIDADE ESTADUAL DE FEIRA DE SANTANA

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ANELITA DE JESUS ROCHA

RESPOSTAS MOLECULARES, HISTOLÓGICAS E HISTOQUÍMICAS DE CULTIVARES DE BANANEIRA DESAFIADAS COM ISOLADOS DE *FUSARIUM OXYSPORUM* F. SP. *CUBENSE* COM DIFERENTES NÍVEIS DE VIRULÊNCIA

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Tese apresentada ao Programa de Pós-graduação em Biotecnologia, da Universidade Estadual de Feira de Santana como requisito parcial para obtenção do título Doutora em Biotecnologia.

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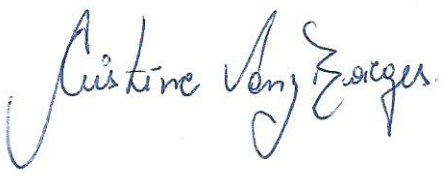

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RESUMO

A banana é um dos alimentos mais consumidos no mundo e uma das principais commodities para exportação em vários países. Nos últimos anos, uma das maiores limitações para a produção da fruta é a murcha de Fusarium causada pelo fungo habitante de solo *Fusarium oxysporum* f. sp. *cubense* (Foc), principalmente devido a cepa altamente agressiva do patógeno, chamada raça 4 tropical (TR4), cuja epidemia está em proporções pandêmicas. Devido aos hábitos de natureza saprofítica e por apresentar muitos mecanismos evolutivos, resultando em alta diversidade genética, o manejo de Foc em áreas infestadas é difícil. O melhoramento genético para o desenvolvimento de cultivares resistentes é a estratégia mais viável e eficiente. Assim, este trabalho propôs inicialmente uma revisão sistemática da literatura gerada nos últimos dez anos sobre o melhoramento genético de *Musa* spp. para resistência a murcha de Fusarium. Para tanto, um protocolo foi gerado e seguido de forma a obedecer às normas descritas nos itens de relatório preferidos para revisões sistemáticas e meta-análises (PRISMA). Após os processos de buscas por artigos em diferentes bases acadêmicas, usando uma *string* de busca padronizada e critérios de inclusão e exclusão pré-definidos, 95 artigos foram incluídos na análise sistêmica da revisão. Com isso, verificou-se que as informações obtidas após o sequenciamento do *Musa* spp. são uma fonte para obtenção de cultivares resistentes, principalmente pela avaliação dos dados do transcriptoma da bananeira após a infecção com Foc. A revisão destaca ainda as fontes de resistência à Foc raça 1 (R1) e Foc TR4 em germoplasma de *Musa* spp. e a técnica de transgenia como abordagem mais frequente no melhoramento da cultura. Este trabalho objetivou ainda realizar a análise da interação do patógeno Foc com o seu hospedeiro *Musa* spp. em nível molecular, histológico e histoquímico, na qual três isolados de Foc representativos da raça 1 (R1), raça subtropical 4 (ST4) e o isolado 229A, putativo ST4, foram inoculados em duas cultivares do tipo Prata (Prata-Anã e BRS Platina) e em uma cultivar do tipo Cavendish (Grande Naine). Os resultados mostraram sete genes induzidos em 'BRS Platina', bem como aumento de compostos como celulose, compostos fenólicos e cristais de oxalato de cálcio, sugerindo papéis importantes nas interações entre a bananeira resistente e Foc ST4 e R1. Essas respostas de defesa foram suprimidas ou reduzidas, principalmente pelo isolado com maior virulência em 'Prata-Anã' e ‘Grande Naine’. Assim, a cultivar BRS Platina será alvo de novas pesquisas para verificar sua interação com Foc TR4. Em conclusão, este trabalho oferece uma compilação de dados e novas perspectivas sobre o melhoramento genético de *Musa* spp. para resistência a murcha de Fusarium.

Palavras-chave: *Musa* spp.; *Fusarium oxysporum* f. sp. *cubense*; melhoramento genético; RT–qPCR.

ABSTRACT

Banana is one of the most consumed foods in the world and one of the main commodities for export in several countries. In recent years, one of the biggest limitations for fruit production is Fusarium wilt caused by the soil-dwelling fungus *Fusarium oxysporum* f. sp. *cubense* (Foc), mainly due to the highly aggressive strain of the pathogen, called tropical race 4 (TR4) whose epidemic is in pandemic proportions. Due to its saprophytic habits and because it presents many evolutionary mechanisms, resulting in high genetic diversity, the management of Foc in infested areas is difficult. Genetic improvement to obtain resistant cultivars is the most viable and efficient strategy. Thus, this work initially proposes a systematic review of the literature generated in the last ten years on the genetic improvement of *Musa* spp. for resistance to Fusarium wilt. To this end, a protocol was generated and followed in order to comply with the standards described in the preferred report items for systematic reviews and meta-analyses (PRISMA). After searching for articles in different academic databases, using a standardized search string and predefined inclusion and exclusion criteria, 95 articles were included in the systemic analysis of the review. With this, it was verified that the information obtained after the genome sequencing of *Musa* spp. is a source for obtaining resistant cultivars, mainly by evaluating the transcriptome data of banana after infection with Foc. The review also highlights the sources of resistance to Foc race 1 (R1) and Foc TR4 in germplasm of *Musa* spp. and transgenesis technique as the most frequent approach to crop improvement. This work also aims to analyze the interaction of the pathogen Foc with its host Musa spp. at molecular, histological and histochemical levels, where three isolates of Foc representative of race 1 (R1), subtropical race 4 (ST4) and the isolate 229A, putative ST4, were inoculated in two cultivars of the Prata type (Prata-Anã and BRS Platina) and in a cultivar of the Cavendish type (Grand Naine). The results showed seven genes induced in 'BRS Platina', as well as increased presence of cellulose, phenolic compounds and calcium oxalate crystals, suggesting their important roles in the incompatible interactions between resistant banana and Foc ST4 and R1. But, these defense responses were suppressed or reduced mainly by an isolate with higher virulence in 'Prata-Anã' and 'Grand Naine'. Thus, the cultivar BRS Platina will be the target of further research to verify its interaction with Foc TR4. In conclusion, this work offers a compilation of data and new perspectives on the genetic improvement of *Musa* spp. for resistance to Fusarium wilt.

Keywords: *Musa* spp.; *Fusarium oxysporum* f. sp. *cubense*; genetic improvement; RT-qPCR.

SUMÁRIO

	INTRODUÇÃO GERAL	12
	REFERÊNCIAS	15
	CAPÍTULO 1 – Improvements in the Resistance of the Banana Species to Fusarium Wilt: A Systematic Review of Methods and Perspectives	19
1.1	INTRODUCTION	20
1.2	MATERIALS AND METHODS	22
1.3	RESULTS	23
1.4	DISCUSSION	42
1.5	CONCLUSIONS	47
	REFERENCES	48
	CAPÍTULO 2 – Molecular, histological and histochemical responses of banana cultivars challenged with Fusarium oxysporum f. sp. cubense with different levels of virulence	55
2.1	INTRODUCTION	56
2.2	RESULTS	58
2.3	DISCUSSION	66
2.4	MATERIALS AND METHODS	71
2.5	CONCLUSIONS	73
2.6	**REFERENCES**	74
	CONCLUSÃO GERAL	79

INTRODUÇÃO GERAL

Bananas e plátanos (*Musa* spp.) são frutas comestíveis que evoluíram no sudoeste asiático e oeste do pacífico pela hibridação intraespecífica entre as espécies selvagens *Musa acuminata* Colla, com genoma A, e *M. balbisiana* Colla, com genoma B, que resultou em vários grupos genômicos (Simmonds e Shepher, 1955; De Langhe et al., 2009). Atualmente, as bananas são amplamente distribuídas nos subtrópicos como alimento básico para milhões de pessoas, contribuindo para a segurança alimentar e o desenvolvimento econômico de diversos países, com mais de 1000 variedades existentes (De Le et al., 2009; Davey et al., 2013).

Com a evolução das bananas cultivadas para consumo, os patógenos causadores de doenças também coevoluíram, e se tornaram cada vez mais eficientes em infectar o hospedeiro, dentre os quais destaque para *Fusarium oxysporum* f. sp. *cubense* (Foc), agente causal da murcha de Fusarium, considerado uma das grandes ameaças à produção global da fruta (Ploetz e Pegg, 1997; Fourie et al., 2011; Maryani et al., 2019). Foc é classificado em três raças, distinguidas pela capacidade de infectar diferentes cultivares, sendo a raça 1 (R1) capaz de causar doença em Gros Michel (AAA) e outras cultivares do tipo Silk (AAB), a raça 2 (R2) capaz de infectar as bananas de cocção do tipo Bluggoe (ABB), e a raça 4 (R4) que causa doença em cultivares do subgrupo Cavendish (AAA), e às cultivares que são suscetíveis a R1 e R2 (Waite e Stover, 1960; Ploetz, 2006).

Foc R4 foi dividido em raça 4 subtropical (ST4), que infecta cultivares ‘Cavendish’ nos subtrópicos expostas a condições de estresse, como baixas temperaturas e seca, predispondo as plantas à infecção; e Foc raça 4 tropical (TR4), que infecta cultivares Cavendish em condições tropicais e subtropicais, sem a necessidade de estresses predisponentes (Bancroft, 1876; Buddenhagen, 2009; Ploetz et al., 2015; Mostert et al., 2017; Dita et al., 2018). Além da classificação em raças, existem 24 Grupos de Compatibilidade Vegetativa (VCGs) que, por anastomose de hifas, conseguem formar heterocários estáveis, cujo material genético pode ser compartilhado por indivíduos compatíveis (Aguayo et al., 2017). Foc ST4 inclui os VCGs 0120, 0122, 0123, 0126, 0129, 01210, 01211, 01215 e 01219 e o Foc TR4 foi caracterizado como um único clone do patógeno que corresponde apenas aos VCGs 01213/16 e que é altamente agressivo, sendo o principal responsável pelas epidemias em bananas

‘Cavendish’ nos trópicos (Buddenhagen, 2009; Fourie et al., 2009; O’Donnell et al., 2009; Mostert et al., 2017; Dita et al., 2018; Maryani et al., 2019).

No geral, Foc é um patógeno habitante de solo que penetra através das raízes primárias ou por meio de pontos de abertura natural ou ferimentos (Li et al., 2011; Pegg et al., 2019; Rocha et al., 2020; Niwas et al., 2022). O patógeno progride em direção aos feixes vasculares causando o sintoma reflexo de murcha, inicialmente observado nas folhas velhas e posteriormente nas folhas jovens, que se dobram junto ao pseudocaule, finalizando com o colapso da planta (Stover, 1962; Fourie et al., 2009; Ploetz et al., 2015; Ploetz, 2018). Internamente as plantas apresentam uma coloração marrom ou pardo-avermelhada no rizoma que pode ser vista pelo corte transversal, ou como pontuações em um corte longitudinal do pseudocaule, sinalizando a oclusão dos vasos xilemáticos. Em muitos casos, é possível observar uma rachadura do pseudocaule, quando o patógeno cresce em direção as bainhas das folhas (Stover e Simmonds, 1987; Dita et al., 2010).

O ciclo de vida do Foc baseia-se nos mecanismos inerentes aos patógenos causadores de murcha vascular, habitantes de solo, sendo representado pela estratégia hemibiotrófica, com um período inicial no qual este se aproveita dos tecidos vivos do hospedeiro e completa o seu ciclo com um período necrotrófico, causando a morte do hospedeiro (Dita et al., 2018; Warman e Aitken, 2018). Seu hábito saprófita permite que possa sobreviver em restos culturais por períodos de pousio, e dificulta o manejo do solo baseado em rotação de culturas, principalmente porque o gênero *Fusarium* afeta muitas espécies de plantas cultivadas, além de poder sobreviver como endofítico, até mesmo em plantas daninhas (Hennessy et al., 2005; Waman et al., 2013; Perez-Vicente et al., 2014; Catambacan e Cumagun, 2022). Além disso, a produção de estruturas de sobrevivência, denominadas clamidósporos, são importantes meios de perpetuação do fungo em solos por longos períodos de tempo (Ploetz et al., 2015; Dita et al., 2018; Warman e Aitken, 2018; Ploetz, 2018; Pegg et al., 2019).

Não há medidas químicas eficientes para o controle do patógeno Foc em solos infestados e nem tratamentos químicos sistêmicos que possam conter a infecção após a colonização dos tecidos, devido, principalmente, à saúde do solo e a variabilidade patogênica do Foc (Siamak and Zheng, 2018; Anderson e Aitken, 2021). Pesquisas voltadas para o controle biológico têm avançado, com foco na obtenção de produtos biológicos a base de microrganismos benéficos que podem ser aliados ao manejo integrado, embora as informações sobre sua eficácia a longo prazo ainda sejam

limitadas, sendo a maioria dos estudos de curto prazo e, principalmente, *in vitro* (Siamak and Zheng, 2018; Bubici et al., 2019; Catambacan e Cumagun, 2021).

Nesse sentido, o melhoramento genético é alvo de muitos programas em diferentes centros de pesquisa da banana no mundo, que estão focados em obter cultivares resistentes ao Foc, por meio do melhoramento tradicional, baseado em cruzamentos, ou pelo uso de ferramentas biotecnológicas, como transgenia, variação somaclonal, mutagênese ou edição de genomas (Saraswathi et al., 2016; Dale et al., 2017; Ferreira et al., 2020; Rocha et al., 2021). Assim, informações obtidas após o sequenciamento do genoma da bananeira diploide selvagem 'Pahang' estão em uso, visando alcançar mais dados do transcriptoma da bananeira infectada por Foc e entender melhor o mecanismo molecular da resistência à murcha de Fusarium, aplicáveis no melhoramento genético (D'Hont et al., 2012; Li et al., 2013).

Mesmo com uma quantidade substancial de dados sobre as respostas de defesa da bananeira na interação Foc x *Musa* spp., os mecanismos da resistência ainda não foram completamente elucidados. Além disso, ainda não existe uma cultivar disponível que seja imune ao Foc TR4, ou com um nível de resistência basal desejável. Por isso, todos os esforços dirigidos para a compreensão dos mecanismos de ataque do patógeno, bem como das respostas de defesa da bananeira, podem trazer contribuições valiosas ao melhoramento genético, principalmente pela identificação de padrões globais de expressão gênica, influenciados pela infecção de diferentes raças.

Portanto, este trabalho tem por objetivos: 1) realizar uma revisão sistemática da literatura publicada nos últimos dez anos sobre o melhoramento genético da bananeira com foco em murcha de Fusarium; 2) Avaliar o processo infeccioso de isolados de *Fusarium oxysporum* f. sp. *cubense* nas raízes de cultivares de bananeira em nível histológico e histoquímico e 3) Avaliar o processo infeccioso de isolados de *Fusarium oxysporum* f. sp. *cubense* nas raízes de cultivares de bananeira em nível molecular pela quantificação da expressão de genes de resistência por qRT-PCR ao longo do tempo. Esses dados trarão novas contribuições para o desenvolvimento de cultivares de bananeira resistentes a murcha de Fusarium e ampliarão o conhecimento sobre os mecanismos envolvidos na interação planta-patógeno.

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CAPÍTULO 1

Improvements in the Resistance of the Banana Species to Fusarium Wilt: A Systematic Review of Methods and Perspectives

Artigo - Improvements in the Resistance of the Banana Species to Fusarium Wilt: A Systematic Review of Methods and Perspectives, publicado na revista científica Journal of Fungi, disponível em: https://doi.org/10.3390/jof7040249

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Review

Improvements in the Resistance of the Banana Species to Fusarium Wilt: A Systematic Review of Methods and Perspectives

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Abstract: The fungus *Fusarium oxysporum* f. sp. *cubense* (FOC), tropical race 4 (TR4), causes *Fusarium* wilt of banana, a pandemic that has threatened the cultivation and export trade of this fruit. This article presents the first systematic review of studies conducted in the last 10 years on the resistance of *Musa* spp. to *Fusarium* wilt. We evaluated articles deposited in different academic databases, using a standardized search string and predefined inclusion and exclusion criteria. We note that the information on the sequencing of the *Musa* sp. genome is certainly a source for obtaining resistant cultivars, mainly by evaluating the banana transcriptome data after infection with FOC. We also showed that there are sources of resistance to FOC race 1 (R1) and FOC TR4 in banana germplasms and that these data are the basis for obtaining resistant cultivars, although the published data are still scarce. In contrast, the transgenics approach has been adopted frequently. We propose harmonizing methods and protocols to facilitate the comparison of information obtained in different research centers and efforts based on global cooperation to cope with the disease. Thus, we offer here a contribution that may facilitate and direct research towards the production of banana resistant to FOC.

Keywords: *Musa* spp.; *Fusarium oxysporum* f. sp. *cubense*; genetic improvement; resistance; state-of-the-art

1. Introduction

Dessert bananas and plantains are very popular fruits worldwide. In 2018, approximately 116 million tons of bananas and 40 million tons of plantains were produced [1]. In terms of exports, bananas are among the most traded fruits globally, with almost 23 million tons (except for plantains) exported in 2017, representing almost 20% of global production [1]. Approximately 11.3 million hectares are dedicated to banana and plantain production worldwide, and there are more than 1000 varieties produced and consumed locally [2]. The Cavendish banana, which accounts for about 47% of global production, is the most traded [2]. In African regions, plantains comprise a significant and essential component, contributing considerably to food security and income generation for more than 70 million Africans [3–5]. Similarly, in Latin America and the Caribbean, 62% of total banana and plantain production (20 million tons) is consumed locally, and approximately

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6.8 million tons of plantains are produced, of which 72% are traded on international markets, indicating the enormous importance of these crops for local food and food security throughout the region [6,7].

Among banana improvement programs' objectives are achieving cultivars resistant to abiotic stressors, such as salinity [8,9] and drought [10–12]. Another major challenge for the global production of Musaceae species is the development of cultivars resistant to biotic stressors, represented by their primary pests, the banana root borer (*Cosmopolites sordidus*) and the nematodes *Meloidogyne* spp., *Pratylenchus coffeae*, and *Radopholus similis* [13–19], and disease-causing pathogens, including banana bunchy top virus (BBTV) [20,21], *Xanthomonas vasicola* pv. *musacearum* causing bacterial wilt [22–25], *Pseudocercospora fijiensis* causing black Sigatoka [26–29], and *Fusarium oxysporum* f. sp. *cubense* (FOC) causing *Fusarium* wilt [30–33]. FOC is one of the main biotic stress factors affecting bananas and *Fusarium* wilt is considered the most destructive and widely spread disease in the banana-producing regions around the world [34,35]. The causal agent is a soilborne fungus apparently considered hemibiotrophic; therefore, it initially establishes in a biotrophic relationship interacting with live plant cells of the host, and then in its necrotrophic phase, the host's tissues are dead [30]. Frequently FOC persists in cultivated areas for years due to it is survival phase when it then interacts as saprophytic in cultural remains or produces resting spores known as chlamydospores besides surviving and multiplying in alternative hosts [30,35,36]. The disease is characterized by yellowing of the young leaves, and pseudostem splitting, and eventually death of the plant [30,37,38].

Fusarium wilt epidemics caused by race 1 (FOC R1), which occurred in Central America, caused the devastation of the susceptible "Gros Michel" cultivar plantations and was one of the most severe in the history of the crop in the Americas. For this reason, Gros Michel was replaced by cultivars of the subgroup Cavendish that are resistant to FOC R1 [39–41]. However, in the late 1980s, a highly virulent strain of FOC-infected Cavendish cultivars and spread to Asia, Africa, Indonesia, and more recently to South America [30,41,42]. Currently, *Fusarium* wilt can be considered a pandemic disease because of the spread of the tropical race 4 (FOC TR4) strain [43,44].

Chemical control is unfeasible and minimally effective, and it can be harmful to human health and the environment. Although still in its initial stages, biological control demonstrates promising results [31,45]. Low efficacy of the biological control is attributed to inherent factors to the dynamics of the disease's primary inoculum, especially production of chlamydospores, which persists in cultivated areas, such as the capacity to survive in crop remains as an endophytic fungus in alternative hosts [30,36,46]. In addition, the genetic variability of the pathogen, resulting in new strains capable of infecting resistant cultivars, is another factor that limits the use of methods of disease management and control [47–49]. Therefore, efforts on the genetic improvement to achieve resistance to FOC R1 and FOC TR4 have been focused on finding resistant cultivars through traditional methods of germplasm selection or the generation of new cultivars by hybridization, genetic transformation, somaclonal variation, or mutation induction [31,50,51].

Until now, there are reviews available in the literature about *Fusarium* wilt related to epidemiology and disease management [30,35,41,52–54], biological control [45,55], genetic breeding for resistance [56,57] and one review about genomic aspects of *Musa* spp. for stress resistance [58].

The systematic reviews were mainly developed because of the need for rapid responses to human health issues, and nowadays, this tool has contributed to several study areas [59–61]. However, to our best knowledge, no systematic reviews on the genetic improvement of *Musa* spp. to resist Fusarium wilt have been published; only studies related to water stress in *Musa* spp. [62] and banana consumption [63]. Therefore, to provide detailed information on the subject and to collaborate with the information gathered so far, we propose a systematic approach to the studies on *Musa* spp., with a focus on genetic improvement for resistance to the FOC pathogen, through a systematic review of studies conducted over the last 10 years.

2. Materials and Methods

The free software State of the Art by Systematic Review (StArt) v.3.3 beta 03, developed by the Federal University of São Carlos (UFSCar), was used to perform a systematic review. This tool offers systematized answers to questions directed toward the objective of the review. The review process was performed in three stages—planning, execution, and summarization—according to the review flowchart in Figure 1, which followed the model proposed by Santos et al. [62].

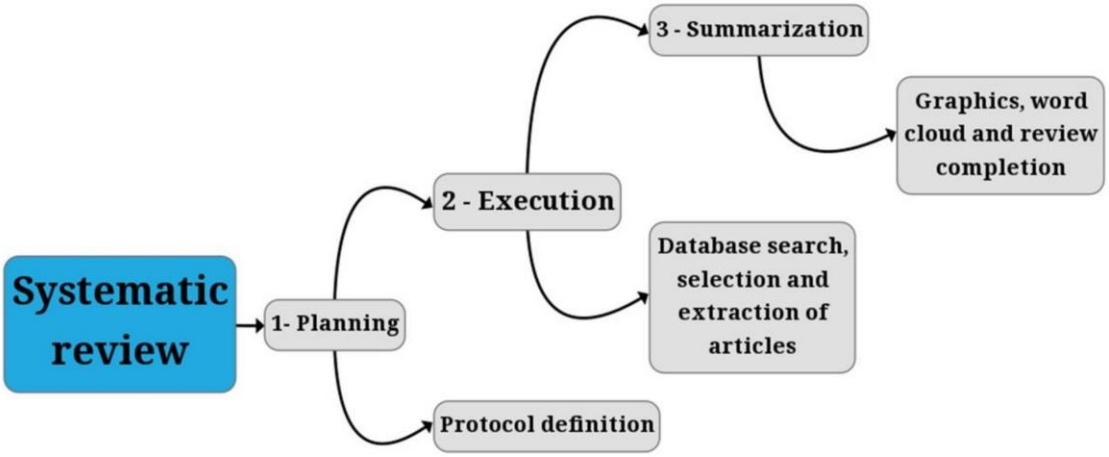

Figure 1. General systematic literature review flowchart. Source: author's compilation.

2.1. Planning

A protocol to be followed during the review process was formulated, in which the title, objective, keywords, research questions, research sources, and inclusion/exclusion criteria of articles were defined during their selection and extraction. The StArt protocol is available for download at https://doi.org/10.5281/zenodo.4555385 (accessed on 22 February 2021). The research questions of the review are listed in Table 1.

Table 1. List of questions about the genetic improvement of *Musa* for resistance to *Fusarium* wilt to be answered by a systematic review of studies carried out in the last ten years.

Research Questions
Q1: What are the known sources of resistance (germplasm) to *Fusarium oxysporum* f. sp. *cubense*?
Q2: Which breeding programs work on the resistance to *Fusarium* wilt with respect to cultivar development?
Q3: Which genes are reported associated with resistance to *Fusarium oxysporum* f. sp. *cubense* in *Musa* spp.
Q4: What breeding techniques are associated with overcome *Fusarium* wilt?
Q5: Which biotechnological tools are used for assisted selection for resistance to *Fusarium oxysporum* f. sp. *cubense*?
Q6: Which germplasm collections have information with the potential for genetic improvement to *Fusarium* wilt?
Q7: What is the frequency of studies by country, and which programs of improvement work with crossbreeding in order to develop resistant cultivars?
Q8: Are there scales to assess the disease? What is the difference between them?
Q9: How often is the banana genome used?

To answer question 7, when there was no mention in the text of the location where the study was conducted, the search criteria within the article were standardized to the corresponding author's mailing address to obtain information from which country the studies originated.

2.2. Execution: Search

Electronic surveys were conducted on the following databases, aiming to identify publications made available between January 2010 and December 2020: Scopus (http://www.scopus.com/), Web of Science (http://apps.isiknowledge.com), PubMed Central (https://www.ncbi.nlm.nih.gov/pmc/about/intro/), Springer (https://www.springer.com/br); Coordination for the Improvement of Higher Education Personnel Portal Journal (http://www.periodicos.capes.gov.br/), and Google Scholar (https://scholar.google.com.br/schhp?hl=en&as_sdt=0,5), using a standardized search string with the following keywords: *Musa* spp. and bananas and plantains and *Fusarium* wilt or *Fusarium oxysporum* f. sp. cubense or Panama disease and genetic resistance and markers and genes. This set of terms was used for research in all fields within the articles. The Boolean operators AND and OR were used to differentiate the search terms. Search results in each base were imported into BIBTEX, MEDLINE, or RIS formats, compatible with StArt. Relevant documents not found or published after the selection stage started were added manually. We did not consider using the name *Fusarium odoratissimum* proposed by Maryani et al. [64] in our standardized search due to the low number of published articles using this new suggested nomenclature, and this would limit the number of recovered articles in the database.

2.3. Execution: Selection and Extraction

In the selection stage, the articles that contained the terms adopted in the search string in the title, abstract, or keywords were accepted. In the extraction stage, where the number of articles was restricted, a single criterion to include articles was adopted, as follows: (I) articles that answer the protocol's questions (Table 1). The criteria used to exclude articles in the extraction stage were (E) review articles, (E) theses, dissertations, manuals, and book chapters, (E) articles outside the subject, (E) articles published in event annals, (E) articles on genetic diversity of FOC, (E) articles on disease management strategies, and (E) articles on first reports of FOC. These criteria were considered to restrict the selected articles to the focus of this review since they do not answer the proposed questions about improving the resistance of *Musa* spp. to FOC. The preferred reporting items for systematic reviews and meta-analyses (PRISMA) checklist is presented for download at https://doi.org/10.5281/zenodo.4313617 (accessed on 9 December 2020).

2.4. Analysis of the Articles

The process of analyzing the articles was based on the calculation of the frequencies of articles related to each of the research questions. Subsequently, graphs, word clouds, and tables were prepared.

3. Results

3.1. Screening of Studies

The article screening process is represented by the flow chart in Figure 2. PubMed Central contributed the largest number of articles to this review, with 806 (50%) of the total, followed by Web of Science with 361 (22%) and Google Scholar with 319 (20%). The other databases, namely Scopus, Springer, and Coordination for the Improvement of Higher Education Personnel Portal Journal, contributed 69 (4%), 26 (2%), and 8 (0.5%) articles, respectively. Moreover, 22 (1.2%) articles that were not obtained automatically were added manually (Figure 2).

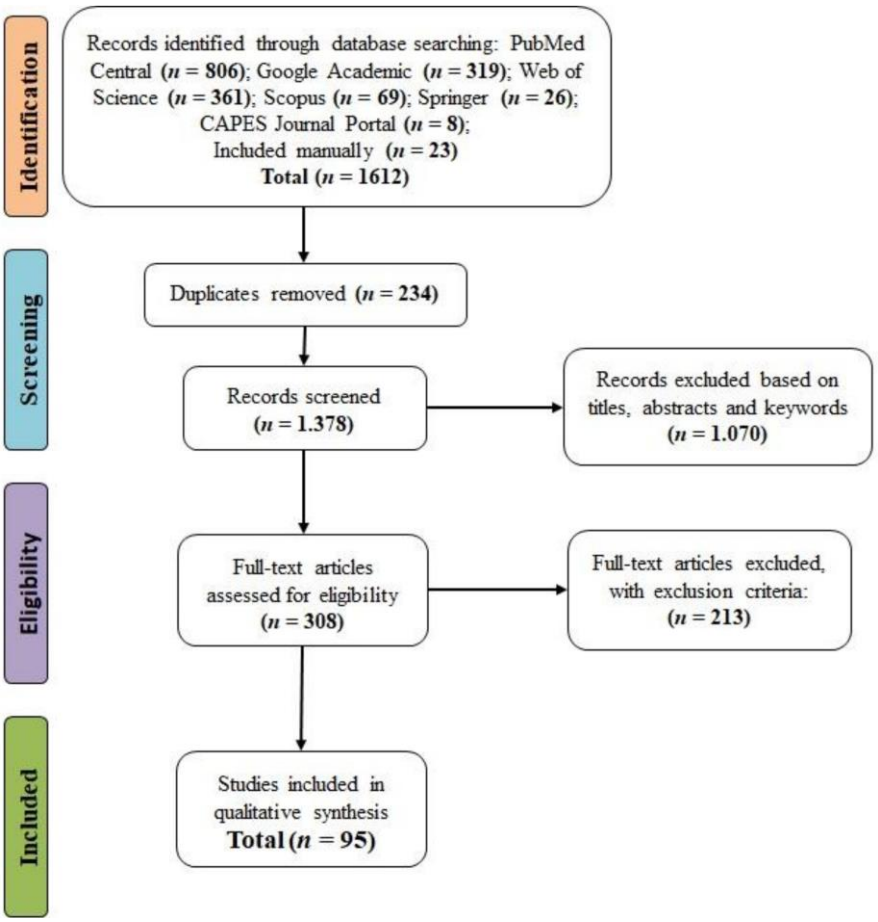

Figure 2. Flow diagram (preferred reporting items for systematic reviews and meta-analyses (PRISMA)). The selection process of studies for inclusion or exclusion in the systematic review on genetic improvement of banana for resistance to *Fusarium* wilt. *n* = number of studies. The flow diagram was based on a model available at http://prisma-statement.org/PRISMAStatement/FlowDiagram. CAPES: Coordination for the Improvement of Higher Education Personnel (accessed on 9 December 2020).

We identified 1612 articles from the database tracking, of which 234 were duplicated, and 1377 were eliminated in the selection process by reading the title, abstracts, and keywords, which did not fit the purposes of the research. In the extraction stage, 308 articles were analyzed. After reading the articles entirely, 213 were eliminated; hence, 95 were selected to compose the systematic review (Figure 2). The articles selected to compose the systematic review are available for consultation and download at https://doi.org/10.5281/zenodo.4555343 (accessed on 22 February 2021) and its origin and database in Table S2.

A word cloud was generated from the keywords of the 95 articles for this review. As expected, there was a predominance of the articles with the keywords, "*Fusarium oxysporum* f. sp. *cubense*", "Fusarium wilt", "Musa", "banana", "disease", "resistance", "race", tropical, and TR4 (Figure 3). Other keywords that had a remarkable frequency in the word cloud were "gene", "Panama", "transformation or transgenesis", "plant", "infection, "green fluorescent protein (GFP)", "protein", "SCAR", "Acuminata", "species", "Cavendish", and "polymerase chain reaction (PCR)" (Figure 3).

Figure 3. Word cloud generated from article keywords of selected articles to compose a systematic review on breeding *Musa* to *Fusarium* wilt. The word cloud was created in a free online generator (https://www.wordclouds.com/, accessed on 16 August 2020), based on the frequency of each keyword.

3.2. Known Origin Sites

Among the 95 articles, 53% were from China, followed by India (15%), Australia (12%), Brazil (7%), Malaysia (5%), Indonesia (4%), Uganda (4%), and other countries with a contribution of 1% (Figure 4). In the selected articles, 10 improvement programs located in different countries were mentioned, containing information with the potential for genetic improvement for the resistance of *Musa* spp. to *Fusarium* wilt (Figure 4).

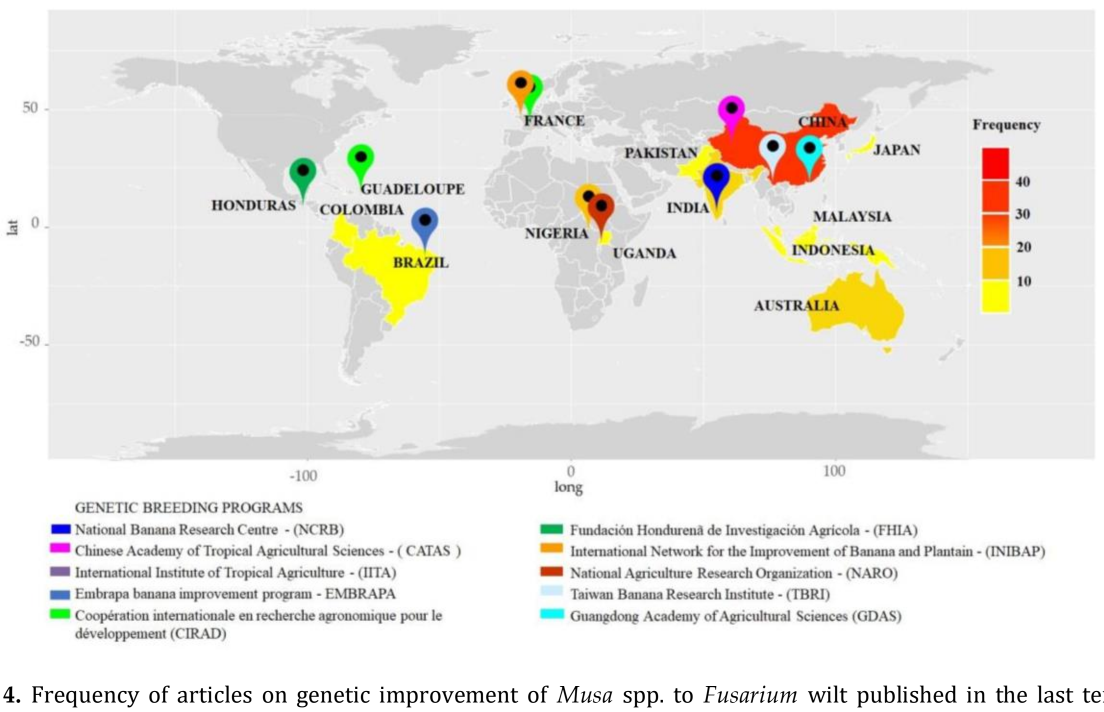

Figure 4. Frequency of articles on genetic improvement of *Musa* spp. to *Fusarium* wilt published in the last ten years in different countries and genetic breeding programs of the banana mentioned. The light yellow tones indicate a frequency below 10% of the articles considered in this review; the intermediate tones indicate frequencies between 10 and 30%, and the intense red tones indicate frequencies above 40%. The location icons indicate the locations of Musa breeding programs identified by the colors. The map was plotted in R, using the packages maps, ggmap, geosphere, Eurostat, GADMTools, country code and ggplot2. lat: latitude; long: longitude.

Among the improvement programs cited, those that worked with crossbreeding to develop resistant cultivars were as follows: Honduras Foundation for Agricultural Research (Fundación Hondureña de Investigación Agrícola—FHIA), located in Honduras; Centre Africain de Recherches sur Bananiers et Plantains (CARBAP) in Cameroon; the International Institute of Tropical Agriculture (IITA) in Nigeria and Uganda; National Improvement Program of the Brazilian Agricultural Research Corporation (EMBRAPA) in Brazil; National Banana Research Center (NCRB) in India; National Research Organization (NARO) in Uganda; and Centre de Coopération Internationale en Recherche Agronomique pour le Développement (CIRAD) in Guadeloupe, French Antilles (Figure 4). In contrast, the improvement programs Taiwan Banana Research Institute (TBRI) and Guangdong Academy of Agricultural Sciences (GDAAS) in China worked with somaclonal variants and biotechnology.

3.3. Main Methods and Tools

Concerning the fungal races, the highest number of articles addressed specific studies with FOC TR4 (57%), 25.8% of the studies dealt only with FOC R1, and 10.1% of the articles performed comparative studies between FOC TR4 and FOC R1 (Figure 5A). Other studies with lower numbers conducted studies on subtropical race 4 (FOC STR4) (3.2%), FOC subtropical race 4 (STR4) and Foc TR4 (2.2%), and FOC R1 and FOC STR4 (1%) (Figure 5A). The highest frequency of articles was related to in silico (42.1%) and in vitro (32.6%) studies, followed by studies performed only in the greenhouse (12.6%), in the greenhouse and the field (5.3%), in the field only (4.2%), in the glasshouse (2.1%), and in other places (1%) (Figure 5B).

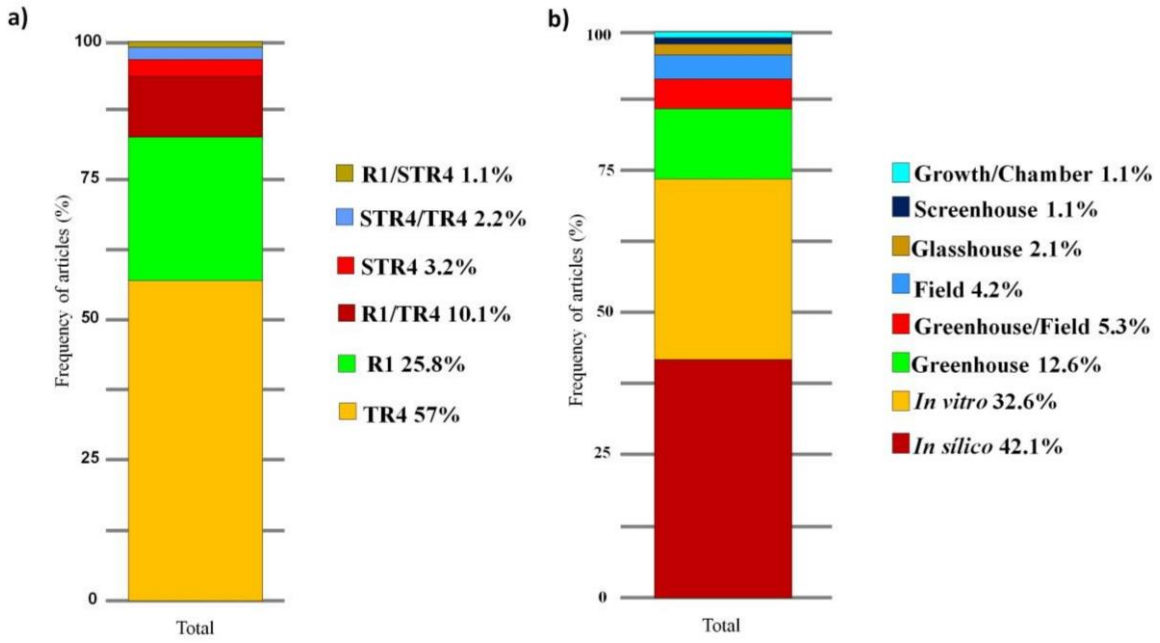

Figure 5. Stacked bar chart of the frequency of articles with different races of *Fusarium oxysporum* f. sp. *cubense* in the past ten years (**a**). Places of achievement of work in articles on the improvement of banana plants to *Fusarium* wilt carried out in the last 10 years (**b**). R1: race 1; STR4: subtropical race 4; TR4: tropical race 4.

To evaluate *Fusarium* wilt symptoms, 26 scales were cited, divided among rhizome-discoloration symptoms, leaf-yellowing symptoms, and pseudostem division (Table 2). We found that 37% (n = 36) of studies adopted a scoring scale for external or internal *Fusarium* wilt symptoms (Table 2). According to the highest frequency of articles, the most-used scoring grades were from 1 to 6 for rhizome-discoloration and leaf-yellowing symptoms

and from 1 to 3 for pseudostem division (Table 2). The most frequently cited scales were those of [65–68].

Table 2. Scales of grades for assessing symptoms of *Fusarium oxysporum* f. sp. *cubense* reported in articles on banana breeding to *Fusarium* wilt conducted in the last ten years.

Article	Internal Symptoms	External Symptoms		Scale Reference
	Rhizome Discoloration	Yellowing of the Leaf	Pseudostem Division	
	Degrees of Scale			
Yip et al. [69]	0–3			[69] *
Orr et al. [70]	1–6			[71]
Chen et al. [72]	1–8			[68]
Warman and Aitken [46]	1–6			[66]
Baharum et al. [73]	1–8			[68]
Zhang et al. [74]	0–4	0–4		[75,76]
Zuo et al. [77]	1–5			[77] *
Ribeiro et al. [78]	0–5	0–4		[67,79]
Wei et al. [80]		0–4		[80] *
Garcez et al. [81]	0–5	0–5		[67,82]
Li et al. [75]	0–3	0–3		[75] *
Ghag et al. [83]		1–6		[66]
Smith et al. [84]	1–6			[65,85]
Mohandas et al. [86]	1–6	0–5		[65,87]
Ting et al. [88]		0–5		[88] *
Paul et al. [89]		1–5	1–3	[89] *
Sun et al. [90]	1–5	1–5		[64,91]
Wu et al. [92]		1–6		[92] **
Ssali et al. [93]		1–6		[94]
Li et al. [95]	0–4	0–5		[95] *
Ghag et al. [96]		1–6		[96] *
Saraswathi et al. [97]	1–5	1–5		[66,91]
Ghag et al. [98]		1–6		[83]
Sun et al. [76]		0–4		[76] *
Wu et al. [99]		1–6		[99] **
Magambo et al. [100]		1–5	1–3	[68]
Smith et al. [101]	1–6			[65]
García-Bastidas et al. [102]	1–6	1–4		[102] *
Arinaitwe et al. [31]	1–5	1–6	1–3	[71]
Cheng et al. [103]	1–8			[68]
Gonçalves et al. [33]	1–5	1–6		[67,104]
Buregyeya et al. [105]	1–6		1–3	[94]
Sunisha et al. [106]		1–5	1–3	[89]
Rocha et al. [107]	1–5	1–4		[104]
Ahmad et al. [108]	1–6	1–4		[102]

* use their own scale; ** in vitro.

Among the main methods used for obtainment or characterization of plants resistant to *Fusarium* wilt, gene expression analysis represented 33% of the selected articles,followed by transgenesis (16%), symptomatology (13%), and resistance induction (11%) (Figure 6). In related articles, the other methods were classified as molecular markers (5%), symptomatology associated with the agronomic characterization of banana genotypes (5%),in vitro mutagenesis (4%), enzyme activity (3%), protein analysis and expression (3%), hybridization by crossbreeding (2%), and methods of somaclonal variation, clone selection, and somatic embryogenesis, each with a 1% frequency (Figure 6).

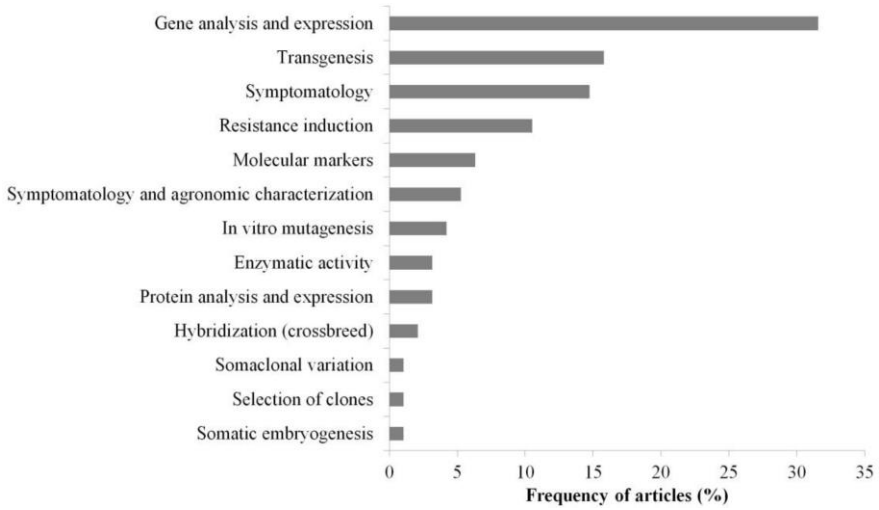

Figure 6. Banana plant breeding techniques used to supplant *Fusarium* wilt in articles published in the last 10 years.

Among the tools used for the analysis and characterization of plants resistant to *Fusarium* wilt, the frequency of articles that employed analysis of reverse transcription-PCR (RT–qPCR) and PCR was the highest (35%). Analyses using bioinformatics tools were in 23% of the articles, and tissue culture represented 13% (Figure 7). Other tools adopted included the genetic transformation of the fungus with the GFP gene, the infection process by FOC strains (7%), banana transcriptome (7%), and phylogenetic analysis (7%). In addition to these tools, there was also a portion of articles using histochemistry and/or histology (6%) and other tools with a lower frequency (Figure 7).

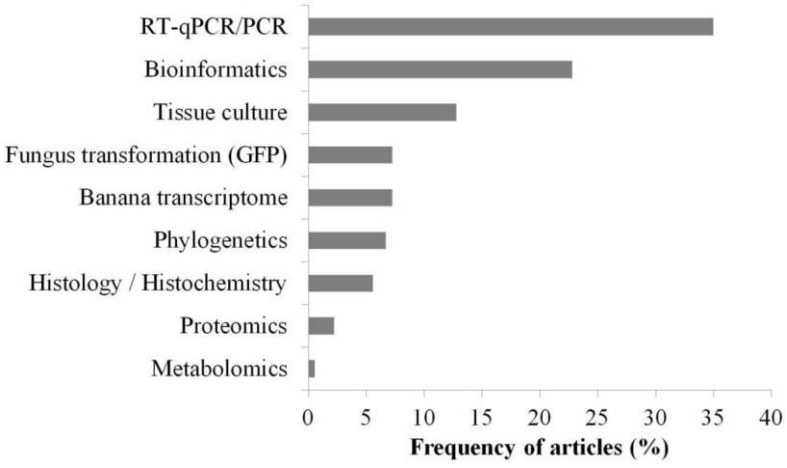

Figure 7. Frequency of articles associated with the main tools used in studies on banana plant breeding to *Fusarium* wilt in the last 10 years. The frequency considered that more than one tool was used per article. RT–qPCR/PCR: reverse transcription-PCR/polymerase chain reaction/ GFP: green fluorescent protein.

Some articles used molecular markers associated with wilting resistance: Silva et al. [109], Wang et al. [110], and Wang et al. [51]. Among the markers associated with the resistance to FOC TR4, seven were from sequence characterized amplified region (SCAR)-type. One marker was associated with the susceptibility to FOC R1 [111] (Table 3). One random amplified polymorphic DNA (RAPD) molecular marker associated with the resistance to FOC R1 was found by Ghag et al. [98] (Table 3).

Table 3. Molecular markers associated with banana breeding strategies to *Fusarium* wilt in articles carried out in the last ten years.

Name	Type	Function	Citation
ScaU1001	SCAR	Resistance to FOC TR4	[109]
SuscPD	SCAR	Susceptibility to FOC 1	[111]
Lipoxygenase (gene)	RAPD	Resistance to FOC 1	[98]
ScaU1001 ScaS0901	SCAR	Resistance to FOC TR4	[110]
SC1/SC2 SC3/SC4 SC5/SC6 SC7/SC8	SCAR	Resistance to FOC TR4	[51]

SCAR: sequence characterized amplified region; RAPD: random amplified polymorphic DNA.

3.4. Resistance Sources

In the set of selected articles, many sources of resistance to *Fusarium* wilt were found for different FOC races (Table 4). Of the sources reported as resistant, 38% were triploid (AAA genome), 33% were diploid (AA genome), 12% were triploid (AAB genome), and 8% were tetraploid (AAAB genome); other genomes reported had a frequency of less than 5% (Figure 8 and Table 4).

Table 4. Sources of resistance to *Fusarium oxysporum* f. sp. *cubense* characterized in articles on the improvement of banana to *Fusarium* wilt carried out in the last ten years.

Musa Germplasm	*Musa* Genome	Race	Level of Tolerance or Resistance to Races	Institution and Location/Country Where Germplasm Was Screened	Known Use to Mitigate Fusarium Impact	References
M53	AA	Race 1	R	Embrapa cassava and fruit growing, Brazil	In breeding programs	[33,78,107]
Birmanie	AA	Race 1	R	Embrapa cassava and fruit growing, Brazil	In breeding programs	[78,107]
PA Songkla	AA	Race 1	R	Embrapa cassava and fruit growing, Brazil	In breeding programs	[78]
Pirua	AAA	Race 1	R	Embrapa cassava and fruit growing, Brazil	Brazil	[78]
Imperial	AAA	Race 1	R	Embrapa cassava and fruit growing, Brazil	Brazil	[78]
Poyo	AAA	Race 1	R	Embrapa cassava and fruit growing, Brazil [78], DAFF, Australia [84]	Brazil, Africa	[78,84]
BRS Vitória	AAAB	Race 1	R	Embrapa cassava and fruit growing, Brazil	Brazil	[107]
Ambei	AA	Race 1	R	Embrapa cassava and fruit growing, Brazil	In breeding programs	[78]
Walebo	AAA	Race 1	R	Embrapa cassava and fruit growing, Brazil	Brazil	[78]

Table 4. *Cont.*

Musa Germplasm	*Musa* Genome	Race	Level of Tolerance or Resistance to Races	Institution and Location/Country Where Germplasm Was Screened	Known Use to Mitigate Fusarium Impact	References
Kongo FRF 1286	AAA	Race 1	R	Embrapa cassava and fruit growing, Brazil	Brazil	[78]
Pisang Nangka	AAB	Race 1	R	Embrapa cassava and fruit growing, Brazil	Brazil, Africa, Australia	[78]
Pisang Jaran	AA	Race 1	R	Embrapa cassava and fruit growing, Brazil	In breeding programs	[78]
Tjau Lagada	AA	Race 1	R	Embrapa cassava and fruit growing, Brazil [33,78]	In breeding programs	[33,78]
Mangana	AA	Race 1	R	Embrapa cassava and fruit growing, Brazil	In breeding programs	[78]
Pisang Pipit	AAA	Race 1	R	Embrapa cassava and fruit growing, Brazil	In breeding programs	[78]
Pisang Rojo Uter	AA	Race 1	R	Embrapa cassava and fruit growing, Brazil	In breeding programs	[78]
2803-01	AA	Race 1	R	Embrapa cassava and fruit growing, Brazil	In breeding programs	[78]
GN. P. Formoso	AAA	Race 1	R	Embrapa cassava and fruit growing, Brazil	In breeding programs	[109]
Pisang Tongat	AA	Race 1	R	Embrapa cassava and fruit growing, Brazil	In breeding programs	[78]
Mchare cultivars	AA	Race 1	R	Stellenbosch University, South Africa (Arusha, Tanzania)	Africa	[112]
Mchare hybrids	AA	Race 1	R	Stellenbosch University, South Africa (Arusha, Tanzania)	Africa	[112]
NARITA hybrids	AA	Race 1	R	Stellenbosch University, South Africa (Kawanda, Uganda)	Africa	[112]
Figo Cinza	ABB	Race 1	R	Embrapa cassava and fruit growing, Brazil [78] Banana Germplasm Bank of the Itajaí Research Station [111]	Brazil	[78,111]
M-61	AAA	Race 1	R	Embrapa cassava and fruit growing, Brazil	In breeding programs	[78]
Nanicão Magario	AAA	Race 1	R	Embrapa cassava and fruit growing, Brazil	Brazil	[78]
Buitenzorg	AA	Race 1	R	Embrapa cassava and fruit growing, Brazil	In breeding programs	[78]
BRS Platina	AAAB	Race 1	R	Embrapa cassava and fruit growing, Brazil [33,78,107] Itajaí Research Station, Brazil [111]	Brazil	[33,78,107, 111]
Nanica	AAA	Race 1	R	Embrapa cassava and fruit growing, Brazil	Brazil	[78,107, 109]
Pisang Ustrali	AAB	Race 1	R	Embrapa cassava and fruit growing, Brazil	In breeding programs	[78]
Markatooa	AAA	Race 1	R	Embrapa cassava and fruit growing, Brazil	In breeding programs	[78]
Robusta	AAA	Race 1	R	Embrapa cassava and fruit growing, Brazil	In breeding programs	[78]
BRS Pacovan Ken	AAAB	Race 1	R	Embrapa cassava and fruit growing, Brazil	Brazil	[78,107]
BRS Princesa	AAAB	Race 1	R	Federal Institute of the Triangulo Mineiro, Brazil [74], Embrapa cassava and fruit growing, Brazil [33,107]	Brazil	[33,81,107]

Table 4. *Cont.*

Musa Germplasm	*Musa* Genome	Race	Level of Tolerance or Resistance to Races	Institution and Location/Country Where Germplasm Was Screened	Known Use to Mitigate Fusarium Impact	References
BRS Japira	AAAB	Race 1	R	Federal Institute of the Triangulo Mineiro, Brazil [81] Embrapa cassava and fruit growing, Brazil [107]	Brazil	[81,107]
BRS Tropical	AAAB	Race 1	R	Federal Institute of the Triangulo Mineiro, Brazil	Brazil	[81]
Grand Naine	AAA	Race 1	R	Embrapa cassava and fruit growing, Brazil [33,78,107,109], Federal University of Santa Catarina, Brazil [111]	Cavendish for export	[33,78,107, 109,111]
Nanicão	AAA	Race 1	R	Embrapa cassava and fruit growing, Brazil [78,111], Federal University of Santa Catarina, Brazil [111]	Brazil	[78,109, 111]
SCS452 Corupá	AAA	Race 1	R	Federal University of Santa C tarina, Brazil	Brazil	[111]
Zellig	AAA	Race 1	R	Federal University of Santa C tarina, Brazil	Brazil	[111]
Figo	ABB	Race 1	R	Embrapa cassava and fruit growing, Brazil [78], Federal University of Santa C atarina, Brazil [111]	In breeding programs	[78,111]
FHIA-17	AAAA	Race 1	R	D FF, Australia	Honduras, Brazil	[84]
SH-3640.10	AAAB	Race 1	R	DAFF, Australia	Honduras, Brazil, Mozambique, Cameroon	[84]
Long Tavoy	*	Race 1	R	University of Malaya, Kuala Lumpur, Malaysia	In breeding programs	[31]
Kasaska	*	Race 1	R	University of Malaya, Kuala Lumpur, Malaysia	In breeding programs	[31]
Monyet	*	Race 1	R	University of Malaya, Kuala Lumpur, Malaysia	In breeding programs	[31]
Mwitu Pemba	*	Race 1	R	University of Malaya, Kuala Lumpur, Malaysia	In breeding programs	[31]
Hom Thong Mokho	AAA	Race 1	R	Department of Agriculture and Fisheries (DAF), Queensland, Australia	Australia	[101]
Mambee Thu	AA	Race 1	R	Embrapa cassava and fruit growing, Brazil	In breeding programs	[78]
PV03-79	AAAB	Race 1	R	Embrapa cassava and fruit growing, Brazil	In breeding programs	[78]
Terra Maranhão	AAB	Race 1	R	Embrapa cassava and fruit growing, Brazil	Brazil	[107]
Williams	AAA	Race 1	R	DAFF, Australia [101], Federal University of Santa Catarina, Brazil [111]	Cavendish for export	[101,111]
Williams	AAA	STR4	SS	University of Queensland, Australia	Cavendish for export	[72]
SH-3217	AA	STR4	R	University of Queensland, Australia	In breeding programs	[72]
Ma250	AA	STR4	R	University of Queensland, Australia	In breeding programs	[72]
Pisang Bangkahulu	AA	STR4	R	University of Queensland, Australia	In breeding programs	[72]
M61 Guadelope	*	STR4	SS	University of Queensland, Australia	In breeding programs	[72]

Table 4. *Cont.*

Musa Germplasm	*Musa* Genome	Race	Level of Tolerance or Resistance to Races	Institution and Location/Country Where Germplasm Was Screened	Known Use to Mitigate Fusarium Impact	References
CAM-020	AAA	STR4	S	University of Queensland, Australia	In breeding programs	[72]
SH-3142	AA	TR4	SS	(IFTR-GDAAS), Guangzhou, China	In breeding programs	[77]
FHIA-1 ("Gold Finger")	AAAB	TR4	S	GDAAS, Guangzhou, China	Australia, Brazil, Mexico, Colombia, EUA	[75]
GCTCV-119	AAA	TR4	HR	Guangdong Academy of Agricultural Sciences, Guangzhou, China	China, Taiwan, The Philippines, Mozambique.	[92]
M61 Guadeloupe	*	TR4	R	University of Queensland, Australia	In breeding programs	[72]
CAM-020	AAA	TR4	R	University of Queensland, Australia	In breeding programs	[72]
Ibwi E	AAA	TR4	R	(IFTR-GDAAS), Guangzhou, China	EAHBs	[77]
Igitsiri (Intuntu)	AAA	TR4	R	(IFTR-GDAAS), Guangzhou, China	EAHBs	[77]
Ingagara	AAA	TR4	R	(IFTR-GDAAS), Guangzhou, China	EAHBs	[77]
Inkira	AAA	TR4	R	(IFTR-GDAAS), Guangzhou, China	EAHBs	[77]
Intokatoke	AAA	TR4	R	(IFTR-GDAAS), Guangzhou, China	EAHBs	[77]
Kazirakwe	AAA	TR4	R	(IFTR-GDAAS), Guangzhou, China	EAHBs	[77]
Mbwazirume	AAA	TR4	HR	(IFTR-GDAAS), Guangzhou, China	Africa	[77]
Akpakpak	AAB	TR4	HR	(IFTR-GDAAS), Guangzhou, China	Africa	[77]
Curaré Enano	AAB	TR4	R	(IFTR-GDAAS), Guangzhou, China	Africa	[77]
Obino l'Ewai	AAB	TR4	R	(IFTR-GDAAS), Guangzhou, China	Africa	[77]
Obubit Ntanga	AAB	TR4	R	(IFTR-GDAAS), Guangzhou, China	Africa	[77]
Orishele False Horn	AAB	TR4	HR	(IFTR-GDAAS), Guangzhou, China	Africa	[77]
Pisang Ceylan	AAB	TR4	R	(IFTR-GDAAS), Guangzhou, China	Africa	[77]
Pisang Rajah	AAB	TR4	R	(IFTR-GDAAS), Guangzhou, China	Africa	[77]
Musa itinerans	*	TR4	HR	(IFTR-GDAAS), Guangzhou, China	In breeding programs	[77]
CIRAD930/DH Pahang	AA	TR4	HR	(IFTR-GDAAS), Guangzhou, China	In breeding programs	[77]
NBA 14	AA	TR4	R	(IFTR-GDAAS), Guangzhou, China	In breeding programs	[77]
Banksii	AA	TR4	R	(IFTR-GDAAS), Guangzhou, China	In breeding programs	[77]
Maia Oa	AA	TR4	R	(IFTR-GDAAS), Guangzhou, China	In breeding programs	[77]

Table 4. *Cont.*

Musa Germplasm	*Musa* Genome	Race	Level of Tolerance or Resistance to Races	Institution and Location/Country Where Germplasm Was Screened	Known Use to Mitigate Fusarium Impact	References
Zebrina	AA	TR4	SS	(IFTR-GDAAS), Guangzhou, China	In breeding programs	[77]
Pa (Rayong)	AA	TR4	R	(IFTR-GDAAS), Guangzhou, China	In breeding programs	[77]
Figue Rose	AA	TR4	R	(IFTR-GDAAS), Guangzhou, China	In breeding programs	[77]
Khai (Kamp-engpeth)	AA	TR4	R	(IFTR-GDAAS), Guangzhou, China	In breeding programs	[77]
Tani	BB	TR4	R	(IFTR-GDAAS), Guangzhou, China	In breeding programs	[77]
Pisang Klutuk Wulung	BB	TR4	R	(IFTR-GDAAS), Guangzhou, China	In breeding programs	[77]
Musa beccarii Callimusa	*	TR4	R	(IFTR-GDAAS), Guangzhou, China	In breeding programs	[77]
Musa laterita Rhodochlamys	*	TR4	R	(IFTR-GDAAS), Guangzhou, China	In breeding programs,	[77]
Musa maclayi ssp.	*	TR4	R	(IFTR-GDAAS), Guangzhou, China	In breeding programs,	[77]
Khai Thong Ruang	AAA	TR4	R	(IFTR-GDAAS), Guangzhou, China	In breeding programs	[77]
Kamaramasenge	AB	TR4	R	(IFTR-GDAAS), Guangzhou, China	In breeding programs	[77]
Rukumamb	AAB	TR4	R	(IFTR-GDAAS), Guangzhou, China	Australia, Papua New Guinea	[77]
Thap Maeo	AAB	TR4	R	(IFTR-GDAAS), Guangzhou, China	Brazil, Honduras	[77]
Foconah	AAB	TR4	R	(IFTR-GDAAS), Guangzhou, China	In breeding programs	[77]
Poingo	AAB	TR4	R	(IFTR-GDAAS), Guangzhou, China	In breeding programs	[77]
FHIA-21	AAAB	TR4	R	(IFTR-GDAAS), Guangzhou, China [77], DAFF, Australia [84]	In breeding programs	[77,84]
Blue Java	ABB	TR4	R	(IFTR-GDAAS), Guangzhou, China [77], Embrapa cassava and fruit growing, Brazil [107]	China, Africa, Brazil	[77,107]
Namwa Khom	ABB	TR4	HR	(IFTR-GDAAS), Guangzhou, China [77], DAF, Australia [101]	China, Africa, Thailand	[77,101]
FHIA-02	AAAA	TR4	R	DAFF, Australia	Africa, Brazil, Colombia, Honduras	[72,84]
SH-3362 ("Pita-16")	*	TR4	R	DAFF, Australia	In breeding programs	[72]
M. yunnanensis	*	TR4	R	South China Agricultural University	Wild germplasm	[75]
M. basjoo	*	TR4	R	South China Agricultural University	Wild germplasm	[75]
M. nagensium	*	TR4	R	South China Agricultural University	Wild germplasm	[75]

Table 4. *Cont.*

Musa Germplasm	*Musa* Genome	Race	Level of Tolerance or Resistance to Races	Institution and Location/Country Where Germplasm Was Screened	Known Use to Mitigate Fusarium Impact	References
M. ruiliensis	*	TR4	R	South China Agricultural University	Wild germplasm	[75]
M. velutina	*	TR4	R	South China Agricultural University	Wild germplasm	[75]
Nantianqing	AAA	TR4	MR	Dongguan Banana Vegetable Institute, China	China	[51]
Dongjiao 1	AAA	TR4	MR	Dongguan Banana Vegetable Institute, China	China	[51]
Kangku 1	AAA	TR4	R	Dongguan Banana Vegetable Institute, China	China	[51]
G6-2	AAA	TR4	R	Dongguan Banana Vegetable Institute, China	China	[51]
Yueke 1	AAA	TR4	MR	Dongguan Banana Vegetable Institute, China	China	[51]
Nongke 1	AAA	TR4	MR	Dongguan Banana Vegetable Institute, China	China	[51]
Kangku 5	AAA	TR4	HR	Dongguan Banana Vegetable Institute, China	China	[51]
Nantianhuang	AAA	TR4	MR	Dongguan Banana Vegetable Institute, China	China	[51]
BXM51	AAA	TR4	MR	Dongguan Banana Vegetable Institute, China	China	[51]
Yueyoukang 1	AAA	TR4	R	South China Agricultural University	China	[113]
Pisang Gajih Merah	AAA	TR4	SS	University of Queensland, Australia	Australia	[72]
GCTCV-218 Formosana	AAA	TR4	R	University of Queensland, Australia and Northern Mozambique	China, Taiwan, Philippines and Mozambique.	[5,72]
FHIA-01 ("Goldfinger")	AAAB	Race 1/STR4	R	DAFF, Australia [84], FHIA, Honduras [93]	Africa, Australia, Honduras	[84,93]
Tuu Gia	AA	Race 1/TR4	HR	(IFTR-GDAAS), Guangzhou, China	In breeding programs	[77]
Pisang Lilin	AA	Race 1/TR4	R	(IFTR-GDAAS), Guangzhou, China	In breeding programs	[77]
Borneo	AA	Race 1/TR4	R	National Agricultural Research Laboratories (NARL) [31] (IFTR-GDAAS), Guangzhou, China [80] and Wageningen University and Research, Wageningen, Netherlands [102]	In breeding programs	[31,77,102]
Pisang Berlin	AA	Race 1/TR4	R	(IFTR-GDAAS), Guangzhou, China [77], Embrapa cassava and fruit growing, Brazil [78]	In breeding programs	[77,78]
Zebrina GF	*	Race 1/TR4	R	University of Malaya, Kuala Lumpur, Malaysia [31], IFTR-GDAAS, Guangzhou, China [77]	In breeding programs	[31,77]

Table 4. *Cont.*

Musa Germplasm	*Musa* Genome	Race	Level of Tolerance or Resistance to Races	Institution and Location/Country Where Germplasm Was Screened	Known Use to Mitigate Fusarium Impact	References
Pahang	AA	Race 1/STR4/TR4	HR	University of Queensland, Australia [72], Yunnan Agricultural University, Kunming, China [74,114] and IFTR-GDAAS, Guangzhou, China [77]	In breeding programs	[72,74,77, 114]
Calcutta-4	AA	Race 1/STR4/TR4	HR	University of Queensland, Australia [66] and (IFTR-GDAAS), Guangzhou, China [72]	In breeding programs	[72,77]
Ma851	AA	STR4/TR4	R	University of Queensland, Australia	In breeding programs	[72]
Ma852	AA	STR4/TR4	R	University of Queensland, Australia	In breeding programs	[72]
Calcutta4-IV9	AA	STR4/TR4	R	University of Queensland, Australia [66] and IFTR-GDAAS, Guangzhou, China [72]	In breeding programs	[72,77]
SH-3362	AA	STR4/TR4	R	University of Queensland, Australia	In breeding programs	[72]
SH-3142	AA	STR4/TR4	R	University of Queensland, Australia	In breeding programs	[72]
Madang Guadeloupe	AA	STR4/TR4	R	University of Queensland, Australia	In breeding programs of	[72]
FHIA-1 ("Gold Finger")	AAAB	STR4/TR4	R	University of Queensland, Australia	Australia, Brazil, Mexico, Colombia, EUA	[72]
FHIA-25	AAB	STR4/TR4	R	University of Queensland, Australia [72], (IFTR-GDAAS), Guangzhou, China [77], Wageningen University and Research, Wageningen, Netherlands [102]	Africa, Latin America and Australia (Honduras, Colombia, Brazil, Jamaica, Mozambique)	[72,77,102]
GCTCV-119	AAA	STR4/TR4	R	University of Queensland, Australia and Northern Mozambique	China, Taiwan, The Philippines, Mozambique	[5,72]
Ma850	AA	ST4/TR4	R	University of Queensland, Australia	In breeding programs	[72]
Pisang Jari Buaya	AA	STR4/TR4	R	University of Queensland, Australia [72] and (IFTR-GDAAS), Guangzhou, China [77]	In breeding programs	[72,77]
FHIA-18	AAAB	STR4/TR4	R	University of Queensland, Australia [72], IFTM Brazil [81], DAFF, Australia [84], Federal University of Santa Catarina, Brazil [111]	Africa, Latin America and Australia (Honduras, Colombia, Jamaica, Mozambique)	[72,81,84, 111]

R, SS, MS, S, and HS abbreviate resistant, slightly susceptible, moderately susceptible, susceptible, and highly susceptible. EAHBs = East African Highland Bananas; IFTR-GDAAS = Institute of Fruit Tree Research, Guangdong Academy of Agricultural Sciences; EMBRAPA = Brazilian agricultural research corporation; DAFF = Department of Agriculture, Fisheries and Forestry.

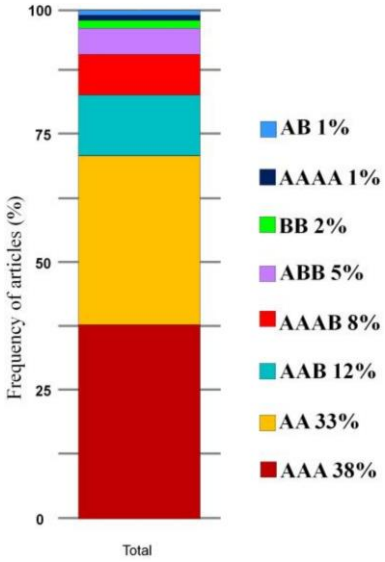

Figure 8. Frequency of genomes associated with sources of resistance to *Fusarium* wilt in studies on banana breeding carried out in the last ten years.

The resistance sources reported that are exclusively related to FOC TR4 included the diploid cultivars, Pahang, Calcutta-4, Zebrina, Pisang Lilin, Malaccensis, Jari Buaya, and Tuu Gia, all with a higher frequency, according to the word cloud (Figure 9A). Besides these, other cultivars have also been reported as resistant to FOC TR4 in field tests, such as the hybrids FHIA-01, FHIA-02, SH-3748, SH-3362, FHIA-25, SH-3142, and SH-3362 (Figure 9A). According to genome frequency data related to resistance sources, most genotypes reported as resistant to FOC TR4 are AA diploid genomes (45%), AAA triploid genomes (21%), and AAB triploid genomes (18%) (Figure 9B).

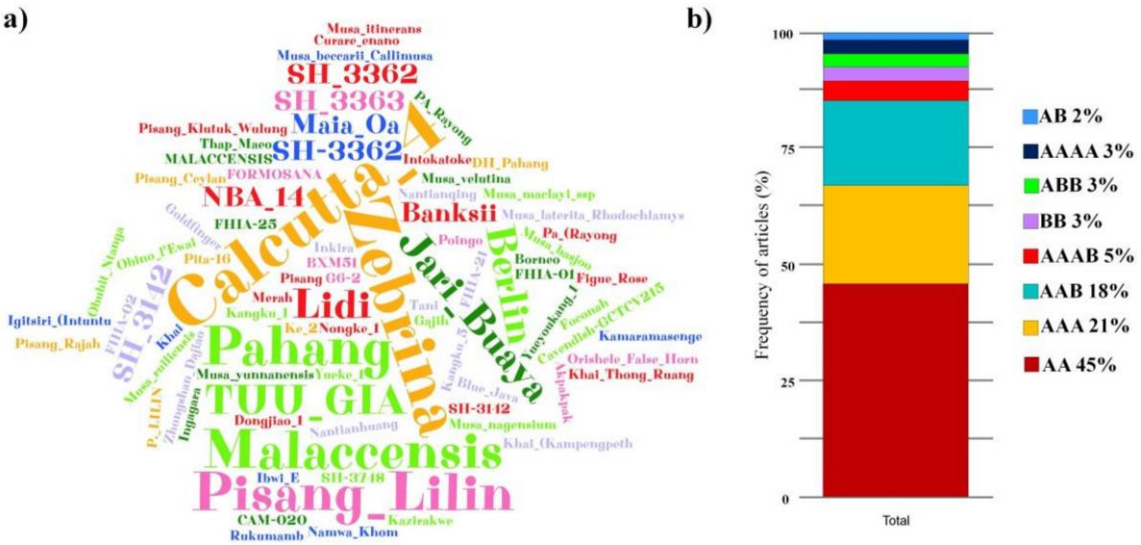

Figure 9. Sources of resistance to *Fusarium oxysporum* f. sp. *cubense* tropical race 4 (TR4) in studies on the improvement of banana plants to *Fusarium* wilt in the last ten years. (**a**) Word cloud of the frequency of cited sources of resistance. (**b**) Frequency of genomes related to the sources of resistance cited.

3.5. Gene Expression Analysis

Figure 10A shows the gene categories present in studies on gene analysis and expression. The highest frequency of articles found genes associated with pathogenesis and defense (57%) (Figure 10A). Other genes, studied at a lower frequency, are related to RNAs (12%), hormone biosynthesis (10%), kinases (9%), transcription factors (6%), genes related to autophagy (4%), and starch biosynthesis (2%). A summary of the main genes related to each category can be found in Table S1.

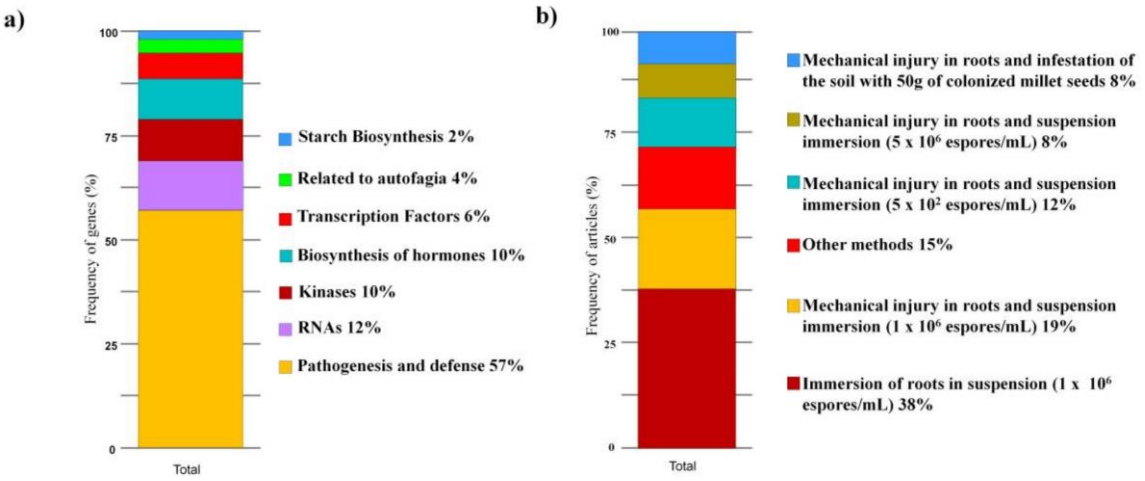

Figure 10. Gene expression studies of banana plants infected with *Fusarium oxysporum* f. sp. *cubense* in articles carried out in the last ten years. Categories of genes associated with the frequency of articles (**a**) and frequency of methods used for inoculation of plants to check gene expression (**b**).

Methods for host plant inoculation to analyze gene expression after FOC infection are not standardized among the analyzed articles (n = 27), with several methods adopted (Figure 10B). The highest frequency of articles related to the inoculation method with conidia suspension at a concentration of 1 x 10^6 spores mL^{-1} (38%), followed by the inoculation method by mechanical damage to the roots and then immersion in suspension at a concentration of 1 x 10^6 mL^{-1} spores (19%). Other methods that were present in a single article represented 15% cumulatively (Figure 10B). The method of mechanical root damage and immersion in suspension at a concentration of 5 x 10^2 spores mL^{-1} represented 12% of the articles and the methods of mechanical root damage and immersion in suspension at a concentration of 5 x 10^6 spores mL^{-1} and mechanical root damage and soil infestation with 50 g of colonized millet seeds represented 8% of the articles (Figure 10B). Therefore, we observed that the main differences were related to whether the roots were wounded and the spore concentration adopted in each case regarding the inoculation method.

Table 5 is from the study by Wang et al. [115], which was modified, to show all the selected articles that evaluated the banana transcriptome infected by FOC TR4 and FOC R1. These studies observed the changes in expression of defense-related genes related to different enriched pathways, from gene annotation pathways, namely Gene Ontology (GO) annotation and the Kyoto encyclopedia of genes and genomes-based pathway analysis (KEGG-PATH). According to most transcriptome studies, the pathways activated after FOC infection were related to phenylpropanoid biosynthesis, sugar biosynthesis, cell wall modifications, flavonoid biosynthesis, and plant hormone signal transduction (Table 5). The main genes related to the above-mentioned pathways are listed in Table S1.

Table 5. Transcriptomic studies involving banana plants infected with *Fusarium oxysporum* f. sp. *cubense* in articles about the improvement of banana to *Fusarium* wilt, carried out in the last ten years *.

Article	Banana Variety	Plant Growth Stage	Race	Sampling (after Infection)	Pathways Enriched for Differentially Expressed Genes
Wang et al. [115]	Banana "Brazil" (susceptible) and "Formosana" (tolerant)	4.5 months	FOC TR4	48 h	Flavonoid biosynthesis, flavone and flavonol biosynthesis, alpha-linolenic acid metabolism, starch and sucrose metabolism and phenylpropanoid biosynthesis.
Wang et al. [116]	Banana "Brazil"	60 d	FOCTR4	0, 2, 4, 6 days	Phenylalanine metabolism, phenylpropanoid biosynthesis, drug metabolism—cytochrome P450, alpha-linolenic acid metabolism, amino sugar and nucleotide sugar metabolism.
Li et al. [37]	Banana "Brazil"	50 d	FOC 1 and FOC TR4	3, 27, 51 h	PR proteins, phytoalexins and phenylpropanoid synthesis, cell wall modifications, biosynthesis via ethylene signaling.
Li et al. [117]	Banana "Brazil" and "Nongke Nº 1" (resistant)	Plants with four or five leaves	FOC TR4	48, 96 h	Perception of PAMP by PRRs, hormone biosynthesis and signaling, transcription factors, cell wall modification, flavonoid biosynthesis, programmed cell death, PR proteins
Bai et al. [113]	Banana "Brazil" and "Yueyoukang 1" resistant	8 weeks (plants with five leaves)	FOC TR4	0, 5, 1, 3, 5, 10 days	PR proteins, transcription factors, cell wall modification, phenylpropanoid biosynthesis, plant hormone signal transduction.
Zhang et al. [114]	*Musa acuminata* Pahang and Brazilian		FOC STR4	at 14 days	PR proteins, transcription factors, cell wall modification, phenylpropanoid biosynthesis, plant hormone signal transduction.
Sun et al. [32]	*Musa acuminata* " Guijiao 9" and Williams	6 months	FOC TR4	At 6 days	Membrane-bound intracellular organelle, cell wall and cytoplasm, ions, transcription factor and oxidoreductase activity, plant–pathogen interaction, plant hormone signal transduction, phenylpropanoid biosynthesis and flavonoid biosynthesis.
Fei et al. [118]	Cavendish banana	3 months	FOC 1 and FOC TR4	At 28 days	Cell components, molecular function and biological process.
Cheng et al. [103]	*Musa acuminata* cv. Tianbaojiao	11 weeks	FOC TR4	5, 10, 25 h	Auxin-activated signaling pathway, cellular response, auxin stimulation, phenylpropanoid catabolic process, lignin catabolic process, lignin metabolic process, via peroxisomes.
Song et al. [119]	Brazilian banana and señorita banana	In the five-leaf stage	FOC 1 and FOC TR4	In the five-leaf stage	Cellular process, metabolic process and binding of organelles and nucleic acids or proteins, regulation of biological processes and transcription factors.
Li et al. [120]	Cavendish banana and Brazilian (BX)	90 days	FOC TR4	27 h, 51 h	Secondary metabolite biosynthesis, plant–pathogen interaction, phenylpropanoid biosynthesis and phenylalanine metabolism, fatty acid metabolism, glycerolipid and glycerophospholipid metabolism
Niu et al. [121]	Yueyoukang1 and Baxijiao	2 weeks	FOC TR4	24 h	Cell wall biosynthesis and degradation, cell polysaccharide metabolic process, chitinase activity, pectinesterase activity and xyloglucan activity, fructose and mannose metabolism, sphingolipid metabolism, butanoate metabolism, porphyrin and chlorophyll metabolism, carotenoid and ribosome biosynthesis.

* modified table by Wang et al. [115].

3.6. Studies on the Achievement and Evaluation of Hybrids and on Genetic Inheritance of Musa spp.

The studies related to crossbreeding to obtain resistant hybrids or those focused on evaluating the genetic inheritance in *Musa* spp., as well as the parental lineages used and their genealogies, are listed in Table 6. Ssali et al. [93] produced hybrids from crossbreeding the resistant diploid TMB2X8075 (originated from the cross between SH3362 (AA) and Calcutta 4 (AA)) and Sukali Ndizi (AAB), which is also resistant to FOC R1 and 4, to evaluate the inheritance of the resistance of *Musa* spp. to FOC R1 in three F2 populations. Concerning the progeny, the authors found that 115 were susceptible, and 48 were resistant. Similarly, in the study by Arinaitwe et al. [31], crossbreeding between Monyet (*Musa acuminata* ssp. *Zebrina*) and Kokopo (*Musa acuminata* ssp. *Banksii*) were performed to identify suitable banana germplasm to generate a segregating F1 population and to understand the mode of inheritance of resistance to FOC R1 (Table 6).

Table 6. Evaluation of hybrids and genetic inheritance studies in articles about the improvement of banana plants to fusarium wilt carried out in the last ten years.

Hybrids	Parentage
	Article
	Ssali et al. [93]
F2 progenies	Diploid TMB2X8075 ("SH3362" (AA) × "Calcutta 4" (AA) × Sukali Ndizi (AAB)
	Arinaitwe et al. [31]
F1 progenies	Monyet (*Musa acuminata* ssp. *Zebrina*) × Kokopo (*Musa acuminata* ssp. *Banksii*)
	Ahmad et al. [108]
	Musa acuminata ssp. *Malaccensis* (selfed)
	Gonçalves et al. [33]
CNPMF0038	((M53 × Madu)) × ((Malaccensis × Tjau Lagada))
CNPMF0496	((M61 × Pisang Lilin)) × ((Terrinha × Calcutta 4))
CNPMF0513	((M61 × Pisang Lilin)) × ((M53 × Kumburgh))
CNPMF0519	Self-fertilization (wild diploid Tambi)
CNPMF0534	((Calcutta 4 × Madang)) × ((Borneo × Guyod))
CNPMF0536	((Calcutta 4 × Madang)) × ((Borneo × Guyod))
CNPMF0542	((SH3263)) × ((Malaccensis × Sinwobogi))
CNPMF0557	((M61 × Pisang Lilin)) × ((Malaccensis × Tjau Lagada))
CNPMF0565	((Calcutta 4 × Pahang) × (Borneo × Madang)) × Khae
CNPMF0572	((Khai × (Calcutta 4 × Madang)) × ((Calcutta 4 × Madang))
CNPMF0612	((M53 × Madu) × Madu)) × SH3263
CNPMF0731	((Malaccensis × Madang)) × ((Tuugia × Calcutta 4))
CNPMF0767	((Malaccensis × Madang)) × ((Khai × (Calcutta 4 × Madang))
CNPMF0811	((Khai × (Calcutta 4 × Madang)) × ((Calcutta 4 × Pahang) × (Borneo × Madang))
CNPMF0978	((Calcutta 4 × Madang)) × ((Terrinha × Calcutta 4))
CNPMF0993	((Borneo × Guyod) × (Tuugia × Calcutta 4)) × ((Khai × (Calcutta 4 × Madang))
CNPMF0998	((Borneo × Guyod)) × ((Borneo × Guyod) × SH3263)
CNPMF1102	((Jari Buaya × (Calcutta 4 × Madang)) × ((Borneo × Guyod) × (Tuugia × Calcutta 4))
CNPMF1105	((Borneo × Guyod) × (Calcutta 4 × Heva)) × ((Calcutta 4 × Madang))
CNPMF1171	((Malaccensis × Madang)) × ((M53 × (Tuugia × Calcutta 4))
CNPMF1272	((Borneo × Guyod) × (Calcutta 4 × Heva)) × ((Tuugia × Calcutta 4))
CNPMF1286	((Calcutta 4 × Madang)) × ((Terrinha × Calcutta 4))

Table 6. *Cont.*

Hybrids	Parentage
CNPMF1323	((Malaccensis × Sinwobogi)) × ((Calcutta 4 × Heva))
CNPMF0241	((Pacovan × improved diploid))
CNPMF0282	((Pacovan × improved diploid))
CNPMF0351	((Prata Anã × improved diploid by FHIA))
CNPMF0897	((Prata Anã)) × ((Malaccensis × Sinwobogi) × (Zebrina × Heva))
CNPMF0898	((Prata Anã)) × ((Malaccensis × Sinwobogi) × (Calcutta 4 × Galeo))
CNPMF0906	((Prata Santa Maria × improved diploid))
CNPMF0908	((Silk × improved diploid))
BRS Princesa	((Yangambi × M53))

The study by Ahmad et al. [108] is the first report of the genetic basis of resistance to FOC R1 in bananas using heterozygous wild banana *Musa acuminata* ssp. *malaccensis* (AA) to generate a mapping population and investigate the inheritance of resistance to FOC R1 and FOC TR4 through genetic mapping. This study demonstrated that resistance to FOC R1 is inherited as a single gene and that *M. acuminata* ssp. *malaccensis* is fertile and can be a potential parent to create resistance to *Fusarium* wilt.

Among the hybrids studied by Gonçalvez et al. [33], the improved diploids (CNPMF0038, CNPMF0513, CNPMF0767, and CNPMF1171) and the tetraploid hybrid BRS Princesa were considered moderately resistant (Table 6). All other hybrids evaluated in their study were considered resistant to *Fusarium* wilt caused by FOC R1. Gonçalvez et al. [33] mostly used improved male diploid parents, resulting from crossbreeding with diploids resistant to FOC R1 and FOC TR4, such as Calcutta 4 and M53.

3.7. Transgenesis

In the articles reporting the use of transgenesis (n = 14), the tool for genetic transformation was mediated by *Agrobacterium tumefaciens*, using embryogenic cell suspension culture. One exception is a method proposed by Subramaniam et al. [122], who, in addition to agroinoculation, developed a biolistics method. In this study, we used a table developed by Poon and Teo [123] as a model to show information about the works of this systematic review related to transgenesis (Table 7). In Table 7, we observed that most studies used the *Rasthali* cultivar (AAB) for genetic engineering.

Table 7. Genes used transgenics in studies on genetic improvement of banana to *Fusarium* wilt in the last ten years.

Gene	Sources	Function	Banana Cultivar	References
Ferredoxin (Atfd3) and ferredoxin-like protein (pflp)	*Capsicum annuum*	Antimicrobial peptide	cv. Pei Chiao (AAA)	[69]
Petunia floral defenses (PhDef1 and PhDef2)	*Petunia hybrida*	Antimicrobial peptide	cv. Rasthali (AAB)	[124]
Onion—Ace-AMP1	*Allium cepa*	Antimicrobial peptide	cv. Rasthali (AAB)	[88]
Endochitinase (chit42)	*Trichodermaharzianum*	Antifungal activity	cv. Furenzhi (AA)	[125]
Defensin (Sm-AMP-D1)	*Stellaria media*	Antimicrobial peptide	cv. Rasthali (AAB)	[126]
Small interfering RNAs(siRNAs)/(ihpRNA)	–	Silencing of vital fungal genes	cv. Rasthali (AAB)	[83]
(MusaDAD1, MusaBAG1 eMusaBI1)	*Musa acuminata*	Cell death is highly induced by FOC infection	cv. Rasthali (AAB)	[96]
Cell death (Bcl-Xl, Ced-9 e Bcl-23)	*Caenorhabditiselegans*	Antiapoptosis	cv. Grand Naine	[89]
Cell death (Ced9)	*Caenorhabditiselegans*	Antiapoptosis	cv. Sukali Ndizi (*Musa* ssp. AAB)	[100]
Pathogenesis-reported (MaPR-10)	*Musa acuminata* ssp. *malaccensis*	Pathogenesis (PR)	*M. acuminata* cv. Berangan	[73]
(RGA2) and (Ced9)	*Musa acuminata* ssp. *malaccensis* /*Caenorhabditis elegans*	Resistance analog/antiapoptosis	cv. Grand Naine	[127]
Chitinases and 1.3-glucanase	*Oryza sativa*	Disease tolerance	cv. Rasthali (AAB)	[122]
Synthesis of ergosterol (ERG6)	–	Silencing of vital fungal genes	Cavendish	[128]
Small interfering RNAs–ihpRNA	–	Silencing of vital fungal genes	cv. Rasthali (AAB)	[129]

Among the genes used for transgenesis, there was a considerable frequency of studies using transgenes as the antiapoptosis gene (Ced9) from the nematode *Caenorhabditis elegans* (Table 7). Two genes derived from *Musa acuminata* ssp. *malaccensis*, one related to pathogenesis (MaPR-10) and the other a resistance analog (RGA2), were also successfully used in this case as cisgenes. Other cell death genes, MusaDAD1, MusaBAG1, and MusaBI1, from *Musa acuminata* were also efficient, particularly MusaBAG1. In addition to these, the RNA interference technology enables the silencing of vital genes of FOC when employing small interfering RNA (siRNA) and intron-containing hairpin RNA (ihpRNA) (Table 7).

Four antimicrobial peptides from the plant species *Capsicum annuum*, *Petunia hybrida, Allium cepa*, and *Stellaria media* and an antifungal activity gene from *Trichoderma harzianum* were also successfully used to obtain resistant transgenic banana plants (Table 7).

3.8. Induction of Resistance

Among the inducers, the biocontrol agents *Bacillus subtilis*, *Trichoderma* spp., and *Penicillium citrinum* were the most reported for exploring induction of systemic resistance (Table 8). Chemical induction agents were also reported, such as the plant hormones abscisic acid (ABA), methyl jasmonate (MeJA), and salicylic acid (SA), in addition to benzothiadiazole (BTH). Other studies explored induced systemic resistance with the FOC pathogen in different ways (Table 8).

Table 8. Resistance-inducing agents in banana plants reported in studies on improvement to *Fusarium* wilt in the last ten years.

Inductor	Application	References
Bacillus subtilis	Inoculation of plants with suspension in a greenhouse	[130]
Trichoderma asperellum	Inoculation of plants with suspension in a greenhouse	[131]
Abscisic acid (ABA), ethephon, methyl jasmonate (MeJA) and salicylic acid (SA)	Root treatment with inductor solutions	[132]
Penicillium citrinum	Inoculation of plants with suspension in a greenhouse	[88]
Bacillus subtilis	Treatment with in vitro fermented culture filtrate and inoculation of plants with suspension in a greenhouse	[90]
Benzothiadiazole (BTH)	Spraying leaves and roots	[133]
Interaction with dead FOC pathogen	Inoculation of plants with suspension in a greenhouse	[134]
Methyl jasmonate (MeJA)	Exogenous solution treatment in soil and leaves	[76]
A strain of FOC 1 incompatible with inducing resistance against the tropical race 4 TR4	Systemic resistance acquired by in vitro inoculation	[99]
Isolates of *Trichoderma* spp. (*T. koningii*, *T. viride*, *T. harzianum*)	Biomass, liquid culture and culture filtrate	[135]

4. Discussion

4.1. Screening of the Studies

The studies analyzed were restricted to genetic improvement and in line with the questions proposed in the protocol formulated for this review. For this reason, articles on FOC genetic diversity, specific management strategies, and first reports of the disease were not considered in the analyses (Figure S1). Literature reviews were also excluded from the research to avoid bias since they could overestimate data, as many articles would be repeated.

Thus, the exclusion criteria used during the extraction stage of the article screening process revealed that many specific studies on FOC genetic diversity (n = 72) were generated in the last ten years, as well as many articles that escaped the proposed subject of this review (n = 47) and several literature reviews (n = 26) (Figure S1). Although these papers were excluded by the criteria, they revealed important aspects of the direction of research on *Fusarium* wilt in the last 10 years, considering the search string used.

The considerable amount of data on the genetic diversity of FOC generated in recent years was primarily in response to the need to understand the population structure of the pathogen in different locations and the evolutionary mechanisms of the fungus that culminate in the emergence of new races [48,136,137]. In fact, the potential for public investment in research that addresses the dissemination of the FOC TR4 can generate high returns and substantially delay the spread of this disease [138].

4.2. Locations of Knowledge Generation

A substantial amount of data on banana genetic improvement for resistance to *Fusarium* wilt was evaluated in this systematic review, the majority (50%) from China. This is a consequence of the number of projects to control *Fusarium* wilt in China, involving institutions, such as the Guangdong Academy of Agricultural Sciences (GDAAS), Chinese

Academy of Tropical Agricultural Sciences (CATAS), Fujian Agriculture and Forestry University, Hainan Academy of Agricultural Sciences, and Guangzhou Institute of Agricultural Sciences [139]. In addition, China is among the countries in Southwest Asia where banana plants were domesticated [140], in which bananas are one of the fruits with the oldest consumption record, and the country that ranks second among the top 10 banana producersworldwide [2].

Besides China, India (16.7%), Australia (10.4%), and Brazil (7.3%) have also contributed to the improvement in research on *Musa* spp. India is the largest banana producer globally, whereas Brazil ranks fourth among banana producers [2]. Furthermore, these countries host important research institutions, which work on banana improvement for the development of resistant hybrids from germplasm collections, such as the Brazilian collections of the National Research Center for Banana (NRCB) and EMBRAPA. Australia was the first country to report and describe *Fusarium* wilt and one of the first countries facing major problems with FOC TR4, which led to the end of the Cavendish banana industry in the Northern Territory in 2015 [35,141,142].

Overall, in recent years, a major stimulus for the growth in studies on *Fusarium* wilt has been the emergence of FOC TR4 as the most devastating threat to bananas worldwide. A clear demonstration of this is the estimate that 17% of the current banana cultivation area, with an annual production of 36 million tons worth approximately US $10 billion at current prices, could be lost over the next 20 years because of *Fusarium* wilt, which would necessitate investment in research aimed at improving the crop in this scenario [138,143].

4.3. Gene Expression Analysis

The genome of the diploid species *Musa acuminata* ssp. *malaccensis*, which is the ancestor of most banana triploid cultivars, has been sequenced [144]. In the present study, 50% of the articles used the banana genome as a tool for analysis. For this reason, the highest frequency of articles was related to in silico studies (45%), as part of gene expression analyses (30%) that mostly performed RT–qPCR and PCR analyses (34%), as well as bioinformatic analyses (23%).

Identifying genes related to host defense is one of the first steps to understand the underlying mechanism of resistance to diseases in plants [145]. Concerning FOC, knowledge of global gene expression patterns, influenced by infection of different races, has enhanced our understanding of host responses to infection. Moreover, the availability of banana transcriptomes was highly useful to improve the annotation of the banana genome and for biological research [37]. Based on the knowledge of the global patterns of gene expression influenced by infection of FOC R1 and FOC TR4, Li et al. [37] found a large number of simple nucleotide polymorphisms (SNPs) and short insertions and deletions (in- dels), which previously had not been annotated in the Musa genome. Other transcriptomic studies observed the regulated expression of defense genes, cell wall-modifying genes, and a phytoalexin, flavonoids, lignin biosynthesis genes and jasmonic acid and other plant hormones and transcription factors [37,113–117,146,147].

The lack of standardization pertaining to the inoculation methods for evaluating gene expression should be questioned to develop a universal method for plant host inoculation so that the results could be equated and compared. It should be noted that a striking difference between these methods is the generation of wounds in the roots before exposing them to a suspension with the fungus, which in fact does not reflect a similar situation in the field, except when there are interactions with other microorganisms in the soil, such as nematodes [107]. Therefore, we consider that the inoculation methods adopted in most studies should be reconsidered because they are primarily related to the mechanical opening of wounds made with sterile needles or crushing the roots. In addition, differences related to the concentration of spores in the infection process generated marked changes in the plant response to infection and in gene expression, especially when a high inoculation pressure is considered, such as at the concentration of 5×10^6 spores mL^{-1} associated with wound generation. Consistent with this information, we know that *F. oxysporum* is

considered a hemibiotrophic pathogen because it begins its infection cycle as a biotroph and later changes to a necrotroph and as the gene expression changes that occur in the roots. Host responses may be prioritized to the perception of the pathogen, preventing the penetration of the root tissue during the biotrophic stage, which would not be possible to notice in previously injured tissue [46,148]. Therefore, we suggest that a standardized method should be adopted regarding the inoculation method of host plants to verify gene expression, aimed mainly to simulate situations in the system of banana cultivation, considering the mechanisms of dissemination of FOC that usually occur by movement and deposition of contaminated soil [30].

4.4. Studies on Resistance Sources

Although available edible banana cultivars originate from *M. acuminata* (genome A) and *Musa balbisiana* (genome B), genome B has been associated with the best vigor and tolerance to biotic and abiotic stresses and is, therefore, a target for *Musa* spp. improvement programs [149,150]. The AAA triploid genomes frequently occurred when considering all resistance sources related to both FOC R1 and FOC TR4 (Figure 8). In the studies with FOC TR4, the highest frequency of resistant genotypes were related to AA diploid genomes (Figure 9). This demonstrates that FOC TR4-related resistance sources are still mostly composed of wild diploids that have not yet been exploited for triploid cultivars, as in FOC R1, which already has a large panel of resistant cultivars available.

Thus, we have shown that some wild relatives of edible bananas, such as *M. itinerans*, Pahang, Calcutta 4, DH Pahang and Tuu Gia (Figure 9A), are valuable resources of resistance genes to FOC TR4 [77]. These data continue to be reaffirmed based on recent RNA-seq analyses that revealed aspects of the key responses of the relative resistance of wild banana to FOC TR4, where it could be seen that many differentially expressed genes were found in the resistant wild relative *Musa acuminata* ssp. *Burmanicoides* compared to the susceptible cultivar "Brazilian (AAA)" [147].

An example of banana resistant to FOC R1 and TR4 are the triploid banana referred to as East African Highland bananas (EAHB), which a recent study has revealed that Mchare and Matooke hybrids resistant to FOC R1 can replace susceptible cultivars in areas of production severely affected by the fungus and are important resources for the generation of resistant banana [112].

The genetic basis of resistance to FOC R1 in banana has been studied in three articles, of which Arinaitwe et al. [31] and Ahmad et al. [108] suggested that resistance to *Fusarium* wilt in *Musa* spp. is conditioned by a single dominant locus of resistance, contradicting Ssali et al. [93], who concluded that the gene was recessively inherited. However, the conclusions by Ahmad et al. [108] were based on genetic analyses that included mapping studies and not just segregation data based on phenotypic characters.

4.5. Main Methods and Tools Adopted

One of the most-used tools (12%), together with the symptomatological assessments to understand Musa x FOC interaction processes, is the genetic transformation of different FOC isolates with the GFP gene. This method allows researchers to follow the movements of the fungus within the tissues and compare the colonization path used by different FOC races [37,46,72,137,151]. A FOC STR4 strain, transformed with the GFP gene, was used to monitor the movement of the pathogen in two susceptible cultivars, Cavendish Williams (Musa AAA) and Lady Finger (Musa AAB) [46]. Those authors detected the presence of FOC on the roots, rhizome, and outer leaf sheaths of the pseudostem before the appearance of external symptoms. Another study using this method verified that, in some cases, the banana rhizome plays an important role as a barrier to the pathogen, preventing its migration to the rest of the plant [103].

The studies carried out in greenhouses corresponded to 13% of the articles, those in greenhouses and in the field to 5%, and those only in the field to 3%. The articles focused only on assessing *Fusarium* wilt symptoms are few since, overall, this type of

evaluation is complementary to several other analyses as a safe phenotypic confirmation of resistance. Most of the evaluation methods cited are related to the quantification of the severity of *Fusarium* wilt by visual categorization of the cross-sections based on the level of discoloration of the vascular tissue of the rhizome and the pseudostem of the root tissue, according to the scales mentioned in Table 2 [65–67,71,77,94].

The greatest difference found between the rating scales adopted for analysis and confirmation of banana resistance to FOC is related to the scoring grades for the disease's severity. A universal scoring scale should be adopted, especially for comparison purposes between studies from different banana research centers, to avoid discrepant results, for example, when evaluating hybrids resulting from crossbreeding, plants obtained by transgenesis, resistance-induction, or other methods.

Although there are few studies with somaclonal variation (1%), this is a tool that presents promising results. The Cavendish somaclone GCTCV-218 for commercial cultivation under the name of Formosona, generated in 2004 by the Taiwan Banana Research Institute, is known to be tolerant to FOC TR4 and two other somaclonal variants of Cavendish called GCTCV-53 and GCTCV-119 [152]. In a recent study, tests with these Cavendish banana somaclones in northern Mozambique revealed that GCTCV-119 was more resistant to FOC TR4, but GCTCV-218 produced better bunches [5]. Another recent study obtained, through different combinations of plant regulators in a culture medium, two somaclones of the cultivar Prata-Anã, namely T2-1 and T2-2, which presented resistance to FOC race 1 in a greenhouse, characterizing an important result for the banana cultivation in Brazil since the pathogen FOC R1is present in most banana plantations and this cultivar is preferred by Brazilian consumers [153].

In the articles analyzed, transgenesis was the most-used method (14%), followed by resistance induction (10%), hybridization (4%), in vitro mutagenesis (4%), and somaclonal variation, clone selection, and somatic embryogenesis (1%). Although the transgenic method has a limitation related to the production of embryogenic cell suspensions, a time-consuming process, some protocols have facilitated their implementation [127,154]. Among the cited protocols, the most-used have been proposed by Ganapathi et al. [154], which included the establishment of embryogenic cell cultures from thin sections of the shoot tip of cultivated Rasthali (AAB) banana cultivar in vitro and by Khanna et al. [155], which proposed transformation mediated by *Agrobacterium tumefaciens* assisted by centrifugation (CAAT) from male flower embryogenic cells suspensions of the Cavendish (AAA) and *Lady Finger* (AAB) cultivars. A protocol established by Yip et al. [69] proposes the substitution of embryogenic cell suspensions for meristematic tissue, where they use multiple shot clump (MSC) of Pei Chiao (AAA) and Gros Michel (AAA) bananas induced from shoots in the rhizome in MS medium; this could be another feasible option for banana cultivars where suspension cultures are difficult to establish. Another protocol was proposed by Subramaniam et al. [122] using the biobalistic gun method for the transformation of the 'Rastali' (AAB) banana cultivar. In addition, the availability of banana genes (cisgenes) and genes from other appropriate sources (transgenes) allowed the development and evaluation of transgenic plants (Table 6).

Conventional resistance improvement methods using hybridization between fertile diploids and crossbreeding with triploid or tetraploid cultivars are efficient. However, they have some limitations concerning the polyploid nature of the cultivars and the low female fertility, as well as the long life cycle leading to a long reproductive cycle [156,157]. Other challenges are related to the need for a large space, which results in high costs and limited knowledge about resistance genetics [31,158,159].

Transgenic methods permit the addition of a single gene or several genes to a highly desirable cultivar quickly [81,124–126]. Due to the sterility of these cultivars, the flow of transgenes and the crossing of modified genes between wild Musa species are unlikely; therefore, genetically modified (GM) bananas could be compatible with organic agriculture [159]. In addition, although no genome editing data associated with obtaining *Fusarium* wilt-resistant cultivars were identified in this study, the potential for using the

CRISPR/Cas9, a genome-editing tool for the development of disease-resistant banana varieties, also has been reported. The use of genome editing (GE) with the availability of a whole-genome sequence and its potential applications to develop disease-resistant bananas opens new areas of research [160–165]. Although there are no published data in banana breeding, another potential tool to be applied is resistance gene enrichment sequencing (RenSeq), a technology that enables the discovery and annotation of pathogen resistance gene families in plant genome sequences. The use of this high-throughput technique was well demonstrated in wheat (*Triticum estivum*) [164] and potato (*Solanum tuberosum*) [165].

These data encourage discussions on the current status of biosafety regulations and laws on the marketing of GM products that face some challenges because of the regulation of these products in several countries [162,163]. Furthermore, their outlook indicated that investments in GM banana plantations would bring few beneficiaries, given the assumption that countries with export-oriented banana production would not adopt GM varieties because of political and consumer concerns [138]. In this sense, it seems reasonable to invest more in improvements based on crossbreeding, considering that there are sources of resistance to *Fusarium* wilt caused by FOC R1 and FOC TR4, which enables the selection of resistant hybrids within the progeny generated.

Using an ex-ante quantitative risk index model, Staver et al. [138] showed that investments in different research areas assessed to address the threat and projected losses from FOC TR4 would provide positive returns and contribute to a reduction in poverty. Moreover, there would be superior returns in poverty reduction, especially in Africa, in the face of investments in the research areas related to the conventional improvement of cultivars resistant to *Fusarium* wilt, as well as in the research area related to improving exclusion and surveillance, as well as measures to eradicate or contain the disease, with 850,000 and 807,000 people lifted out of poverty in each case, respectively.

4.6. Perspectives

In this study, we found that several articles in the last 10 years have focused on a variety of analyses to improve our understanding and identification of genetic, molecular, biochemical, or structural mechanisms of banana resistance to FOC, based on a set of tools. Based on these articles, we also showed that there are sources of resistance to FOC R1 and FOC TR4 in banana germplasms and that the data generated in these studies are the basis for obtaining cultivars resistant to *Fusarium* wilt. Moreover, they can contribute significantly to the expansion of resistant cultivars, including those for export. Although there is not yet a banana cultivar resistant to FOC TR4 that can replace the cultivars of the Cavendish subgroup, from the resistance sources found in different studies, it would be possible to develop a "type" similar to the Cavendish cultivars resistant to FOC TR4 or other races.

Concerning the improvement methods, there is a growing incentive for new precise and efficient genetic technologies, and the use of the CRISPR/Cas9 genome editing tool will also contribute to obtaining banana cultivars with FOC resistance in a short span of time. Other tools, which explore acquired and induced systemic resistance, also emerged as important means to achieve resistance to the pathogen, supported by experiments on tissue culture. Meanwhile, conventional improvement seeks to overcome the challenges inherent to the plant species by offering seemingly more appropriate measures with a focus on family-based agriculture of banana production systems worldwide. Nevertheless, the debate concerning various improvement methods should not be focused on just one method since all of them contribute to improving the crop, and the existence of different scenarios of banana production should be considered for the use of each method.

Furthermore, it is important to emphasize that the results obtained in this study are linked to the keywords used in the search string. The use of different terms could lead to the inclusion and exclusion of other articles in the systematic review and, consequently, lead to other methods and conclusions.

5. Conclusions

Improvement programs of *Musa* spp. have sought to reinforce their methods through new technologies and accumulate knowledge on resistance to *Fusarium* wilt. The genome sequencing of Musa is a widely used data source for improving the identification and analysis of resistance-related genes. The production of transgenic bananas has been explored, leading to the need for social exposure regarding the acceptance of such products. Although the use of genome editing tools, such as CRISPR/Cas9, to obtain resistance to *Fusarium* wilt in banana plants has not been performed, it is a method with promising prospects. In this review, we highlighted sources of resistance to FOC (R1 and TR4) based on diploids resistant to *Fusarium* wilt, which is the starting point for genetic improvement. Therefore, we confirm that genetic improvement is the best strategy for combating *Fusarium* wilt by expanding resistant cultivars to producers. From the data collected inour systematic review, we believe that future research efforts can be based on integrating the knowledge obtained thus far to obtain results with greater applicability and direct the next steps in research to produce banana species resistant to *Fusarium* wilt. We suggest that future studies address the following questions: How can we exploit germplasm sources resistant to FOC R1 and FOC TR4 in improvement programs? Could the standardization of protocols for plant inoculation facilitate the comparison of data regarding gene expression analysis? Should a universal scoring scale contemplating the disease's external and internal symptoms be elaborated based on existing scales? Can existing molecular markers be used
in a standard-assisted selection protocol for resistance to FOC R1 and FOC TR4?

In addition, strategies based on the integration of knowledge from different *Musa* spp. improvement research centers should be adopted for cooperative efforts so that different improvement programs can cooperate on a global scale. Considering that the current banana export scenario is based exclusively on a single group, strategies should be considered to ensure the agribusiness export's sustainability, prioritizing the production of other cultivars resistant to FOC.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/jof7040249/s1, Figure S1. Frequency of articles by exclusion (E) and inclusion (I) criteria, used in the study extraction phase to composing a systematic review of the resistance of *Musa* spp. to *Fusarium* wilt; Table S1. Summarization of the main genes identified in the analysis of articles on the improvement of banana to *Fusarium* wilt carried out in the last ten years (additional to Figure 9B); Table S2. Origin and database of articles selected to compose a systematic review on the improvement of banana to *Fusarium* wilt.

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CAPÍTULO 2

Molecular, histological and histochemical responses of banana cultivars challenged with *Fusarium oxysporum* f. sp. *cubense* with different levels of virulence

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Molecular, histological and histochemical responses of banana cultivars challenged with *Fusarium oxysporum* f. sp. *cubense* with different levels of virulence

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Abstract: Fusarium wilt caused by *Fusarium oxysporum* f. sp. *cubense* (Foc) is the most limiting factor in the banana agribusiness worldwide. Therefore, studies regarding pathogen attack mechanisms and especially host defence responses in this pathosystem is of outmost importance for genetic breeding programs in the development of Foc resistant banana cultivars. In this study the analysis at the molecular, histological and histochemical levels in the *Musa* spp. x Foc interaction, was performed. Three Foc isolates representative of race 1 (R1), subtropical race 4 (ST4) and isolate 229A which is a putative ST4, were inoculated in two Prata-type cultivars (Prata-Anã and BRS Platina) and one cultivar of the Cavendish type (Grand Naine). Of seven genes related to plant–pathogen interactions, five were overexpressed in 'BRS Platina' 12 hours after inoculation (HAI) with Foc R1 and ST4 but had reduced or negative expression after inoculation with Foc 229A according to RT–qPCR analyses. While hyphae, mycelia and spores of the Foc 229A isolate grow towards the central cylinder of the Grand Naine and Prata-Anã cultivars, culminating in the occlusion of the xylem vessels, the BRS Platina cultivar responds with increased presence of cellulose, phenolic compounds and calcium oxalate crystals, reducing colonization within 30 days after inoculation (DAI). In general, these data indicate that the cultivar BRS Platina has potential for use in banana breeding programs focused on resistance to Foc tropical race 4 (TR4) and aggregating information on the virulence relationships of the Foc pathogen and the defence responses of banana plants after infection.

Keywords: Fusarium wilt, *Musa* spp., gene expression, plant resistance.

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1. Introduction

Bananas are the most commercialized fresh fruit in the world, and its productive sector serves as an essential source of employment and income for thousands of rural families in developing countries. However, an eminent threat to fruit production is the Fusarium wilt caused by the soil-borne fungus *Fusarium oxysporum* f. sp. *cubense* (EF Smith) Snyder and Hansen (Foc) [1, 2, 3]. Although it originated in Southwestern Asia, where it co-evolved with bananas, the pathogen was identified by the first time in Australia in 1876 from where it spread causing the destruction of more than 40,000 ha of the cultivar Gros Michel [4, 5, 6]. At the time,

the cultivar Gros Michel was replaced by cavendish cultivars, resistant to race 1 of the pathogen, which began to substantially make up the export trade of the fruit [7, 8]. However, around 1990, Cavendish plantations began to be heavily affected by tropical race 4 (TR4), a new highly virulent strain that devastated Southeast Asian plantations and has spread to several regions of the world [7].

Currently tropical race 4 is responsible for the destruction of banana plantations and creating unsuitable soil conditions on almost all continents and is present in Asia, Africa, Indonesia and South America [9, 10, 11, 12]. Conversely, the subtropical race 4 (ST4) was characterized by causing symptoms in Cavendish cultivars in subtropical regions when there are environmental stress factors, such as temperature extremes or water deficit; events increasingly common due to climate changes occurring in recent decades [7, 13, 8, 3].

In infested fields, there is still a lack of economically viable measures for disease management. This is because Foc is a soil-borne pathogen that produces chlamydospores, resistant spores that remain viable in the soil for many years; a saprophytic habit allows this pathogen to survive in dead organic matter in the absence of a host [9, 10, 14, 15]. The use of chemical treatments raises environmental and health concerns and although biological control is expanding, it´s efficacy has not yet been demonstrated in the field in the long term [16, 17]. Therefore, genetic improvement and selection for resistance is the best option for an effective and sustainable management. Many breeding programs of *Musa* sp. in different research centers around the world are focused on obtaining resistant cultivars from crosses.

These research centers include the Fundación Hondureña de Investigación Agrícola - FHIA (Honduras), Center Africain de Recherches sur Bananiers et Plantains - CARBAP (Cameroon), International Institute of Tropical Agriculture - IITA (Nigeria and Uganda), Empresa Brasileira de Pesquisa Agropecuária - EMBRAPA (Brazil), National Banana Research Center - NCRB (India), National Research Organization - NARO (Uganda) and the Center de Coopération Internationale en Recherche Agronomique pour le Dé enveloppement - CIRAD (Guadeloupe). Other programs, such as the Taiwan Banana Research Institute (TBRI) and the Chinese Academy of Agricultural Sciences (GDAAS) in China have obtained resistant cultivars through somaclonal variation and biotechnology [12].

Thus, information obtained after sequencing the genome of wild diploid banana 'Pahang' is in use, aiming to achieve more data from the transcriptome of Foc-infected banana and to better understand the molecular mechanism of Fusarium wilt resistance applied in banana genetic breeding [18, 6]. Knowledge of plant–pathogen interaction genetics focused on understanding both pathogen attack mechanisms and plant defence responses, has been explored in many studies, mainly from transcriptome data of banana infected with Foc and differentially expressed genes (DEGs) [19, 20, 21, 22, 12]. Thus, many studies seek to differentiate the defence responses of banana plants to infection by different Foc isolates, mainly to understand the susceptibility of the Cavendish cultivars to Foc TR4 [23].

It is well known that pathogens can secrete effectors to regulate the immune response of plants, while plants can also produce specific receptor-like protein kinases (RLKs) to recognize and fight pathogen infection [24, 25, 26]. Many RLK genes have been associated with responses of banana plants to infection to Foc TR4, and other genes, such as chitin receptor elicitor kinase 1 (CERK1), flagellin-sensitive 2 (FLS2), serine/threonine-protein kinase (PBS1), transcription factor WRKY 22 (WRKY22), pathogenesis-related proteins (PR-1), chitinase, lipoxygenases (LOX), jasmonate (ZIM), domain protein (JAZ), glutathione-S-transferase (GST) and cellulose synthase, may contribute to differentiate the virulence between the Foc R1 and TR4 races and account for the difference in the host resistance response [21, 27, 23].

Although there is a substantial amount of data on banana defense responses in the interaction with the Foc pathogen, knowledge of the pathogenic molecular mechanism and its interaction with the host has not yet been fully elucidated. In addition, there is no cultivar available that is immune to Foc TR4, or with a desirable baseline resistance level that can replace Cavendish cultivars. Therefore,

all efforts to understand the mechanisms of attack of the pathogen, such as the defense responses, can bring valuable contributions to banana genetic breeding, mainly by identifying global patterns of gene expression, influenced by infection of different races. Therefore, the present study aimed to evaluate the infectious process of Foc isolates with different virulence profiles in the roots of banana cultivars at molecular level by quantifying the expression of resistance genes by qRT-PCR over time and at histological and histochemical levels. These data will bring new contributions to the development of fusarium wilt-resistant banana cultivars and expand knowledge about the mechanisms involved in plant-pathogen interactions.

2. Results

2.1. Expression profile of defence genes in response to different Foc isolates

To verify the interaction of banana cultivars BRS Platina, Grand Naine and Prata-Anã with Foc R1, ST4 and 229A isolates at the molecular level, a total of seven genes potentially involved in the plant-pathogen interaction (ATL, CESA7, CHI, LOX, PI206, WRKY22, PR1) related to disease resistance were selected, and qRT-PCR validation was performed. The set of genes analyzed showed different expression patterns for cultivars infected with different isolates (Figures 1, 2 and 3). In general, the cultivar BRS Platina demonstrated an upregulated expression level for most genes in the first 12 hours after inoculation with Foc R1 and ST4 (Figures 1, 2 and 3). The results of the expression pattern of these genes were consistent and showed correlation with fluorescence microscopy analyses for the detection of cellulose (Figure 4), with scanning electron microscopy analyses (Figure 7, 8) and with the symptomatic analyses (Figure 9), considering that for both analyses the cultivar BRS Platina demonstrated greater defense responses against the different Foc isolates.

The Auxin transporter-like protein 1 (ATL) gene, related to auxin response, was upregulated in the cultivar BRS Platina at all evaluation times, starting with the highest levels at 12 hai, mainly for Foc R1 and ST4 isolates, but for Foc 229A, it was downregulated, mainly at 48 hai (Figure 1). Conversely, in relation to the cultivars Prata-Anã and Grand Naine, the ATL gene was gradually upregulated over time, with the highest levels of expression at 24 or 48 hai (Figure 1). The upregulated expression of ATL in the resistant cultivar at 12 hai mainly for Foc R1 and ST4, indicates a possible involvement in rapid defense responses after infection. The Cellulose synthase gene A catalytic subunit 7 (CESA7), which is part of different classes of proteins related to the synthase cellulose complex, showed an oscillation in the level of expression according to inoculation times, and for the cultivar BRS Platina, inoculated with Foc R1, an upregulated expression was observed only at 12 hai. In relation to Foc ST4, there was an upregulated expression at all evaluation times; for isolate Foc 229A, the expression was upregulated in 12 and 24 hai (Figure 1). In the cultivar Prata-Anã inoculated with Foc R1, the CESA7 gene was only upregulated at 48 hai, in relation to ST4 whose gene expression was upregulated in 12 hai, downregulated in 24 hai and again upregulated in 48 hai. For the cultivar Grand Naine, the CESA7 gene was upregulated at inoculation with Foc R1 with the highest levels of expression being downregulated at 12 hai. As to Foc ST4 and 229A, gene expression was upregulated in 48 hai (Figure 1).

The Chitinase gene (CHI) that encodes enzymes that catalyze chitin hydrolysis offering antifungal activity was upregulated mainly in the cultivar BRS Platina inoculated with Foc R1 in 12 hai and with inoculation with Foc ST4 was upregulated in 12 and 48 hai, but for interaction with Foc 229A the expression level was lower in comparison to the cultivar Prata-Anã (Figure 1). In the cultivar Grand Naine the CHI gene was downregulated in all hours after inoculation with Foc R1 and in inoculation with Foc ST4 and 229A had an upregulated expression at 12 and 48 hai. For the cultivar Prata-Anã inoculated with Foc R1, the expression of the CHI gene in all hours after inoculation was downregulated and for inoculation with Foc ST4 there was an upregulated expression at 12 and 48 hai, while in relation to

inoculation with Foc 229A the CHI gene presented an upregulated expression superior to all cultivars in 12 hai, but this expression was downregulated at 24 and 48 hai (Figure 1). Thus, the CHI gene seems to be associated with a rapid defense response in the cultivar BRS Platina for Foc R1 and ST4, but not for resistance to Foc 229A.

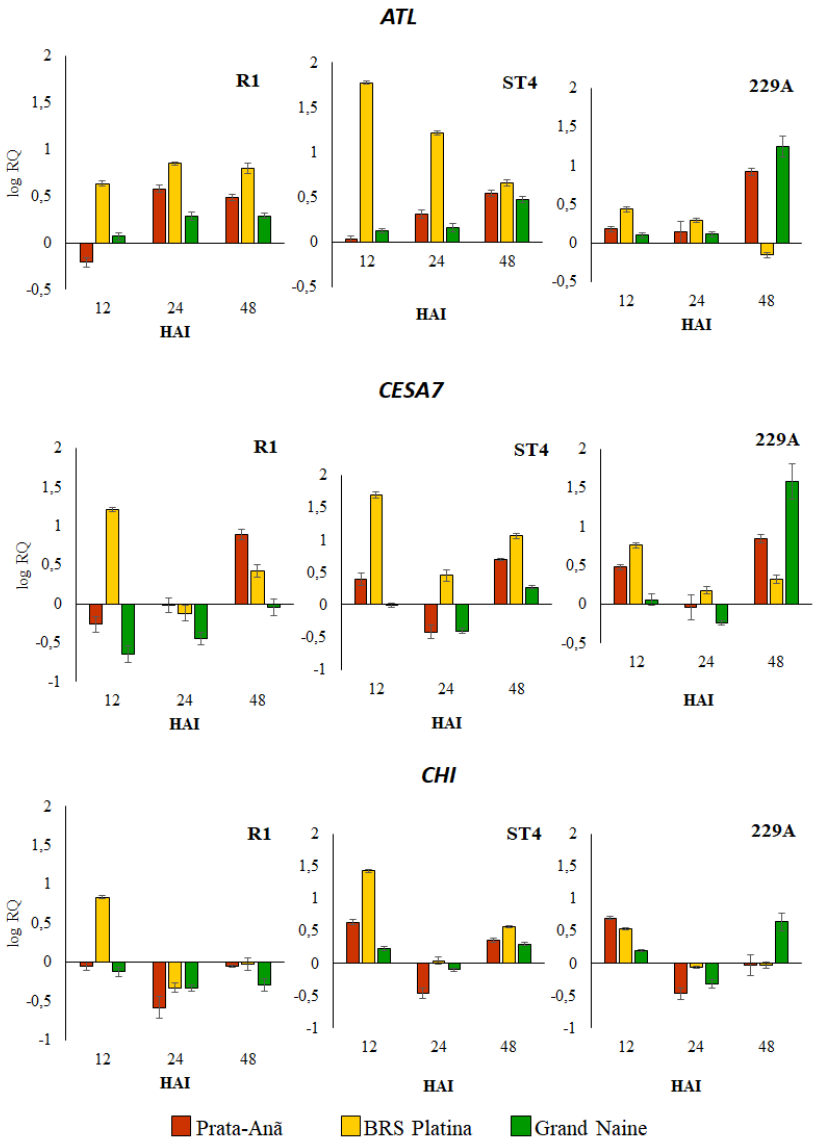

Figure 1. Relative expression levels of three defense-related genes in banana genotypes at 12, 24 and 48 hours after inoculation (HAI) with *Fusarium oxysporum* f. sp. *cubense* races 1 (Foc R1), subtropical 4 (Foc ST4) and isolate 229A. Levels of the relative expression of the defense-related genes Auxin transporter-like protein 1 (ALT), Cellulose synthase A catalytic subunit 7 (CESA7) and chitinase (CHI) were analysed by RT-qPCR. The data represent the means ± standard deviations of three replicates.

The Lipoxygenase gene (LOX) was upregulated in the cultivar BRS Platina inoculated with Foc R1 and ST4 isolates in 12 hai reducing expression over time, unlike the cultivars Grand Naine and Prata-Anã, where the expression was downregulated in all hours after inoculation of these isolates (Figure 2). Regarding inoculation with Foc 229A, the LOX gene was upregulated in the first 12 hai mainly for cultivar Prata-Anã and in 48 hai for the cultivars Prata-Anã and Grand Naine (Figure 2). Similarly, to the expression profile of the ALT and CHI genes, the LOX gene seems to play essential functions in the initial defense responses of cultivar BRS Platina to Foc R1 and ST4, but it does not seem to be related to the resistance response of the cultivar Grand Naine to Foc R1, since its expression was

downregulated. The Putative Disease resistance response protein 206 gene (PI206) was upregulated exclusively in the cultivar BRS Platina inoculated with Foc R1 and ST4 especially in 12 hai, considering that the expression of this gene was downregulated in the cultivars Grand Naine and Prata-Anã for all hours after inoculation of the isolates (Figure 2). At inoculation with Foc 229A there was no expression of the PI206 gene considered upregulated for any of the cultivars (Figure 2).

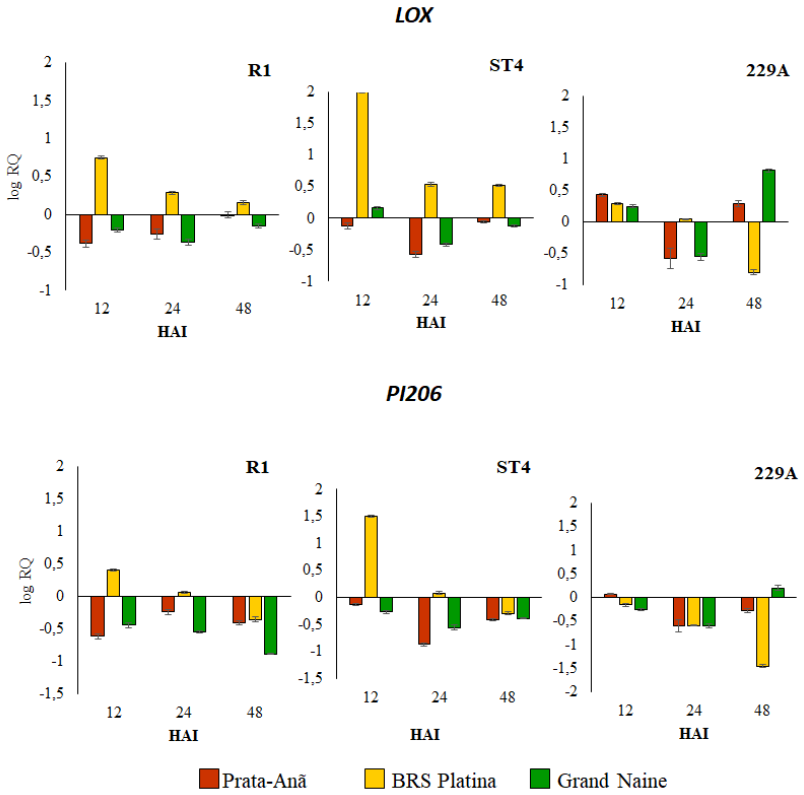

Figure 2. Relative expression levels of three defense-related genes in banana genotypes at 12, 24 and 48 hours after inoculation (HAI) with *Fusarium oxysporum* f. sp. *cubense* races 1 (Foc R1), subtropical 4 (Foc ST4) and isolate 229A. Levels of relative expression of the genes related to the defence lipoxygenase (LOX) and putative disease resistance response protein 206 (PI206) were analysed by RT-qPCR. The data represent the means ± standard deviations of three replicates.

Transcription factor WRKY (WRKY22) was upregulated in the resistant cultivar BRS Platina inoculated with Foc R1 and ST4 at 12 hai and over time the expression was reduced. In the interaction with Foc 229A was upregulated only in 12 hai and the expression was downregulated in 24 and 48 hai (Figure 3). In cultivar Grand Naine inoculated with Foc R1 the expression of the WRKY22 gene was upregulated in 12 hai and downregulated at 24 and 48 hai, already in relation to inoculation with Foc ST4 the expression was upregulated in 12 and 48 hai, and downregulated at 24 hai and in relation to inoculation with Foc 229A the expression was upregulated especially in 12 and 48 hai (Figure 3). In the susceptible cultivar Prata-Anã inoculated with Foc R1, the WRKY22 gene was upregulated in all hours after inoculation, but the expression was lower in comparison to the cultivar BRS Platina. At inoculation with Foc ST4 and 229A the expression was upregulated in 12 and 48 hai and downregulated in 24 hai (Figure 3). The Pathogenesis-related proteins 1 (PR1) gene encodes pathogenesis-related proteins and, in general, had the highest levels of upregulated expression in relation to the other genes analyzed for all cultivars (Figure 3). At 12 hai, all cultivars showed upregulation of the PR1 gene when inoculated with Foc R1 and Foc ST4 and 229A, but the expression levels in the cultivar BRS Platina were higher when inoculated with Foc ST4 and lower in interactions with Foc 229A (Figure 3). For interactions with the Isolate ST4 at 12 hai, the cultivar Prata-Anã presented the highest levels of overexpression, while in the

cultivar Grand Naine, the levels of relative expression were higher only at 48 hai (Figure 3).

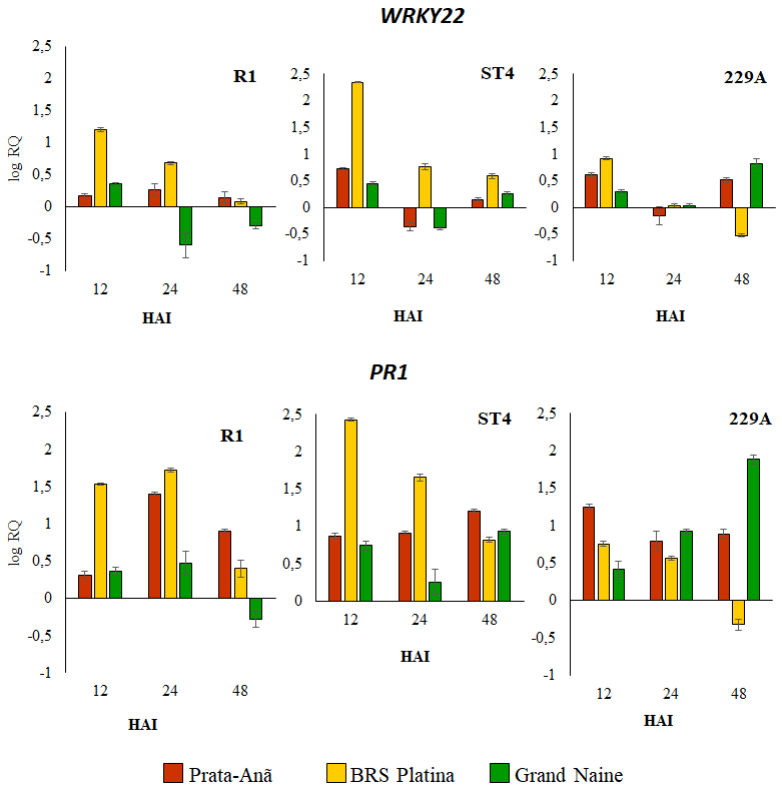

Figure 3. Relative expression levels of three defense-related genes in banana genotypes at 12, 24 and 48 hours after inoculation HAI with *Fusarium oxysporum* f. sp. *cubense* races 1 (Foc R1), subtropical 4 (Foc ST4) and isolate 229A. The relative expression levels of the defense-related gene transcription factor WRKY22 and protein-related pathogenesis 1-like PR1 were analysed by RT-qPCR. The data represent the means ± standard deviations of three replicates.

2.2. Histological and histochemical responses

The production of cellulose was verified by staining samples from all treatments at 12 hai with Calcofluor White dye where positive samples emitted secondary blue–white fluorescence in the parenchyma and central cylinder. For the BRS Platina cultivar, the emission of fluorescence was reflected in the inoculation with isolates Foc R1, ST4 and 229A (Figura 4). These observations were consistent with the CESA7 gene expression data, related to cellulose synthesis, since the cultivar BRS Platina had higher expression of this gene in 12 hai (Figure 1), as well as with the symptomatological analyses, considering the lower level of symptoms of the disease in the cultivar BRS Platina inoculated with the isolates compared to the cultivars Grand Naine and Prata-Anã (Figure 9). Therefore, cellulose is an apparently efficient defense response for the cultivar BRS Platina. However, in the cultivars Grand Naine and Prata-Anã, the fluorescence was efused only after inoculation with Foc ST4. In general, the control samples did not present high fluorescence levels (Figure 4).

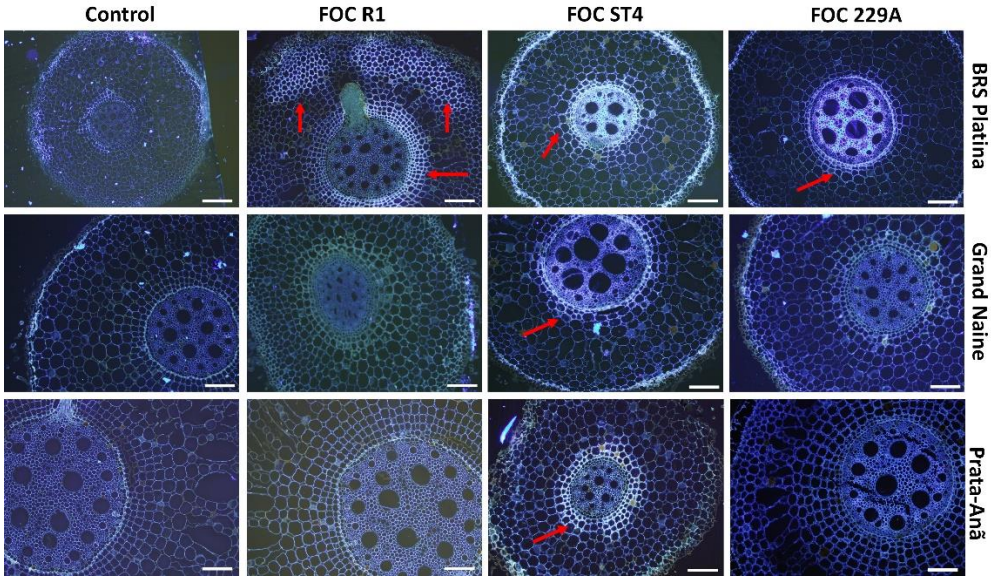

Figure 4. Cross-sectional fluorescence micrographs of roots of banana cultivars with xylem cavities infested by different *Fusarium oxysporum* f. sp. *cubense* strains 12 hours after inoculation (HAI) and stained with Calcofluor White to detect cellulose. Red arrows indicate second grade white-blue fluorescence related to the concentration of cellulose in the tissues. Foc: *Fusarium oxysporum* f. sp. *cubense*. R1: race 1. ST4: subtropical race 4. Bars = 200 μm.

The ferric chloride test to detect phenolic compounds produced scores with a dark brown colour in the rhizomes at 12 hai (Figure 5). All the cultivars showed positive results for the presence of phenolic compounds in samples inoculated with Foc R1, ST4 and 229A, and a strong indication of phenolic compounds in the control samples was observed, which is due to environmental changes or a constitutive basal production of this compound in each cultivar (Figure 5). The presence of phenolic compounds in the cultivar BRS Platina was more significant when inoculated with Isolate ST4 (Figure 5). In the cultivar Grand Naine, the presence of phenolic compounds was higher in the interaction with isolate 229A and in the cultivar Prata-Anã when inoculated with foc R1 and Foc 229A isolates (Figure 5). Considering that the presence of fusarium wilt symptoms is associated with higher oxidation of phenolic compounds, these observations are consistent with the symptomatological analyses performed in 90 dai, considering that the cultivar BRS Platina demonstrated a higher level of symptoms for the Foc ST4 isolate, cultivar Grand Naine for Foc 229A and Prata-Anã cultivar for both isolates (Figure 9).

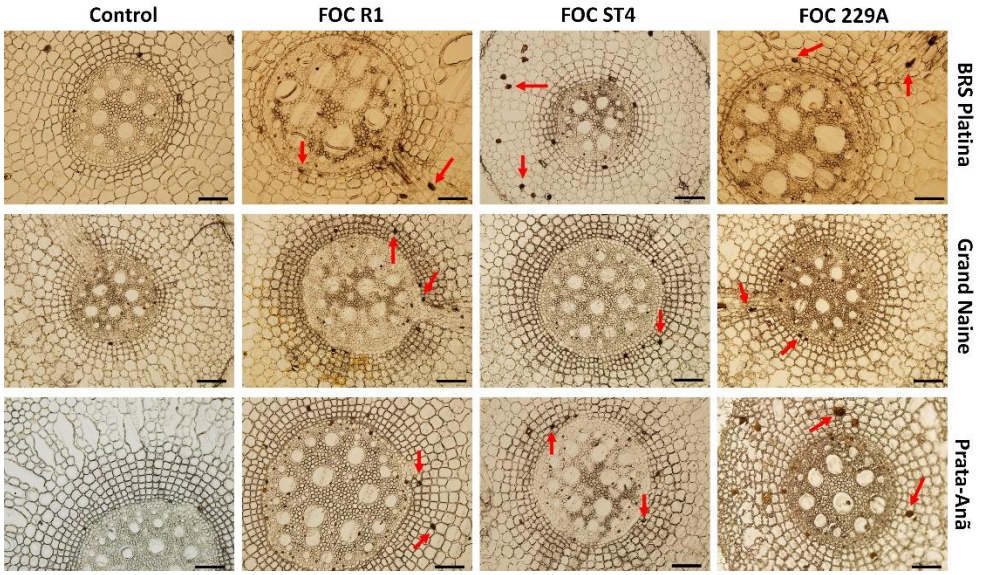

Figure 5. Cross-sectional micrographs of roots of banana cultivars with xylem cavities infested by different *Fusarium oxysporum* f. sp. *cubense* strains 12 hours after inoculation (HAI) and stained with ferric chloride for the detection of phenolic compounds. Red arrows

indicate sections with accumulation of phenolic compounds. Foc: *Fusarium oxysporum* f. sp. *cubense*. R1: race 1. ST4: subtropical race 4. Bars = 200 µm.

With the clarification of the tissues and staining of Foc structures inside the roots at 90 days after inoculation, it was possible to determine that all cultivars were positive for the presence of pathogen structures such as hyphae and spores indicating that penetration occurs inside the roots both in resistant cultivars and susceptible cultivars. Thus, in the resistant cultivar BRS Platina the presence of hyphae inside the tissue in the interaction with Foc R1 and 229A, and spores in the interaction with Foc ST4 (Figure 6), were observed. There were pathogen spores in the cultivar Grand Naine inside the tissues even for the interaction between the cultivar Grand Naine and Foc R1, for which there were no symptoms characterizaing an immunity response (Figure 9). In this cultivar the presence of hyphae and spores in the interaction with Foc ST4 and 229A was also detected (Figure 6). For the cultivar Prata-Anã, hyphae were found in the tissue and there were spores in the interaction with Foc R1, ST4 and 229A (Figure 6).

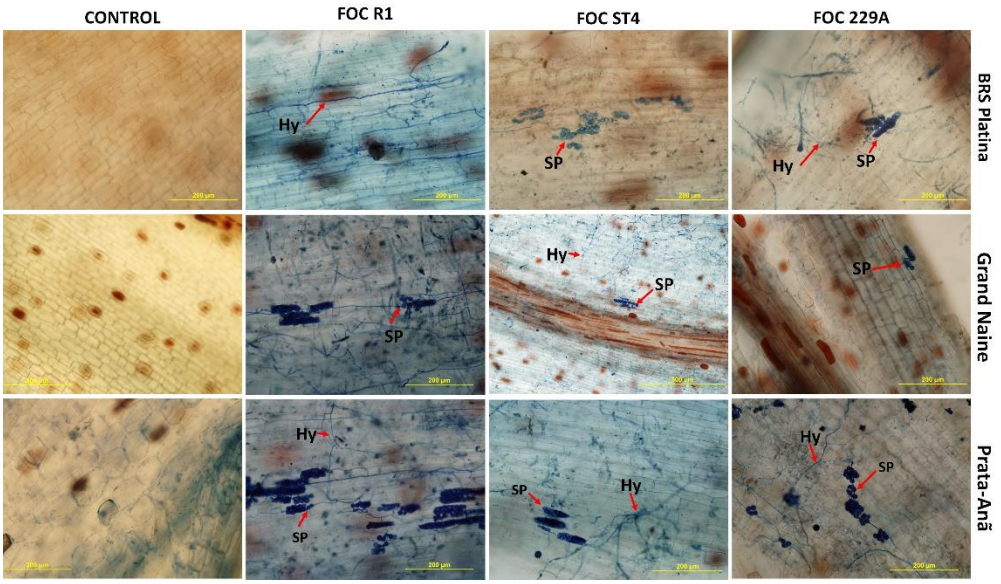

Figure 6. Root clarification and staining of fungal structures with trypan blue dye after infestation by different isolates of *Fusarium oxysporum* f. sp. *cubense* 90 days after inoculation. SP: spores; HY: hyphae. Red arrows indicate hyphae (Hy) and spores (SP). Foc: *Fusarium oxysporum* f. sp. *cubense*. R1: race 1. ST4: subtropical race 4. Bars = 200 µm.

3.3. Analysis of the interaction by scanning electron microscopy

The analysis of scanning electron microscopy indicated that in 48 hai the cultivar BRS Platina responded to infection by Foc ST4 and 229A with intense production of calcium oxalate crystals (Figure 7). This response was not observed in the interaction with Foc R1, but the tissues were intact and without the presence of fungal growth, indicating probable delay in the penetration of this isolate in comparison to the others. No structural defence responses or pathogen growth were observed in the Grand Naine cultivar inoculated with this pathogen at 24 hai (Figure 7). In the Prata-Anã cultivar, growth of fungal mycelium and early obstruction of the conducting vessels was observed in the interactions with Foc R1 and ST4, and in the interactions with Foc 229A, pathogen sporulation was observed, confirming the greater virulence of this isolate by its ability to rapidly grow and reproduce within the tissue in the susceptible cultivar. Structural changes or traces of the pathogen in the control samples of the three cultivars were not observed (Figure 7).

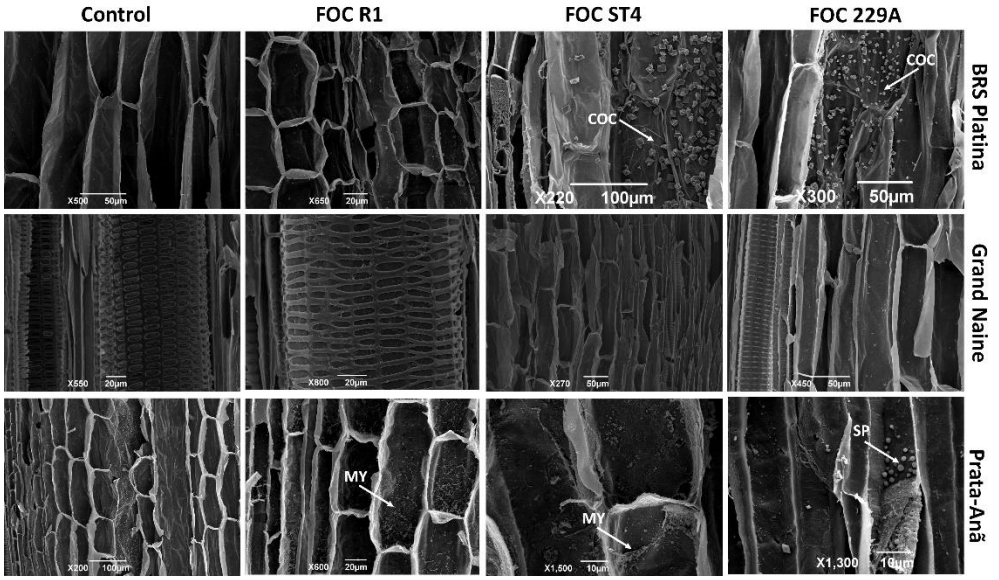

Figure 7. Scanning electron micrographs of longitudinal sections of roots of banana cultivars with xylem cavities infested by different *Fusarium oxysporum* f. sp. *cubense* isolates 48 hours after inoculation (HAI). Foc: *Fusarium oxysporum* f. sp. *cubense*. R1: race 1. ST4: subtropical race 4. COC: calcium oxalate crystals. MY: mycelium. SP: spores.

The interactions of the different Foc isolates with the cultivars over a period of 30 DAI in order to compare the extent of pathogen infection in different cultivars was analysed via SEM. In the 30 DAI, fungal hyphae extensively colonized the epidermis, collenchyma, cortical and medullary parenchyma and vascular bundle of the Prata-Anã susceptible cultivar inoculated with isolates R1, ST4 and 229A, with obstruction of the xylem vessels caused by the presence of large amounts of mycelium and spores linked to late defence responses mainly in the interaction with Foc R1 (Figure 8). These data confirm the susceptible phenotype of the Prata-Anã cultivar observed in the symptom evaluations at 90 DAI (Figure 9) as they may be associated with the downregulated expression of some important resistance genes in the plant-pathogen interaction such as the CHI (Figure 1), LOX and PI206 genes (Figure 2).

In contrast, in the BRS Platina cultivar, the occlusion of conducting vessels was observed only when inoculated with the ST4 isolate (Figure 8), but the pathogen did not reach the central cylinder or xylem vessels, only the parenchyma and collenchyma tissues that surround the central cylinder, demonstrating that there is no growth of the pathogen in a timely manner to reach the rhizome. In this cultivar, occlusion or growth structures were not observed in plants inoculated with Foc R1 and 229A at 30 DAI (Figure 8). This result is consistent with the upregulated expression of important defense genes in this cultivar such as CESA7 and CHI (Figure 1) and LOX and PI206 (Figure 2) which may have culminated in the lowest rates of symptoms in relation to isolates at 90 dai (Figure 9). The Grand Naine cultivar presented tissue occlusion in the parenchyma and collenchyma that extended to the central cylinder, with the presence of fungal mycelium at 30 DAI when inoculated with the ST4 and 229A isolates. The root tissues of this cultivar had no trace of the pathogens in the interactions with the R1 isolate (Figure 8) and this may be related to the resistant profile of race 1 of the pathogen, although in 90 DAI it is possible to identify the presence of hyphae by the method of clarification and staining of fungal structures which indicates a post-penetration defense response in relation to Foc R1 (Figure 6).

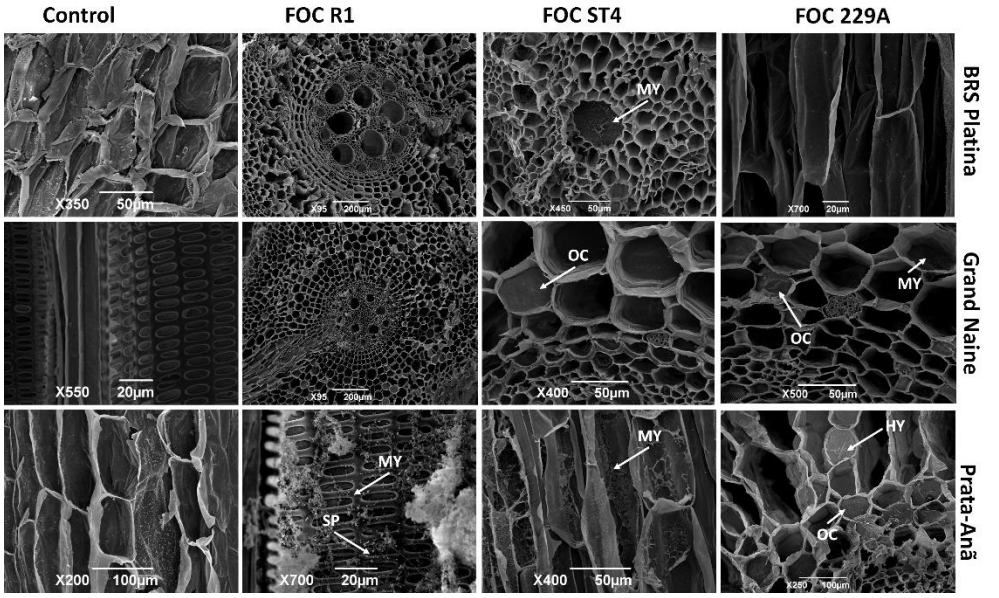

Figure 8. Scanning electron micrographs of cross-sectional and longitudinal sections of roots of banana cultivars with xylem cavities infested by different *Fusarium oxysporum* f. sp. *cubense* strains at 30 days after inoculation (DAI). Foc: *Fusarium oxysporum* f. sp. *cubense*. R1: race 1. ST4: subtropical race 4. MY: mycelium; SP: spores; HY: hyphae.

3.4. Symptoms

In addition to molecular, histological and histochemical analyses and to verify the efficiency of the inoculation method, 10 plants of each cultivar for each isolate were evaluated as to the presence of symptoms in cross-sections of the rhizome at 90 DAI. As expected for a susceptibility profile, the cultivar Prata-Anã presented characteristic symptoms of wilting with the death of some plants mainly when interacting with the Isolate ST4. Symptoms were also evident in the cross-section of the plant rhizome, where a reddish-brown colour was observed with infection points starting from the extremities and reaching the central cylinder until its complete obstruction (Figure 9). It was possible to identify and quantify the aggressiveness of the isolates by the index of internal symptoms that are demonstrated by the heat map in Figure 9, where the colours of intense red indicate higher indices and in the level of intense green, lower indices. For the Prata-Anã cultivar the indices were 90%, 98% and 59% for isolates of Foc R1, ST4 and 229A, respectively (Figura 9). As cavendish cultivars are resistant to Foc R1 in the cultivar Grand Naine, Foc R1 isolate did not cause the disease; the values of the symptom index for the ST4 isolate were 14%, while for isolate 229A were 70% (Figure 9).

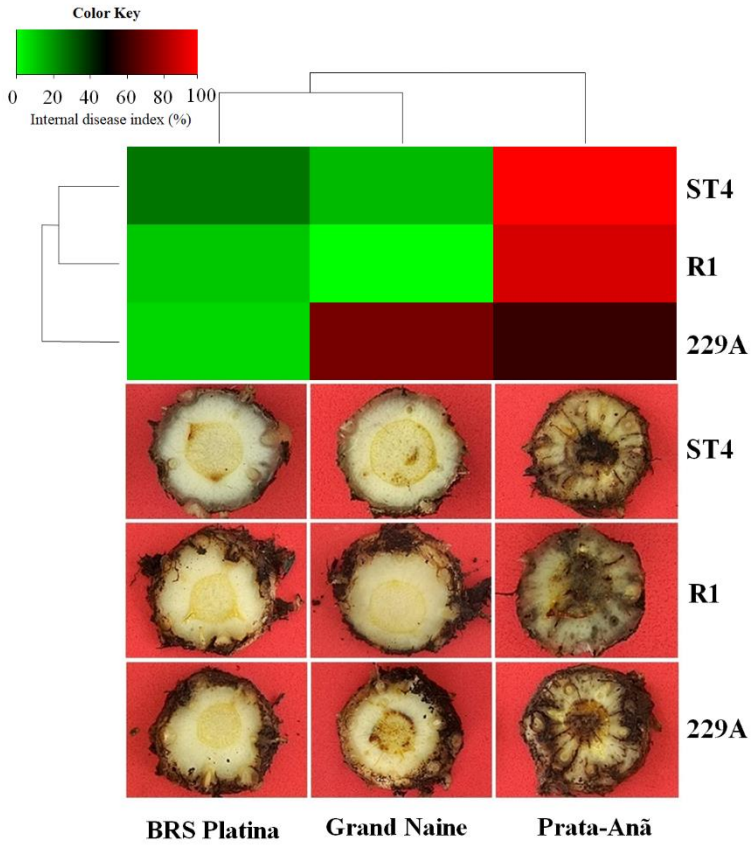

Figure 9. Heat map of internal symptom indices and cross-sections of the rhizomes of three banana cultivars 90 days after inoculation (DAI) with *Fusarium oxysporum* f. sp. *cubense* isolates that differ in virulence. The heat map colours reflect high levels of internal symptoms (intense red) and low levels of internal symptoms (intense green). The disease indices were calculated from the evaluation scores of 10 plants of each cultivar using the Dita et al., (2014) rating scale. Foc: *Fusarium oxysporum* f. sp. *cubense*. R1: race 1. ST4: subtropical race 4.

In the cultivar BRS Platina, there was no discoloration of the rhizome associated with practically any of the isolates. The index of internal symptoms in 'BRS Platina' inoculated with isolate ST4, 229A and Foc R1 was 27%, 9% and 11%, respectively (Figure 9). These data therefore show that in fact the upregulated genes in the cultivar BRS Platina, as well as the cellulose related to cell wall seen by fluorescence microscopy may play important roles in the delay of infection by the pathogen seen by SEM analyses which culminated in this phenotype of resistance to Foc isolates with a lower symptom index of the cultivars Grand Naine and Prata-anã (Figure 9). Overall, heatmap-based cluster analysis showed that the cultivars Grand Naine and Prata-Anã grouped in a single cluster, different from the cultivar BRS Platina, which was much more distant with a disease index of less than 30% considering all isolates (Figure 9). This implies that the Foc 229A isolate was more aggressive in the cultivar Grand Naine than in the cultivars Prata-Anã and BRS Platina, which confirms its virulence profile.

3. Discussion

Seven genes related to plant-pathogen interaction pathways were validated by real-time quantitative polymerase chain reaction (qRT–PCR) from root samples of BRS Platina, Grand Naine and Prata-Anã cultivars following interactions with Foc R1, ST4 and 229A isolates. Differences were observed in the level of gene expression in all the cultivars according to the isolate, where high levels of overexpression were generally associated with interactions with the ST4 isolate, and lower levels were associated with interactions with Foc 229A. These results confirm that differences in the virulence of the isolates may contribute to changes in the host resistance response and that the expression of defence-related genes may alter the efficacy of

pathogen virulence mechanisms in some cultivars [28].

Plant hormones play a key role in the interaction between plant development and the environment in order to create a signal pathway that helps to mold its architecture and at the same time prepare it to respond to certain stresses appropriately. Auxins or its signal pathways, modulate resistance in plants to diseases directly and indirectly. The direct effects of auxins are interference in the circuit of signalization, and indirectly, they may alter the progress of the disease during the plant x pathogen interaction due to its effect on plant development [29, 30]. In the present study the ATL gene which is related to auxin production and signalling, seems to play an important role in interactions of the BRS Platina cultivar with Foc R1 and ST4, considering that its expression was increased at all times, except in the interaction with Foc 229A, for which a reduction was observed. These results are in agreement with the evaluation by Costa [31], who found that this gene was exclusively associated with the 'BRS Platina' genome when inoculated with the isolate Foc R1. In addition, auxins are known to be important phytohormones for plant growth and disease resistance. Some studies have suggested that pathogens could increase auxin biosynthesis in a plant to alter the growth and development of the plant in its favour [30]. However, our symptomatology results indicate that the increased expression of the ATL gene did not contribute to greater susceptibility of the BRS Platina cultivar to the ST4 isolate, nor did its reduction seem to be associated with resistance in the other cultivars, considering the isolate.

Besides offerring structural support and a passive barrier against pathogens, the cell wall controls cell expansion and is involved in the exchange of water and substances during plant development. It is also considered a resovoir of anti-microbial components and a source of signilizing molecules where alterations in the cell wall influence growth and the network of responses to stress, especially during the response to pathogens that survive in the apoplast, such is the case with Fusarium oxysporum in bananas [32]. In the case of chitin β-(1–4)-poly-N-acetyl-D-glucosamine), it is also a structural compound of the exoskeleton of crustaceous and the cell wall of fungi is abundantly distributed [33]. Therefore, chitinase (CHI gene), an enzyme capable of passing through the cell wall o fungi may offer plants good protection against pathogens. The CESA7 gene, which is part of a family of genes required for cellulose biosynthesis in the secondary cell wall, and the CHI gene, which regulates chitinase production, were overregulated in the roots of the banana cultivar BRS Platina at 12 hai with Foc R1 and ST4 and downregulated in the Prata-Anã and Grand Naine cultivars at almost all times after inoculation of the isolates (Figure 2). After infection by pathogens, it was suggested that the plant responds with the synthesis of new carbohydrates, especially callose and cellulose, which are added to the interior of the cell wall adjacent to the infection, in order to contain the advance of the fungus [31, 26].

In this study, both the expression of the CESA7 gene and the presence of cellulose detected by fluorescence microscopy were reduced in the interaction with the 229A isolate, which at the end of the experiment was considered more aggressive in the Prata-Anã and Grand Naine cultivars (Figure 2). Given the aggressiveness of this isolate, this finding may be associated with the fact that fungal and bacterial pathogens can produce cellulases as virulence factors, which have the ability to break down cellulose in the host cell wall [34, 32]. As to the cultivars Prata-Anã and Grand Naine, the presence of cellulose by fluorescent microscopy was not consistent with the data of expression of gene CESA7, being present only after inoculation with Foc ST4 (Figure 4), whereas the expression of gene CESA7 was upregulated in cultivar Prata-Ana inoculated with Foc 229A and was not differential in cultivar Grand Naine inoculated with Foc ST4 (Figure 1). The CESA genes for the respective CESA complexes are highly co-regulated since many CESA genes are reported as playing key roles in cellulose synthesis in the secondary cell wall, such as the CESA8 and CESA6 genes [35]. Although these genes were not evaluated in this study, a hypothesis is that there might have been some changes in the combined regulation of these genes in cultivars Prata-Anã and Grand Naine in the interaction with isolates Foc R1 and 229A, culminating in the absence of

fluorescence in Figure 4.

Chitinases target components of the fungal cell wall, breaking down β-1,3-glucans and chitin, which are important pathogen virulence factors [36, 28]. The CHI gene was more highly expressed in the BRS Platina cultivar than in the Prata-Anã and Grand Naine cultivars at 12 hai, which may be closely related to the resistance of this cultivar in the early stage of infection [31]. In contrast, with the 229A isolate, there was a reduction in the expression of the CHI gene in all the cultivars, which may be related to the greater virulence of this isolate. Ding et al. [36] demonstrated that the null mutation of three mitogen-activated protein kinase (MAP) genes led to a substantial attenuation of fungal virulence in bananas, especially in the regulation of genes encoding the production of chitin, peroxidase, beauvericin and fusaric acid, demonstrating that these genes can actually alter the response mechanisms of infected plants. In another study, Li et al. [28] demonstrated that the CHI gene was more induced in the mutant resistant to Foc TR4 'Nongke No. 1' (NK) than in the susceptible cultivar Baxi (BX) in 27 hai, which may be closely related to the resistance of NK in the inicial phases of infection. Furthermore, this study showed that fungal genes which express chitin synthase (CHS), necessary for *Fusarium oxysporum* pathogenesis had the highest level of expression in BX than in NK and may indicate higher pathogenicity in a susceptible infected cultivar than in a resistant one.

The lipogenases (LOXs) are enzymes of natural occurance amply distributed in plants and animals. These enzymes are capable o dioxygenizing insaturated fatty acids which leads to lipoperoxidation of biological membranes. This process causes the synthesis of signalizing molecules and also may alter cell metabolism. LOXs are known for being involved in the apoptosis (programmed cell death) pathway and permeate the biosynthesis of jasmonic acid (JA) acting as a biomarker of stress against fungi, bacteria, pests and abiotic stresses [37, 38, 39, 40]. In this study, there was upregulation of the LOX (lipoxygenase) gene in the BRS Platina cultivar at all hours after inoculation with isolates R1 and ST4, but for isolate 229A, the regulation was reduced by 12 hai. In the Prata-Anã and Grand Naine cultivars, this expression was downregulated in all evaluations, except at 12 hai and 48 hai (Figure 3). A RT–qPCR analysis performed by Liu et al. [41] revealed that several genes of the LOX family may increase banana resistance to Foc TR4 by regulating the jasmonic acid (JA) pathway. In another study, Li et al. [42] found that the high expression of LOX was related to greater resistance to Foc TR4 in a mutant, and Li et al. [43] also found that LOX1.1-3 and LOX2.3 were significantly induced in a resistant strain of *Musa yunnanensis* during early infection with Foc TR4.

The PI206 gene was overexpressed only in the BRS Platina cultivar inoculated with Foc R1 and ST4 at 12 hai, but there was no upregulation in any of the cultivars inoculated with the 229A isolate (Figure 3). This gene is part of the leucine-rich repeat domain of the nucleotide binding site (NBS-LRR) and was identified exclusively in the 'BRS Platina' genome via RNAseq analysis [31]. NB-LRR genes are the largest group of plant R genes and play important roles in the perception of extracellular immunogenic patterns that trigger defence responses to prevent the spread of a pathogen [44, 45]. An analysis of the entire genome of the LRR-RLP gene family in wild banana *Musa acuminata* ssp. *malaccensis* identified several candidate genes for Fusarium wilt resistance [45]. In other studies, NBS-LRR genes were strongly induced after inoculation of TR4 in resistant cultivars, indicating that these genes play important roles in banana against Foc infection [42, 20, 46].

Transcription factors (TFs) are part of the machinery of the defense response to stress through regulation of a complex system of genes in plants. The WRKY superfamily of TFs is the seventh lartgest in plants with flowers and is a promising candidate for plant breeding due to rigid regulations envolving the specific recognition and link of WRKYs to forward promotors. WRKYs orchestrate molecules in plants and provide multiple simultaneous responses where activation or repression occurs through the recognition of W-box sequences present in promotor sequences of target genes [47, 48]. Many WRKY functions involved in defense responses against biotic and abiotic stress have been studied [49, 50, 51, 52].

In the present study, the WRKY22 gene, belonging to a family of transcription factors known to play an important role in resistance to biotic stress, had a higher relative expression profile at all hours after infection in the BRS Platina cultivar inoculated with Foc R1, ST4 and 229A (Figure 4). It is known that transcription factors are part of many signalling pathways that regulate plant defence responses. The superfamily of WRKY transcriptional regulators in addition to regulating the expression of defence genes, also regulate response pathways to diseases regulated by salicylate and jasmonate [53, 54]. In one study, 7 WRKY genes, including one WRKY22 gene, were involved in the plant–pathogen pathways and were twice as high in the Pahang cultivar as in a Cavendish cultivar, suggesting that the expression of these WRKY genes may be associated with constitutive defence mechanisms [22]. WRKY56 and WRKY75 genes were also associated with the response of the resistant Cavendish banana mutant 'Nongke No 1' to Foc TR4 [42].

Pathogen related proteins are well studied in the literature and PR1, among the 17 families, is the dominant group induced by pathogens or salicilic acid used as a marker of pathogen induced systemic acquired resistance (SAR) [55, 56]. It´s role as an indispensible component of native immune responses in plants under biotic or abiotic stress and its interaction with the inhibition of pathogen effectors are also reported [57, 58]. In our study, the PR1 gene presented a overregulated expresssion profile superior to all the other genes evaluated for all cultivars inoculated with Foc R1 and ST4, except for the 229A isolate, whose expression of the PR1 gene was reduced especially in cultivar BRS Platina (Figure 4). High levels of expression of the PR1 gene in response to Foc TR4 are reported in the literature [59, 60, 22].

In our study, the BRS Platina cultivar responded to the Foc R1, ST4 and 229A isolates with a greater presence of cellulose in its tissues than that observed for the Prata-Anã and Grand Naine cultivars. This result is consistent with the expression data of the CESA7 gene, which is related to cellulose synthesis. These data suggest that cellulose is not an important component in the defence immune response of the Grand Naine cultivar to the Foc R1 isolate. Phenolic compounds seem to be extensively produced in the roots of the Prata-Anã cultivar at 48 hai. It has been suggested that the accumulation of phenolic compounds indicates the sensitivity of the plants to the pathogen and the attempt to protect themselves by the formation of structural barriers [61]. Therefore, this result may be related to the extreme susceptibility of this cultivar during interactions with the studied isolates. On the other hand, phenolic assays indicated that tolerance to Foc ST4 may be linked to the increase in phenolic compounds associated with the cell wall [62]. It is believed that this may also be one of the resources used by the resistant cultivar BRS Platina as a defence response in relation to the ST4 isolate, since there was an increase in this compound.

SEM data showed that calcium oxalate crystals are produced in abundance in the BRS Platina cultivar inoculated with Foc ST4 and 229A and can play important roles in resistance. In contrast, this type of response was not observed in the interactions between any of the isolates with the cultivars Prata-Anã and Grand Naine, and even only 48 hai, pathogen structures such as hyphae, spores and mycelium were found inside the roots of the cultivar Prata-Anã (Figure 7). Although there are few studies reporting the presence of calcium oxalate crystals as a defence response in interactions between *Musa* spp. x Foc, it is believed that these crystals play an important role, especially because the degradation of single crystals can produce reactive oxygen species, which have been extensively related to the response to infection by pathogens and have also been related to the inhibition of infection [63, 31]. In addition, in the staining data of fungal structures, after root clarification, all the isolates penetrated the tissues of susceptible and resistant cultivars, which is in agreement with the evaluations performed by Dong et al. [27], who observed that both Foc R1 and Foc TR4 could penetrate the root epidermis and invade the xylem vessels of Cavendish cultivars.

At 30 DAI, SEM analysis showed that the cultivar Prata-Anã behaves as a plant with a high level of susceptibility to isolates R1, ST4 and 229A; that is, progression of the invasion by the pathogen and clear failures in the responses of defence

culminated in the collapse of the xylem vessels, which was observed at 90 DAI. In contrast, in the interactions of the BRS Platina cultivar with the ST4 isolate, the vessel occlusion in the xylem was limited to the parenchyma and had not yet reached the central root cylinder at 30 DAI, which suggests that the containment of the pathogen advanced over time. Regarding the Grand Naine cultivar, the interaction with isolate 229A resulted in the occlusion of vessels and mycelium, indicating that the pathogen continued to expand, advancing towards the central cylinder, which explains why this isolate contributed 70% of the internal symptom index at 90 DAI for this cultivar. In a study that followed the colonization pathway of Foc ST4 in susceptible banana genotypes, Warman and Aitken [64] also found a 30 dpi Cavendish root sample, with macroconidia forming outside the root surface

All this information considered together allows us to infer that different defence response mechanisms are taken by the BRS Platina, Grand Naine and Prata-Anã cultivars based on the virulence of the isolates. Thus, it is reported that the genes CESA7, ATL, PI206, WRKY22, PR1, CHI and LOX may not play important functions in the first hours of interactions in the immune defence response of the Grand Naine cultivar to the Foc R1 isolate. Other mechanisms may confer race-specific resistance to this cultivar of the Cavendish subgroup, given that the complexity of the defence system of these cultivars has been documented in other studies [6, 42]. According to Dong et al. [23], after infection by Foc R1, the synthesis pathways of lignin and flavonoids are enriched in Cavendish cultivars, and when measuring the expression patterns of defence-associated genes, five genes overregulated were found that can cause hypersensitive cell death after infection.

On the other hand, it was demonstrated that the seven genes studied may play essential functions in the defence response of the BRS Platina cultivar in the interaction with the three Foc isolates, especially in the first hours after inoculation, and these data were supported by SEM and histochemical analyses and symptomatological data. In a previous study, it was determined that the type of resistance response involved in the interaction between the Foc 0801 isolate (race 1) and the BRS Platina cultivar is based on cell wall modifications, such as the formation of a healing zone, the presence of tylose and oxalate crystals, and lignification [31, 65]. Therefore, this shows that this resistance is not the same resistance of Cavendish cultivars to Foc R1 isolates but a quantitative type of resistance that can be very well explored, especially in a culture system based on integrated management. In addition, all the genes included in this study have been reported to be important in the response of banana genotypes to Foc TR4 infection.

When interacting with isolate 229A, which is considered more aggressive than the other isolates, the cultivar BRS Platina and the other cultivars had reduced levels of gene expression, especially for PI206 and PR1. Isolate 229A was initially isolated from the Prata-Anã cultivar in 2014 in farms in the municipality of Miracatu in the state of São Paulo in Brazil [66]. In a recent study that characterized Foc populations of several regions of Brazil [67], other isolates from these same farms were classified as part of clade A, and VCG 0120, possibly the isolate of Foc 229A, was characterized among the group of isolates belonging to VCG 0120, which is usually associated with Foc ST4, with no possibility of this being an isolate of TR4. In addition, its variation in terms of virulence and aggressiveness noted here may represent the existence of genetic diversity, even among individuals of the same race [68, 69, 70].

The better performance of the BRS Platina cultivar can be explained by its genealogy, considering that it is a tetraploid hybrid (AAAB) developed from the cross between the Prata-Anã cultivar (AAB) and the improved diploid M53 (AA) [71, 72]. Diploid M53 was notable for not showing symptoms of Fusarium wilt in the field and for being the parent of other hybrids, such as BRS Princesa, BRS Preciosa and BRS Pacovan Ken, which are already widespread in the domestic market of Brazil because of their good agronomic and sensory characteristics [73, 74, 76]. The M53 hybrid was also characterized as resistant to tropical Foc race 4 in a resistance test that was performed in the Northern Territory (Australia) in an area naturally infested with the pathogen, with no symptoms of Fusarium wilt being

observed during the culture under high inoculum pressures [77]. This information is important because it allows assessments of a possible resistance of the BRS Platina cultivar to TR4. In partnership with the Colombian Agricultural Research Corporation (AgroSavia, Colombia), the BRS Platina cultivar is being quarantined in that country and subsequently will be challenged in the presence of Foc TR4 to confirm its putative resistance.

Although Foc TR4 has not yet been detected in Brazil, its recent introduction in Colombia and Peru further demonstrates the need for prevention of a possible introduction [11, 78]. In addition, there is much damage caused by isolates of the Foc population in Brazil even in the absence of TR4, where it was shown that the average incidence of Fusarium wilt is 11%, causing an estimated productivity loss of 1.8 t ha-1 year-1 [79]. Thus, the data presented here suggest that the BRS Platina cultivar has potential for use in breeding programs focused on resistance to Foc TR4.

4. Material and Methods

4.1. Foc isolates

Three Foc isolates from the biological collection of the Laboratory of Plant Pathology of Empresa Brasileira de Pesquisa Agropecuária – EMBRAPA, were selected for this study. Isolate 0801 was characterized as the standard for race 1 (R1), and isolate 218A is part of the vegetative compatibility group VCG 0120, characterized as subtropical race 4 (ST4) [65]. The 229A isolate was selected due to the percentage of Fusarium wilt symptoms caused in the Grand Naine cultivar in a previous study. In the mentioned study this isolate was considered virulent, defined as the ability to cause disease in a given cultivar [66].

Each isolate was grown in potato dextrose agar culture medium and subsequently multiplied in rice (20 ml of spore suspension in 500 g of autoclaved rice). After growth in this culture medium, the inoculum was adjusted to 10^6 conidia/g of rice.

4.2. Plant material

Three banana cultivars with different responses to Foc were used: the cultivar Prata-Anã (subgroup Prata; AAB), which is susceptible to Foc R1, ST4 and 229A; the cultivar Grand Naine (subgroup; Cavendish; AAA), which is resistant to R1, moderately resistant to ST4 and possibly susceptible to Foc 229A; and the cultivar BRS Platina (subgroup Prata; AAAB), which is resistant to R1 and whose response to ST4 and 229A has not yet been evaluated.

Plantlets of each cultivar grown from tissue culture were acclimated under ideal growth conditions and planted in pots with substrate consisting of pine bark (Tecnomax®) and coconut fibre (5:1; v:v), where they remained for 40 days under adequate irrigation and fertilization.

4.3. Bioassay

For inoculation in the greenhouse, the plants were removed from the pots, and the substrate was carefully removed from the roots. Subsequently, the substrate was infested with 40 g of the inoculum of each Foc isolate separately, and subsequently, the plants were replanted in the pots, placing the roots in contact with the infested substrate. The experiment was conducted in a completely randomized design, with nine treatments: 3 cultivars x 3 Foc isolates.

For each cultivar, 40 plants were inoculated, and only autoclaved rice was deposited in the pots holding control plants. Three root collection times were established for the subsequent analyses (12, 24 and 48 hours after inoculation (HAI)), and a collection was performed at 30 days after inoculation. For each treatment, three biological replicates were collected at each collection time. At the end of the experimental period, 90 days after inoculation, 10 plants from each

treatment remained for evaluation of internal symptoms of Fusarium wilt, using the methods described by Dita et al. [80]. The scores obtained in the evaluation of symptoms were converted into an internal index according to the McKinney [81] formula.

4.4. RNA extraction and cDNA synthesis

Root samples used for total RNA extraction were collected, immediately immersed in liquid nitrogen and stored in an ultrafreezer (-80 °C) until processing. Plants collected at 12, 24 and 48 hai and control plants were used for total RNA extraction using a cetyltrimethylammonium bromide (CTAB 2%) protocol previously described by Zhao et al. [82]. RNA was quantified by comparative analysis on a 1% agarose gel and by spectrophotometry with a Nanodrop ND-2000 device (Thermo Scientific, Waltham, MA, USA). The RNA samples were treated with DNase (RNase TURBOfree-Ambion), and cDNA synthesis was performed with a high-capacity RNA-to-cDNA Kit (Applied Biosystems) following the manufacturer's recommendations.

4.5. Gene expression analysis by quantitative real-time PCR

The genes listed in Table 1, Cellulose synthase A catalytic subunit 7 (CESA7), Auxin transporter-like protein 1 (ATL) and Putative Disease resistance response protein 206 (PI206), were derived from a previous study that analysed the transcriptomic profiles of the cultivars BRS Platina, Prata-Anã and Silk via RNAseq in response to infection by Foc R1. After large-scale analysis of the data obtained by RNAseq, these genes were selected because they are related to plant defence responses and shared among cultivars or exclusive to some cultivars [31]. The genes encoding transcription factor WRKY 22 (WRKY22), pathogenesis-related protein 1 (PR1), chitinase (CHI) and lipoxygenases (LOX) were derived from a study that compared infection processes and gene expression levels in a cultivar of banana (Cavendish) inoculated with Foc 1 and Foc TR4 [27].

Table 1. Primers used in gene expression analysis of the interaction of banana cultivars and *Fusarium oxysporum* f. sp. *cubense* with different levels of virulence.

ID	Gene	Description	Sequence (5'-3')	pb	Reference
GSMUA_Achr5T15720_001	CESA7	*Cellulose synthase A catalytic subunit 7*	F: GAGAATGGAGAACGGGTGCA R: CCCCTCCATGTCTCTCTCCA	108	[31]
GSMUA_Achr8T02300_001	ATL	*Auxin transporter-like protein 1*	F: GGTTCAGCTGCTCCTCCAAT R: AGAACAGCTGCAGGATCACC	161	[31]
GSMUA_Achr8T15700_001	PI206	*Putative Disease resistance response protein 206*	F: AGTACAACGGGAGCAGCTTC R: GATGAGCCTGCTGATGGTGT	128	[31]
XM_009417035.2	WRKY22	*Transcription factor WRKY 22*	F: CGTGACGTACGAAGGAGAGCA R: GGTCAACGCGAAGTCAACCA	95	[27]
XM_009417035.2	PR1	*Pathogenesis-related proteins 1*	F: AGTTATGGACGAGCTACCCG R: GTAGCTGAAGTACTTCCCCTC	77	[27]
XM_009415745.2	CHI	*Quitinase*	F: TACTGGAACTACAACTACGGAGC R: CGTTCTGCTCGAGGTACTC	82	[27]
XM_008803483.2	LOX	*Lipoxygenases*	F: ACGATGCAGACGGTATTGGAGT R: GGTACTGTCCGAAGTTGACG	94	[27]
25SMU	25S	*25S rRNA*	F: ACATTGTCAGGTGGGGAGTT R: CCTTTTGTTCCACACGAGATT	106	[83]

The real-time PCR assays were performed in the Applied Biosystems 7300 Real-Time PCR System (ABI, Foster City, CA, USA) using SYBR Green PCR mix (Ludwig Biotech) with the primers listed in Table 1. The reaction mixture included 1 µl of cDNA, 0.3 µl of each primer (RF), 5 µl of SYBR Green PCR mix and 3.4 µl of nuclease-free water for a total volume of 10 µl in each reaction. The amplification conditions of the reactions were as follows: 50 °C for 2 minutes and 95 °C for 10 minutes followed by 40 cycles of denaturation at 95 °C for 15 seconds and annealing and primer extension at 58 °C for 1 minute. The 25S gene was used as an endogenous reference gene, as previously tested [84, 45]. Each biological replicate was examined in triplicate using a relative quantification analysis and the pairwise fixed

reallocation randomization test method, where the values of the quantification cycle (Ct) were used to calculate the relative quantities by the Formula 2 (ΔΔ TC) [85, 86]. All the values are expressed as the mean ± standard deviation.

4.6. Histological and histochemical analyses

Small root fragments were collected and immediately immersed in Karnovsky's solution [87], where they remained for 48 hours; the fragments were then dehydrated in an increasing ethanol series with an interval of three hours each (30%-100%). Infiltration and blocking were performed with a Historesin kit (hydroxyethyl methacrylate, Leica Heldelberg, Germany). After historesin polymerization, histological sections (8 μm) were obtained with a Leitz 1516 microtome. The sections were mounted on histological slides that were stained with ferric chloride for three hours to detect phenolic compounds [88] and Calcofluor White dye 0.01% to detect cellulose [89]. The histological sections were analyzed and photographed under a B x S1 fluorescence microscope (Olympus Latin America Inc.).

Analysis of root clarification and staining of fungal structures was performed according to a method described Phillips and Haymann [90] with modifications. Briefly, for clarification, the roots were immersed in a 10% KOH (potassium hydroxide) solution at room temperature for 48 hours and then in a 1% HCl solution for 30 minutes, and the dye was used to stain the structures. Trypan blue in 0.05% solution (2:1:1 lactic acid: glycerol: water) was applied for 1 hour. After staining, slides were prepared, and the fragments microphotographed under a light microscope.

4.7. Analysis by scanning electron microscopy - SEM

After dehydration in an ethanol series, root samples were dried in a critical point apparatus (Leica EM CPD 030) using liquid CO2. Samples were fixed to a metallic support (stubs) with double-sided carbon adhesive tape and metallized with gold in JEOL Smart Coater equipment (DII-29010SCTR). The observations and electron micrographs were performed in a JEOL JSM-6390LV scanning electron microscope in the electron microscopy laboratory of the Gonçalo Moniz Institute, Fiocruz, Salvador-BA.

5. Conclusions

In this study, the histological, histochemical and molecular analysis of the roots of the banana cultivars BRS Platina, Grand Naine and Prata-Anã and the differences in the defence response profiles between cultivars infected by these three Foc isolates with different virulence patterns, were compared. Results showed that the CESA7, ATL, PI206, WRKY22, PR1, CHI and LOX genes as well as the increased presence of cellulose, phenolic compounds and calcium oxalate crystals, were induced in 'BRS Platina', suggesting their important roles in incompatible interactions between resistant banana cultivars and Foc ST4 and R1. However, these defence responses were suppressed or reduced mainly by an isolate with greater virulence in 'Prata-Anã' and 'Grand Naine', suggesting that these strategies are not adopted by these cultivars and that this isolate can suppress these defence responses as part of their infection strategies. Additional studies are needed to determine the functions of the genes studied and the corresponding pathways. In conclusion, our study expands the information on compatible and incompatible interactions between banana genotypes and the Foc pathogen and highlights the understanding of the response mechanism of the BRS Platina cultivar to Foc TR4 as the next phase of our research.

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CONCLUSÃO GERAL

Neste trabalho, a ferramenta de revisão sistemática foi aplicada pela primeira vez nos estudos de *Musa* spp. para resistência a murcha de Fusarium, de forma que foram sistematizados e compilados dados de 95 artigos publicados nos últimos dez anos com o tema. Com a revisão sistemática verificou-se que muitos centros de pesquisa em diferentes países se dedicam ao melhoramento genético para desenvolver cultivares resistentes a *Fusarium oxysporum* f. sp. *cubense* (Foc). Esses estudos são principalmente com as informações obtidas após o sequenciamento do genoma de *Musa* spp., principalmente pela avaliação dos dados do transcriptoma da bananeira após a infecção com Foc, para melhorar a compreensão e identificação de mecanismos genéticos, moleculares, bioquímicos ou estruturais de resistência da bananeira, de modo que já foram identificados muitos genes de resistência a murcha de Fusarium. Esses genes, no geral, são utilizados para o desenvolvimento de plantas transgênicas.

A revisão destaca como fontes de resistência a Foc raça 1 (R1) e Foc TR4 alguns diploides como *M. itinerans*, Pahang, Calcutá 4, DH Pahang e Tuu Gia, como ponto de partida para o melhoramento genético. A produção de bananas transgênicas tem sido alvo de muitos estudos e embora não tenha sido realizado o uso de ferramentas de edição de genoma, como CRISPR/Cas9, para obter resistência à murcha de Fusarium em bananeiras, trata-se de um método com perspectivas promissoras.

Os resultados discutidos contribuem para o desenvolvimento de estratégias de melhoramento baseado no germoplasma existente para resistência a murcha de Fusarium e ampliam as discussões sobre a padronização de protocolos de inoculação para expressão gênica, escalas de notas para sintomas da doença e marcadores moleculares existentes para seleção assistida, propondo esforços cooperativos para que diferentes programas de melhoramento possam cooperar em escala global, sobretudo a fim de garantir a sustentabilidade da exportação do agronegócio da banana, priorizando a produção de outras cultivares resistentes à Foc, considerando que o atual cenário de exportação de banana é baseado exclusivamente no subgrupo Cavendish, suscetível à Foc TR4.

Os dados obtidos pela análise da interação do patógeno Foc com o seu

hospedeiro *Musa* spp. em nível molecular, histológico e histoquímico demonstraram que os genes CESA7, ATL, PI206, WRKY22, PR1, CHI e LOX, bem como aumento da presença de celulose, compostos fenólicos e cristais de oxalato de cálcio, desempenham papeis importantes na resistência da cultivar BRS Platina à Foc ST4 e R1. Em comparação com as cultivares Prata-Anã e Grande Naine essas respostas de defesa foram suprimidas ou reduzidas principalmente por o isolado 229A, que apresentou maior virulência.

Assim, foi confirmado que diferenças na virulência de isolados de Foc podem contribuir para alterações na resposta de defesa da bananeira. Ademais, a continuidade dos estudos das funções de genes e vias relacionados, bem como a compreensão do mecanismo de resposta da cultivar BRS Platina à Foc TR4 são passos importantes para as próximas pesquisas.