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PROGRAMA DE PÓS-GRADUAÇÃO EM
BIOTECNOLOGIA**



WANDERLEY DIACISO DOS SANTOS OLIVEIRA

**INDUÇÃO, SELEÇÃO E CARACTERIZAÇÃO DE
VARIANTES SOMACLONAIAS DE BANANEIRA DO
SUBGRUPO CAVENDISH RESISTENTES A *Fusarium*
oxysporum f. sp. *cubense*.**

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Tese apresentada ao Programa de Pós-graduação em Biotecnologia, da
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Tese apresentada ao Programa de Pós-Graduação em Biotecnologia da Universidade Estadual de Feira de Santana, área de concentração em Biotecnologia com ênfase em Recursos Naturais da Região Nordeste, como requisito para obtenção do grau de doutor, tendo sido aprovada pelos (as) membros (as) signatários (as) abaixo.

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"Deus não é homem, para que minta, nem filho do homem, para que se arrependa. porventura diria ele, e não o faria? Ou falaria, e não o confirmaria?"
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RESUMO

A bananicultura desempenha um papel crucial na segurança alimentar de milhões de famílias em todo o mundo, mas encontra-se gravemente ameaçada pela murcha de Fusarium, causada pelo fungo *Fusarium oxysporum* f. sp. *cubense* (Foc), especialmente pela raça tropical 4 do patógeno. Atualmente, não existem métodos de controle plenamente eficazes, sendo o uso de cultivares resistentes a estratégia mais efetiva. Contudo, o melhoramento genético convencional apresenta limitações, o que tem incentivado o uso de abordagens alternativas, como a indução de variação somaclonal. Neste trabalho, exploramos a indução de variação somaclonal mediada por Thidiazuron (TDZ) como estratégia biotecnológica para obter somaclones resistentes à Foc. No Capítulo 1, avaliou-se o potencial do TDZ (1 mg/L - T1 e 2 mg/L - T2) como agente indutor em cultivares suscetíveis: 'Grande Naine' e 'Valery', visando a resistência à Foc raça 4 subtropical (isolado putativo). O tratamento T2 foi o mais eficiente, resultando em sete somaclones de 'Grande Naine' com resistência completa. A estabilidade genética, avaliada por marcadores moleculares (IRAP, REMAP e ISSR), foram predominantemente monomórfico, mas com polimorfismos notáveis em alguns somaclones do tratamento T2 de 'Grande Naine'. A análise histológica validou a resistência, indicando ausência de hifas na maioria dos somaclones resistentes. No Capítulo 2, foi realizada caracterização molecular e histoquímica de 14 somaclones de 'Grande Naine' resistentes à Foc STR4. As análises de IRAP não detectou polimorfismo na maioria dos somaclones comparados a planta controle. Após a inoculação com Foc STR4, cinco somaclones (E10, E13, E14, E15, E16) demonstraram resistência completa. Os genótipos E10, E13 e E15 ativaram significativamente genes de defesa (LOX, PIP2-3, PI206 e PTI6). O somaclone E13 destacou-se com maior deposição de calose e compostos fenólicos nas raízes, inibindo a presença fúngica. Em suma, a indução de variação somaclonal utilizando TDZ se mostrou uma abordagem eficaz para gerar novas variantes de bananeira com resistência completa à Foc. A caracterização genético-molecular e a validação das respostas de defesa destes somaclones confirmam seu potencial imediato para programas de melhoramento, visando a sustentabilidade e estabilidade da produção global de banana.

Palavras-chave: *Musa* spp; Melhoramento genético; Murcha de Fusarium; Somaclones; TDZ.

ABSTRACT

Banana cultivation plays a crucial role in ensuring food security for millions of families worldwide, yet it is severely threatened by Fusarium wilt, caused by the fungus *Fusarium oxysporum* f. sp. *cubense* (Foc), particularly by the Tropical Race 4 (TR4) of the pathogen. Currently, no fully effective control methods exist, and the use of resistant cultivars remains the most effective strategy. However, conventional breeding presents limitations, which has encouraged the adoption of alternative approaches such as the induction of somaclonal variation. In this study, we explored the induction of somaclonal variation mediated by Thidiazuron (TDZ) as a biotechnological strategy to obtain Foc-resistant somaclones. In Chapter 1, we evaluated the potential of TDZ (1 mg/L – T1 and 2 mg/L – T2) as an inductive agent in susceptible cultivars, ‘Grande Naine’ and ‘Valery’, targeting resistance to Foc subtropical race 4 (putative isolate). The T2 treatment proved most effective, resulting in seven ‘Grande Naine’ somaclones exhibiting complete resistance. Genetic stability, assessed through molecular markers (IRAP, REMAP, and ISSR), was predominantly monomorphic, although notable polymorphisms were detected in some T2 somaclones of ‘Grande Naine’. Histological analysis validated the resistance, revealing the absence of fungal hyphae in most resistant somaclones. In Chapter 2, we focused on the molecular and histochemical characterization of 14 ‘Grande Naine’ somaclones resistant to Foc STR4. IRAP analyses confirmed high genetic stability in these somaclones compared to the control plant. Following inoculation with Foc STR4, five somaclones (E10, E13, E14, E15, E16) exhibited complete resistance. Genotypes E10, E13, and E15 showed significant activation of defense-related genes (*LOX*, *PIP2-3*, *PI206*, and *PTI6*). Notably, somaclone E13 displayed enhanced deposition of callose and phenolic compounds in roots, effectively inhibiting fungal colonization. In summary, the induction of somaclonal variation using TDZ proved to be an effective approach for generating new banana variants with complete resistance to Foc. The genetic-molecular characterization and validation of defense responses in these somaclones confirm their immediate potential for integration into breeding programs aimed at ensuring the sustainability and stability of global banana production.

Keywords: *Musa* spp. Genetic improvement. Fusarium wilt. Somaclones. TDZ.

SUMÁRIO

	INTRODUÇÃO GERAL	11
	REFERÊNCIAS	13
	CAPÍTULO 1	17
1.1	INTRODUÇÃO	19
1.2	MATERIAIS E MÉTODOS	21
1.3	RESULTADOS	26
1.4	DISCUSSÃO	34
	REFERÊNCIAS	40
	CAPÍTULO 2	47
2.1	INTRODUÇÃO	49
2.2	MATERIAIS E MÉTODOS	50
2.3	RESULTADOS	54
2.4	DISCUSSÃO	61
	REFERÊNCIAS	65
	CONSIDERAÇÕES FINAIS	68

INTRODUÇÃO GERAL

As bananas (*Musa* spp.) são originárias do sul e sudeste da Ásia, bem como de regiões do Oceano Pacífico. Após sua domesticação e a expansão do seu cultivo para regiões tropicais e subtropicais, elas se tornaram uma das principais commodities agrícolas e um dos alimentos básicos mais importantes do mundo (Heslop-Harrison; Schwarzacher, 2007; Bebbler, 2022).

Apesar da sua ampla adaptação geográfica, a bananicultura enfrenta sérios desafios decorrentes das mudanças climáticas (Abigaba et al., 2024), como estresse hídrico e baixas temperaturas (El-Mahdy et al., 2018; Amnan et al., 2021), além da incidência de pragas e doenças causadas por fungos, nematoides e outros agentes fitopatogênicos que agravam severamente o desenvolvimento e a produtividade desta cultura (Sousa et al., 2024; Musabyemungu et al., 2025). Entre as doenças mais devastadoras estão a Sigatoka-negra, causada pelo fungo *Pseudocercospora fijiensis* (Soares et al., 2021) e a murcha de Fusarium, provocada por *Fusarium oxysporum* f. sp. *cubense* (Foc), patógeno habitante do solo que representa uma ameaça persistente com ampla distribuição (Rocha et al., 2021).

A forma *specialis* (f. sp.) *cubense*, patogênica para *Musa* spp. (Snyder; Hansen, 1940; Chen et al., 2019), apresenta três raças principais, a saber, a raça 1, 2, e 4. Entre essas, a raça 4 é a mais preocupante por afetar todas as cultivares suscetíveis às raças anteriores, incluindo o subgrupo ‘Cavendish’ (AAA) (Waite; Stover, 1960; Ploetz, 2006). Essa raça é dividida em subtropical (STR4), capaz de afetar Cavendish em áreas sujeitas a baixas temperaturas e outros estresses predisponentes, e tropical (TR4), altamente destrutível tanto em ambientes tropicais quanto subtropicais (Batista et al., 2022).

Desde a sua identificação no Sudeste Asiático em 1990, a TR4 espalhou-se por diversos países da Ásia, Oceania, África e, mais recentemente, chegou a América do Sul, com casos confirmados na Colômbia, Peru e Venezuela, colocando em risco a produção de banana em toda região (Chen et al., 2019; García-Batidas et al., 2019; Acuña et al., 2022; Herrera et al., 2023). Esse avanço geográfico evidencia o elevado potencial destrutivo da doença e reforça a necessidade urgente de medidas eficazes de manejo.

Entretanto, diversas estratégias já testadas, como métodos culturais, químicos e biológicos, têm se mostrado ineficazes para o controle duradouro da murcha de Fusarium em solos infestados (Dita et al., 2018). Nesse contexto, a resistência genética tem se consolidado como a alternativa mais promissora para o manejo a longo prazo. Até recentemente, contudo, não havia cultivares comerciais de banana resistentes a Foc TR4 (Santana et al., 2024). Avanços

recentes da Empresa Brasileira de Pesquisa Agropecuária (Embrapa) indicam uma mudança nesse panorama. Duas cultivares desenvolvidas pela instituição, ‘BRS Princesa’ e ‘BRS Platina’, foram confirmadas como resistentes a Foc TR4, representando um marco significativo no combate à doença e abrindo novas perspectivas para o cultivo da bananicultura em nível global (Embrapa, 2025).

Apesar desse progresso relevante, o melhoramento clássico da bananeira ainda enfrenta obstáculos substanciais. A poliploidia, heterozigosidade e esterilidade das cultivares comestíveis dificultam a obtenção de genótipos que aliem resistência à doença com características agronômicas, organolépticas e comerciais desejáveis (Ganapathi et al., 2015; Silva et al., 2022). Diante dessas limitações, abordagens biotecnológicas emergem como alternativas complementares e promissoras. Estratégias como a indução de mutações, a hibridação somática, a indução da variação somaclonal e a edição gênica, vêm sendo investigadas com o objetivo de acelerar o desenvolvimento de novas variedades de bananeira resistentes à murcha de *Fusarium*, ampliando as possibilidades de manejo sustentável da doença (Rebouças et al., 2021; Tripathi et al., 2024; Costa et al., 2025).

A variação somaclonal é definida com variações ocorridas em plantas durante o processo de cultura de células ou tecidos (Larkin; Scowkraft, 1981), envolvendo alterações no fluxo genômico que podem ser genética ou associadas a fatores epigenéticos, que tornam esse processo ainda mais complexo (Krishna et al., 2016). Esta técnica apresenta um elevado potencial para o melhoramento de culturas propagadas vegetativamente, uma vez que permite gerar variabilidade genética em um curto intervalo de tempo, e sem a necessidade de tecnologias avançadas (Ferreira et al., 2023). A eficiência na seleção de variantes somaclonais desejáveis podem ser aumentadas mediante a aplicação de pressão de seleção, tanto em condições *in vitro* quanto em campo (Rebouças et al., 2021; Ferreira et al., 2024).

Embora muitas variantes somaclonais apresentem características indesejáveis, algumas tem demonstrado potencial no melhoramento genético. Isso foi observado em Taiwan, com a seleção do somaclone resistente à Foc TR4, GCTCV-119 (Hwang; Ko, 2004). No Brasil, Ferreira et al. (2020) selecionaram dois somaclones da cultivar Prata Anã, resistente à Foc raça 1. Rebouças et al. (2021) também contribuíram com a seleção de dois somaclones da cultivar Grande Naine com resistência à Foc STR4 e mais recentemente, Ferreira et al. (2024) identificaram 13 somaclones da cultivar Prata Catarina com resistência à Foc STR4.

O cultivo *in vitro* podem aumentar a taxa de mutações, influenciadas sobretudo pela relação nos níveis relativos de auxina e citocinina e pelo número de subcultivos realizados

(Bairu et al., 2011). Essas condições também podem induzir a ativação de elementos transponíveis, contribuindo para o surgimento de variações somaclonais (Krishna et al., 2016). A detecção e caracterização dessas variantes podem ser realizadas por meio de uma gama de ferramentas, aplicadas de forma isolada ou combinada, incluindo análise morfológica, histoquímica, citogenética, bioquímica, bem como molecular/expressão gênica (Krishna et al., 2016; Ferreira et al., 2024).

Diante desse contexto, o primeiro capítulo deste trabalho teve como objetivos: (i) avaliar o potencial do TDZ como indutor de variação somaclonal em cultivares de bananeira do subgrupo Cavendish suscetíveis à Foc raça 4; e (ii) identificar somaclones resistentes com potencial para aplicação em programas de melhoramento genético. O segundo capítulo, por sua vez, buscou: (i) detectar a variabilidade genética em somaclones da cultivar Grande Naine por meio de marcadores IRAP; (ii) analisar a expressão de genes relacionados à defesa e à deposição de compostos pós-formados em resposta à inoculação com Foc STR4; e (iii) identificar, com base nas análises supracitadas, os somaclones mais promissores para utilização em programas de melhoramento. Os resultados obtidos contribuem para o avanço no desenvolvimento de cultivares resistentes à murcha de *Fusarium* e ampliam o entendimento dos mecanismos envolvidos na interação entre somaclones de bananeira e o patógeno.

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CAPÍTULO 1

**Potencial do Thidiazuron como agente indutor de variação somaclonal em bananeira
Cavendish resistente a *Fusarium oxysporum* f. sp. *cubense***

Artigo - Potential of thidiazuron for inducing somaclonal variation in Cavendish banana resistant to *Fusarium oxysporum* f. sp. *Cubense*, submetido na revista científica Plant Breeding.

Potential of thidiazuron for inducing somaclonal variation in Cavendish banana resistant to *Fusarium oxysporum* f. sp. *Cubense*

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Abstract

Banana (*Musa* spp.) is one of the most important agricultural crops worldwide and plays a fundamental role in the food security of millions of people. Fusarium wilt, caused by the soil-borne fungus *Fusarium oxysporum* f. sp. *cubense* (Foc), has a devastating effect on banana crops. The development and use of resistant cultivars represent the most effective strategies for controlling and eradicating this pathogen in infested areas. In this context, the induction of somaclonal variation is a promising tool for overcoming the limitations of conventional breeding by offering new possibilities for obtaining resistant and productive genotypes. In this study, we evaluated the potential of Thidiazuron (TDZ) as an agent for inducing somaclonal variation in banana cultivars susceptible to Foc subtropical race 4 – Foc STR4 (Grand Naine and Valery) and identified resistant somaclones with the potential for use in genetic improvement programs. Two concentrations of TDZ were tested for the induction of

somaclonal variation: 1 mg/L (T1) and 2 mg/L (T2). Treatment T2 was more efficient and generated a greater number of somaclones highly resistant to the pathogen. In the Grand Naine cultivar, four and seven somaclones with complete resistance were obtained in treatment T1s and T2, respectively. In the Valey cultivar, only one resistant somaclone was obtained in treatment T1. Molecular analyses (IRAP, REMAP, and ISSR) indicated a predominance of genetic stability, although some polymorphic bands were observed, especially in Grand Naine - T2. Histological analysis only identified hyphae in the control plants and some somaclones of Grand Naine - T2. These results confirm that biotechnological approaches serve as a complement to conventional breeding and have the potential to promote the development of banana cultivars resistant to Fusarium wilt and thus improve the stability of banana crops.

Keywords: Genetic improvement-Fusarium wilt-*Musa* spp-TDZ-Somaclones.

1 introduction

Banana (*Musa* spp.) is among the world's most important agricultural crops, with global production estimated at approximately 139 million tons in 2023 (FAO, 2025). It is considered essential for the economic and social development of many developing countries based on its important role in the food security of millions of people in these regions (Munhoz et al., 2024). Brazil stands out as one of the main banana producers in Latin America, with an annual production of approximately 6.8 tons. In South America, it is second only to Ecuador, which leads with approximately 7.1 million tons. Globally, the largest producers are India, China, and Indonesia (FAO, 2025). Most of the production consists of bananas of the Cavendish subgroup, which dominates the international market (Martínez et al., 2023).

Edible bananas originated from Asia and are now popular worldwide. Most are triploids which were formed by interspecific hybridization between the diploid parent species *Musa acuminata* Colla (AA) and *Musa balbisiana* Colla (BB) (Kannan et al., 2022). Commercial cultivars are generally seedless and produce fruit by parthenocarpy. They are propagated vegetatively through cuttings obtained from the rhizome or by tissue culture techniques. Although in vitro propagation has facilitated the cultivation of genetically uniform and pathogen-free banana trees, the limited genetic diversity of the varieties increases their vulnerability to abiotic stresses, such as extreme temperatures, and biotic stresses, such as pests and diseases (Hou et al., 2022; Bebber, 2022; Sousa et al., 2024).

Among the threats that limit banana cultivation, Fusarium wilt, caused by the soil-borne fungus *Fusarium oxysporum* f. sp. *cubense* (Foc), is one of the most devastating agricultural

diseases (Dita et al., 2018). The first major banana-related event caused by Foc occurred in the last century with the decimation of thousands of Gros Michel (AAA) plantations in Central America. To circumvent the loss caused by Fusarium wilt, the international banana export trade replaced the Gros Michel cultivar with resistant varieties of the Cavendish subgroup, which currently account for more than 40% of global banana production (Ploetz, 2015).

As a soil-borne fungus, Foc usually infects the root first and then colonizes the vascular tissue, specifically the xylem (Li et al., 2017). As it colonizes the xylem, the pathogen produces spores and mycelial structures that clog the conducting vessels, thus interrupting the flow of water and nutrients (Pegg et al., 2019). This leads to visible symptoms such as yellowing and wilting of leaves and necrosis of the vascular system and eventually causes plant death. Outside of the plant, Foc survives in the form of chlamydospores, which are resistant structures that allow the fungus to persist in the soil or in organic debris for decades until it finds a new host (Wang et al., 2022). Consequently, the incidence of the disease varies depending on the cultivar, environment, and inoculum level, although it can extend to total crop loss in heavily infested fields (Chen et al., 2019).

Foc has three distinct physiological races that affect different banana cultivars, referred to as races 1, 2, and 4. Race 4 is the most virulent and infects cultivars of the Cavendish subgroup, which is resistant to race 1, and cultivars susceptible to races 1 and 2. Race 4 is subdivided into Tropical Race 4 (TR4) and Subtropical Race 4 (STR4). STR4 causes disease in Cavendish plants in subtropical regions under specific environmental stress conditions, such as low temperatures. However, TR4 is highly virulent and can compromise banana cultivation under any circumstances. This race has caused significant losses in crops in Asia, the Middle East, Africa, and, more recently, Latin America (Ploetz, 2015; Pegg et al., 2019).

A detailed understanding of Foc is essential for implementing Fusarium wilt control measures because effective methods of eradicating the pathogen in infested soils have not been developed to date (Ploetz, 2015; Santana et al., 2024). The use of resistant varieties has been identified as the most efficient strategy for managing the disease (Dita et al., 2018), as confirmed in the Cavendish subgroup, in which resistance to Foc race 1 has made it the main group of cultivars used for global export. However, the susceptibility of these cultivars to Foc TR4 is a critical challenge for global banana production, and urgent solutions are required to mitigate the impacts of Foc spread (Ploetz, 2005; Rocha et al., 2021).

Although natural hybridization is widely used in other crops, this approach is limited in edible bananas due to their sterility. Thus, alternative strategies such as somaclonal variation

(Rebouças et al., 2021), mutation induction (Hou et al., 2022), and CRISPR gene editing (Ntui et al., 2020) are being explored with the aim of developing Foc-resistant varieties.

Somaclonal variation is common in tissue cultures and defined as genetic or epigenetic changes that occur spontaneously or are induced in plants grown in vitro, and it is influenced by factors such as the number of subcultures and concentration of growth regulators added to the medium (Krishna et al., 2016). Although undesirable characteristics may emerge, somaclonal variation has been established as a promising strategy in genetic improvement, with reports of its use in several crops (Ferreira et al., 2023).

Among the main plant growth regulators used to induce somaclonal variation, Thidiazuron (TDZ) has attracted interest in recent decades, especially in banana cultivation (Rebouças et al., 2021; Ferreira et al., 2024). This regulator belongs to the phenylurea class and has high cytokinin activity (Dewir et al., 2018). Its effectiveness is associated with the ability to modulate endogenous hormonal pathways and thus stimulate cell dedifferentiation, callus formation, and shoot proliferation. These processes increase the probability of spontaneous mutations or genetic rearrangements and the frequency of somaclonal variation events, which in turn result in new genetic combinations of agronomic interest (Smulders and Klerk, 2011; Wang et al., 2025).

Despite the potential of TDZ, its use in generating somaclonal variants resistant to *Fusarium* wilt remains largely unexplored. Thus, the objectives of the present study were to (i) evaluate the ability of TDZ to induce somaclonal variation in banana cultivars susceptible to Foc race STR4 and (ii) identify potentially resistant somaclones for use in agriculture and breeding programs. The obtained results contribute to the consolidation of biotechnological strategies complementary to conventional methods, with the aim of developing resistant cultivars and strengthening preventive actions in the management of Foc in banana crops worldwide.

2 Material and Methods

2.1 Plant Material

In the present study, 100 micropropagated plants of cultivars of the Cavendish subgroup, namely, Grand Naine and Valery, were used to induce somaclonal variation through tissue culture via direct organogenesis. The explants were grown in vitro in MS medium (Murashige and Skoog, 1962) supplemented with indoleacetic acid (IAA) at a concentration of 1.6 mg/L,

adenine hemisulfate at 80 mg/L, and TDZ at different concentrations, with 1 mg/L in treatment 1 (T1) and 2 mg/L in treatment 2 (T2). The plants were subcultured five times throughout the experiment at intervals of 40 to 60 days for each subculture.

The controls were explants from both genotypes that were subjected to the same protocol and the number of subcultures differed only in the use of 6-benzylaminopurine (BAP) as a growth regulator during the plant multiplication phase. This procedure allowed for comparisons between the treatments with TDZ and the treatments with BAP, which is commonly used for in vitro multiplication of banana plants.

At the end of the subculture period, the plants were acclimatized and transferred to a greenhouse. In this controlled environment, the plants were transplanted into beds (10 m x 1 m) with soil previously infested with the CNPMF 229 isolate of Foc.

2.2 Preparation of the Inoculum

Isolate CNPMF 229 was obtained from the biological collection of the Plant Pathology Laboratory at Embrapa Cassava and Fruit Crops and selected due to its high virulence and ability to aggressively infect banana varieties, including those of the Cavendish subgroup. Under controlled inoculation conditions, isolate CNPMF 229 had virulence characteristics similar to those of isolate CNPMF 218, which is classified as subtropical race 4 (STR4) and originated from the same region as CNPMF 229, as reported by Rocha et al. (2022).

Isolate CNPMF 229 was initially grown on potato dextrose agar (PDA) medium in an incubation chamber at a constant temperature of 28 °C and a 12-hour photoperiod. For the mass multiplication stage of Foc, the isolate was transferred to an autoclaved rice substrate medium. Approximately 500 g of rice was infested with 10 mL of spore suspension that had been prepared in an aqueous solution.

After complete colonization of the substrate by the fungus, the inoculum was serially diluted. Aliquots were taken from each dilution and transferred to Petri dishes containing BDA culture medium. The dishes were then incubated for 48 h and colony-forming units were counted using a stereomicroscope with a colony counter. The final concentration of the inoculum was adjusted to 10⁶ conidia per gram of rice. On average, 40 kg of inoculum was produced per bed.

2.3 Evaluation of Resistance in the Greenhouse

For the first evaluation of resistance to *Fusarium* wilt, 600 somaclones each of the Grand Naine and Valery cultivars were used. Additionally, 100 control plants of each genotype were included in the trial. After 120 days, internal symptoms were evaluated according to the method of Dita et al. (2014) with modifications. Briefly, the original scale of 1 to 5 was replaced by a scale of 0 to 4, with 0 = absence of symptoms, 1 = initial discoloration of the rhizome, 2 = mild discoloration of the rhizome, 3 = necrosis in most of the internal tissues of the rhizome, and 4 = totally necrotic rhizome. The scores were transformed into a disease intensity index (DI) using McKinney's formula (1923).

To analyze the DI of the treatments in the first evaluation, the disease scores assigned to the 600 somaclones of each cultivar were organized into 10 replicates of 60 plants each. The allocation of somaclones to each repetition was performed randomly using Excel software. For the control group, which consisted of 100 plants, the scores were divided into 10 replicates of 10 plants each.

During the evaluation of the first test, the somaclones that did not show symptoms were reintroduced in vitro and multiplied in MS medium supplemented with BAP, with the aim of generating clones available for a second resistance evaluation. At this stage, nine replicates of each somaclone as well as the control plants were transplanted to beds previously infested with the same *Foc* isolate in a completely randomized design. In addition, plants of the Silk cultivar, known for its high susceptibility to the pathogen, were included as a susceptibility standard. Internal symptoms were evaluated 120 days after planting using the aforementioned scale.

In the second evaluation, the scores for the nine clones of each somaclone and control plants were distributed in three replicates of three plants each, which were randomly allocated using Excel. Based on the analysis of internal symptoms and the mean DI values of the three replicates, the following disease categories were established: highly resistant (HR), including plants without symptoms of *Fusarium* wilt; resistant (R), including plants with mild spots around the xylem; moderately resistant (MR), including plants with slight discoloration of the rhizome; susceptible (S), including plants with extensive discoloration of the rhizome; and highly susceptible (HS), including plants with total discoloration of the rhizome.

Statistical analysis of the two assessments was performed using analysis of variance (ANOVA) to determine the statistical differences between DI values (5% significance level).

The means obtained in the first and second resistance assessments were compared using the Tukey and Scott-Knott tests, respectively. The results of the first and second assessments were presented graphically in bar charts and boxplots, respectively, to highlight DI variations between the treatments. All analyses were performed using R software (version 4.4.1, 2024).

2.4 Genomic DNA Extraction

Young leaves from highly resistant banana somaclones selected in the second assessment and control plants were collected and sent to the Molecular Biology Laboratory at Embrapa Cassava and Fruit Crops. DNA extraction was performed using the CTAB protocol, which was modified as described by Ferreira et al. (2019). Next, DNA quantification and quality verification were performed on 1% agarose gel stained with GelRed[®] (Biotium, Inc., 46117 Landing Parkway, Fremont, CA, USA). DNA electrophoresis was run at 80 V for 1 h. Subsequently, DNA visualization was performed using a UV transilluminator.

2.5 IRAP and REMAP Analysis

For the inter-retrotransposon amplified polymorphism technique (PCR-IRAP), long terminal repeat (LTR) primers from Teo et al. (2005) and Baumel et al. (2002) were used (Table 1).

TABLE 1. Inter-retrotransposon amplified polymorphism (IRAP) primers used for the genetic characterization of somaclones from the Grand Naine and Valery cultivars resistant to *Fusarium oxysporum* f. sp. *cubense*.

Primers	Sequence (5'-3')								Reference			
5'LTR2	5'-ATC ATT GCC TCT AGG GCA TAA TTC-3'								Teo et al. (2005)			
3'LTR	5'-TGT TTC CCA TGC GAC GTT CCC CAA CA-3'								Teo et al. (2005)			
Nikita	5'-CGC ATT TGT TCA AGC CTA AAC C-3'								Teo et al. (2005)			
Sukkula	5'-GAT AGG GTC GCA TCT TGG GCG TGA C-3'								Teo et al. (2005)			
LTR6149	5'-CTC GCT CGC CCA CTA CAT CAA CCG CGT TTA TT-3'								Teo et al. (2005)			
LTR6150	5'-CTG GTT CGG CCC ATG TCT ATG TAT CCA CAC ATG TA-3'								Teo et al. (2005)			
C0947	5'-TTG TTC ACC CAC CGT CTA CTT GC-3'								Baumel et al. (2002)			
C0945	5'-GCA AGC TTC CGT TTC CGC-3'								Baumel et al. (2002)			
Combinations of IRAP primers and their respective annealing temperatures (At)												
Prime combination	CO94 + 3'LT	CO94 + Nikit	LTR61 + LTR61	5'LT + CO94	CO94 + LTR61	3'LT + Nikit	CO94 + Niki	3'LTR + LTR61	LTR61 + Nikita	3'LT + 5'LT	Nikit + Sukk	LTR61 + 5'LTR
At	42°C	42°C	42°C	42°C	46°C	46°C	46°C	46°C	46°C	46°C	46°C	46°C

The primers used in the retrotransposon-microsatellite amplified polymorphism (PCR-REMAP) technique were obtained from the combination of an LTR retrotransposon primer and single simple repeat (SSR) microsatellite primers (Kalendar et al., 1999) (Table 2).

TABLE 2. REMAP primers used for the genetic characterization of somaclones from the Grand Naine and Valery cultivars resistant to *Fusarium oxysporum* f. sp. *cubense*.

Primers	Sequence (5'-3')	Reference
SSR		
8081	(GA)9C	Kanledar et al. (1999)
8082	(CT)9G	Kanledar et al. (1999)
8386	(GTG)7C	Kanledar et al. (1999)
8387	(CA)10G	Kanledar et al. (1999)
8565	GT(CAC)7	Kanledar et al. (1999)
LTR		
LTR7286 (R)	GGA ATT CAT AGG ATG GAT AAT AAA CGA TTA TC	Kanledar et al. (1999)
Combinations of REMAP primers and their respective annealing temperatures (At)		
Prime combination	LTR7286 +	LTR7286 +
	8081	8082
At	54°C	54°C
		8387
		8386
		8565
		58°C
		58°C

The PCR-IRAP and REMAP amplification reactions contained 4 µL of template DNA (10 ng), 0.75 µL of MgCl₂ (50 mM), 1.5 µL of 10 x PCR buffer, 1.2 µL of dNTP (2.5 mM), 1.5 µL of each paired primer (10 mM), 0.3 µL of commercial Taq DNA polymerase (5 U µL⁻¹), and nuclease-free water to a final volume of 15 µL per sample. The amplifications were performed in a Veriti[®] 96-well Thermal Cycler (Applied Biosystems) using the following amplification program: initial step at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, annealing temperature (Tables 1 and 2, respectively) for 60 s, and 72 °C for 45 s; final extension at 72 °C for 5 min, and hold at 14 °C ∞. After amplification, all PCR-IRAP reaction products were stained with 3 µL of running buffer solution containing GelRed[®]. The amplified fragments were also stained with GelRed[®] and separated by electrophoresis in 2% agarose gel at 80 v for approximately 3 h. Gel electrophoresis real-time images were captured using the Locus L-PIX EX 25x30 image capture system.

2.6 ISSR Analysis

For the inter-simple sequence (PCR-ISSR) technique, the primers were obtained from the Molecular Biology Laboratory of Embrapa Cassava and Fruit Crops (Table 3).

TABLE 3. ISSR primers used for the genetic characterization of somaclones from the Grand Naine and Valery cultivars resistant to *Fusarium oxysporum* f. sp. *cubense*.

Primer	Name	Sequence	At (°C)
ISSR 92	TriGAC 3'RC	GACGACGACGACGACRC	45
ISSR 72	TriTCC 3'RC	TCCTCCTCCTCCTCCRC	45
ISSR 95	TriGTT 3'RC	GTTGTTGTTGTTGTTRC	45
ISSR 29	TriCAC3'RC	CACCACCACCACCACRC	45

For the inter-simple sequence repeat (ISSR) amplification, the reaction contained 4.0 µL of DNA (10 ng µL⁻¹), 3.5 µL of primer (2 mM) (Table 3), 2.5 µL of MgCl₂ (25 mM), 2.5 µL 10× PCR buffer, 2.0 µL dNTP (2.5 mM), 0.2 µL commercial Taq DNA polymerase (5 U·µL⁻¹), and nuclease-free water to a total volume of 25 µL. The amplifications were performed in a Veriti 96-well (0.2 mL) Thermal Cycler (Applied Biosystems) using the following program: initial step at 94 °C for 3 min; followed by 39 cycles at 94 °C for 40 s, annealing temperature (Ta) (Table 3) for 40 s, and extension at 72 °C for 60 s; and final extension at 72 °C for 5 min.

2.7 Root Clearing Analysis

Root specimens from highly resistant somaclones were collected 120 days after inoculation (DAI). The samples were immediately immersed in modified Karnovsky solution (Karnovsky, 1965) (1% glutaraldehyde, 4% formaldehyde, 0.1 M CaCl₂, and 0.2 M sodium cacodylate buffer, pH 7.2). Forty-eight hours after fixation, the root specimens underwent a dehydration process in a series of increasing ethanol concentrations from 30% to 70%, with 3-hour intervals between each step.

The analyses of root clearing and fungal structure staining were conducted according to the method described by Phillips and Hayman (1970). The roots were first immersed in a 10% potassium hydroxide (KOH) solution at room temperature for 48 h and then in a 1% HCl solution for 5 min. Subsequently, the samples were stained with 0.05% Trypan blue solution (prepared in lactic acid/glycerol/water in a 2:1:1 ratio) for 1 h. After staining, the samples were mounted on slides and the root specimens were microphotographed using a light microscope (Olympus Latin America, Miami, FL, USA).

3 Results

3.1 First Resistance Assessment in the Greenhouse

The first evaluation of the plants in the greenhouse showed significant differences in the resistance response of the somaclones of both genotypes to Foc compared to the control plants.

One hundred and twenty days after planting, most of the somaclones exhibited low internal symptom indices and a predominance of scores 1 and 2 on the symptom scale, while the control plants had a higher frequency of scores 2 and 3 (Figure 1).

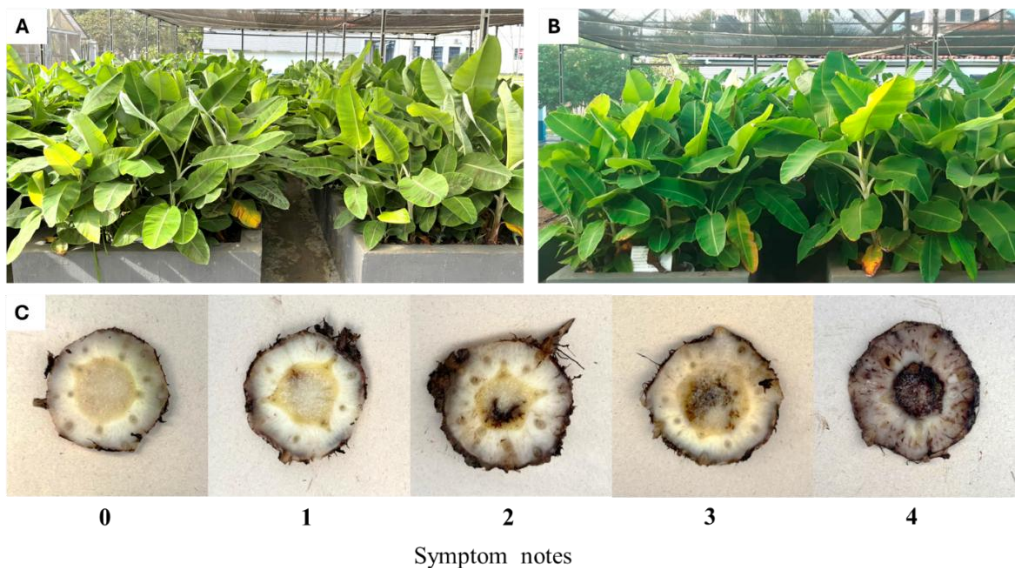


FIGURE 1. Assessment of somaclones in the greenhouse. (A) Somaclones of the Grand Naine cultivar 120 days after planting. (B) Somaclones of the Valery cultivar 120 days after planting. (C) Internal symptoms of Fusarium wilt in rhizomes of banana plants susceptible to Foc 120 days after planting.

In Grand Naine, somaclonal variants showed significant changes compared to the control treatment. In T1, 14 somaclones did not show the presence of internal symptoms (score 0), 519 plants exhibited rhizomes with initial discoloration (score 1), 64 plants showed partial discoloration of the rhizomes (score 2), 3 plants showed necrosis in the rhizomes in most of the internal tissues (score 3), and 0 plants showed complete rhizome necrosis (score 4). In treatment T2, 25 plants had a score of 0, 494 plants had a score of 1, 72 had a score of 2, 9 had a score of 3, and 0 had a score of 4. In the control plants, 0 plants had a score of 0, 9 plants had a score of 1, 53 had a score of 2, 42 had a score of 3, and 3 had a score of 4 (Figure 2A).

The mean DI differed significantly between the treatments and control. However, treatments T1 and T2 did not differ significantly, with both showing significantly lower indices than the control, with mean values close to 28%. In contrast, the control reached a mean DI above 63%. These results demonstrate that the use of TDZ during in vitro multiplication may be associated with a reduction in plant susceptibility to Foc (Figure 2B).

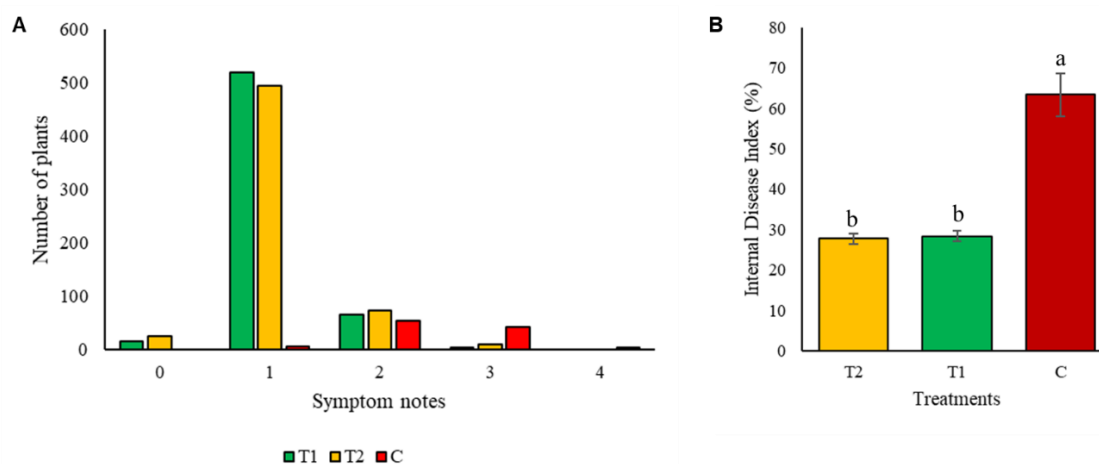


FIGURE 2. Internal symptoms of Fusarium wilt in banana somaclones of the Grand Naine cultivar (AAA) assessed in a greenhouse. (A) Bar chart showing the number of plants with each degree of symptoms according to the classification scale ranging from 0 to 4. (B) Bar chart showing the internal disease symptom index values (ID%). T1: TDZ dose of 1 mg/L; T2: TDZ dose of 2 mg/L. Treatments marked with the same letters did not differ significantly according to Tukey's test ($p>0.05$).

In the Valery cultivar, symptom distribution differed between the treatments. In treatment T1, 8 plants had a score of 0, 525 plants had a score of 1, 58 plants had a score of 2, 9 plants had a score of 3, and 0 plants had a score of 4. In treatment T2, 9 plants had a score of 0, 40 plants had a score of 1, 159 had a score of 2, 26 plants had a score of 3, and 5 plants had a score of 4. In the controls, 0 plants had a score of 0, 9 plants had a score of 1, 46 had a score of 2, 49 had a score of 3, and 5 had a score of 4 (Figure 3A).

With regard to the DI, statistical differences were observed between all treatments, especially between T1 and T2. The control treatment had a high DI, with mean values above 66%. In contrast, treatments T1 and T2 showed substantially reduced indices, with T2 and T1 having DIs of 33% and 27%, respectively. Although both treatments contributed to a significant reduction in disease severity, T1 was significantly more effective than T2 (Figure 3B).

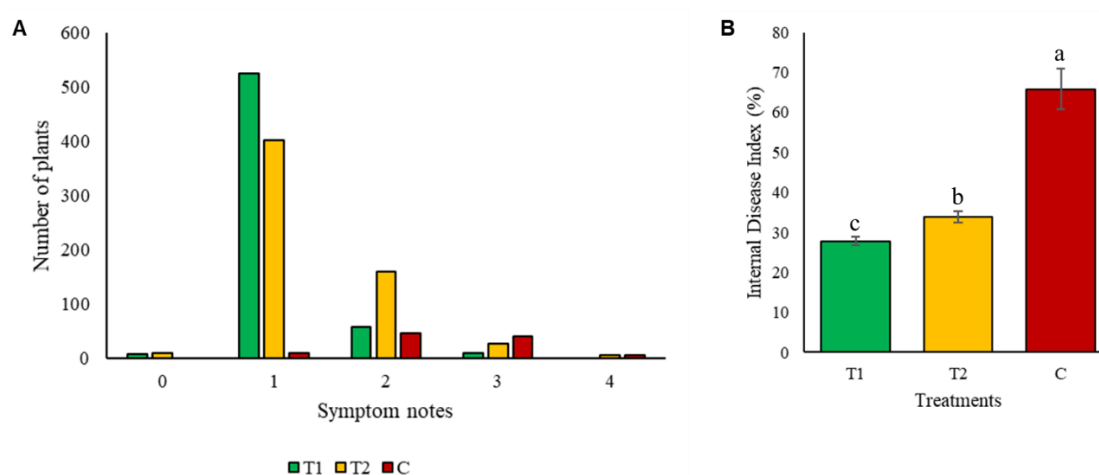


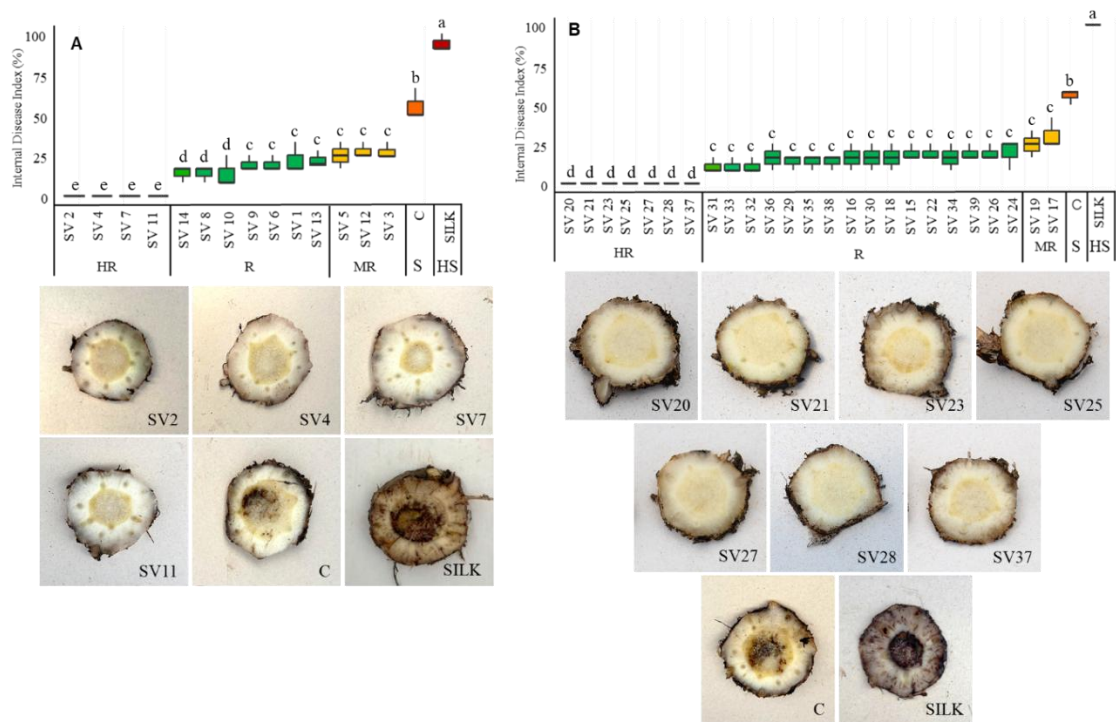
FIGURE 3. Internal symptoms of Fusarium wilt in banana somaclones of the Valery cultivar (AAA) assessed in the greenhouse. (A) Bar chart showing the number of plants with each degree of symptoms according to the classification scale, ranging from 0 to 4. (B) Bar chart showing internal disease symptom indices (ID%). T1: TDZ dose of 1 mg/L; T2: TDZ dose of 2 mg/L. Treatments indicated with the same letters did not differ significantly according to Tukey's test ($p>0.05$).

3.2 Second Resistance Assessment in the Greenhouse

In the second resistance test, nine clones of each somaclonal variant selected in the first trial were evaluated for resistance to Foc. In Grand Naine, a clear distinction was observed between the response of somaclones from treatments T1 and T2 and the control plants. In treatment T1, somaclones SV2, SV4, SV7, and SV11 showed complete resistance, with no symptoms of Fusarium wilt in the nine replicates. In treatment T2, the same level of resistance was observed in somaclones SV20, SV21, SV23, SV25, SV27, SV28, and SV37 (Figure 4).

Another group of resistant somaclones was identified that exhibited spots only around the xylem, with a greater number in T2 than T1: SV14, SV8, SV10, SV9, SV6, SV1, and SV13 in T1; and SV31, SV33, SV32, SV36, SV29, SV35, SV38, SV16, SV30, SV18, SV15, SV22, SV34, SV39, SV26, and SV24 in T2 (Figure 4).

An additional group was characterized as moderately resistant and showed slight discoloration of the rhizome. This group included somaclones SV5, SV12, and SV3 in T1 and SV19 and SV17 in T2. In contrast, the control plants in both treatments were classified as susceptible, with extensive discoloration of the rhizome. The Silk cultivar, used as the susceptibility standard, showed severe symptoms and was classified as highly susceptible to the pathogen (Figure 4).



341

342 **FIGURE 4.** Boxplot of internal disease symptom index values (ID%) in Grand Naine
343 somaclones challenged with isolate CNPMF 229 of *Fusarium oxysporum* f. sp. *cubense*. (A)
344 Somaclones generated in MS culture medium supplemented with 1 mg/L TDZ. (B)
345 Somaclones generated in culture medium supplemented with 2 mg/L TDZ. HR (highly
346 resistant), R (resistant), MR (moderately resistant), S (susceptible), HS (highly susceptible),
347 C (control plant). SILK (highly susceptible control, Apple). Treatments marked with the same
348 letters do not differ significantly according to the Scott-Knott test ($p>0.05$).

349

350 In the evaluation of the Valery cultivar, only somaclone SV1 showed complete resistance to
351 the pathogen and no symptoms of Fusarium wilt. The other identified somaclones from both
352 treatments exhibited similar behavior and were classified as resistant. This group included
353 somaclones SV5, SV3, SV7, SV8, SV4, SV6, and SV2 in T1 and somaclones SV16, SV14,
354 SV10, SV12, SV17, SV11, SV9, SV13, and SV15 in T2. The control plants of Valery
355 developed symptoms in most of the rhizome and were characterized as susceptible to Foc.
356 Similarly, the Silk cultivar exhibited intense necrosis and rapid progression of symptoms,
357 confirming its highly susceptible profile to the pathogen (Figure 5).

358

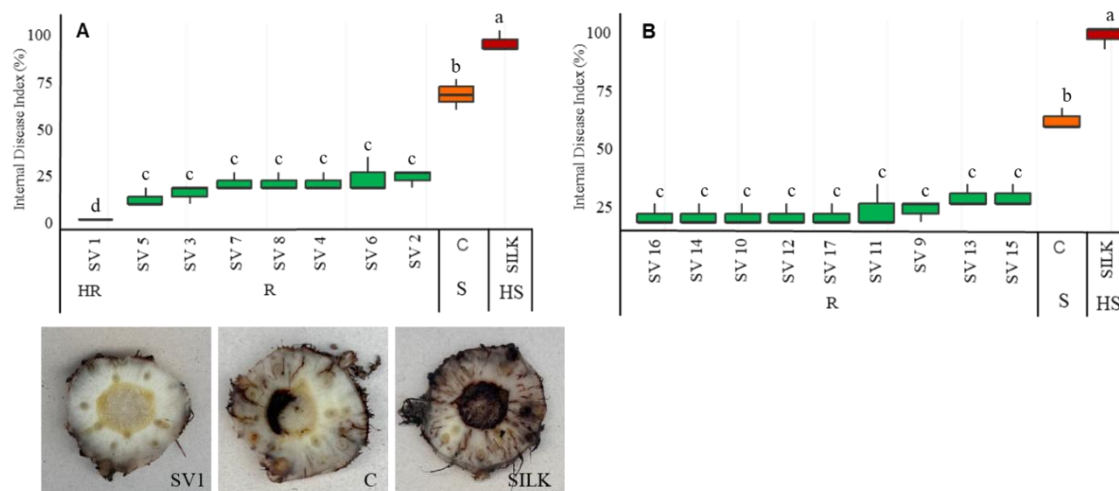
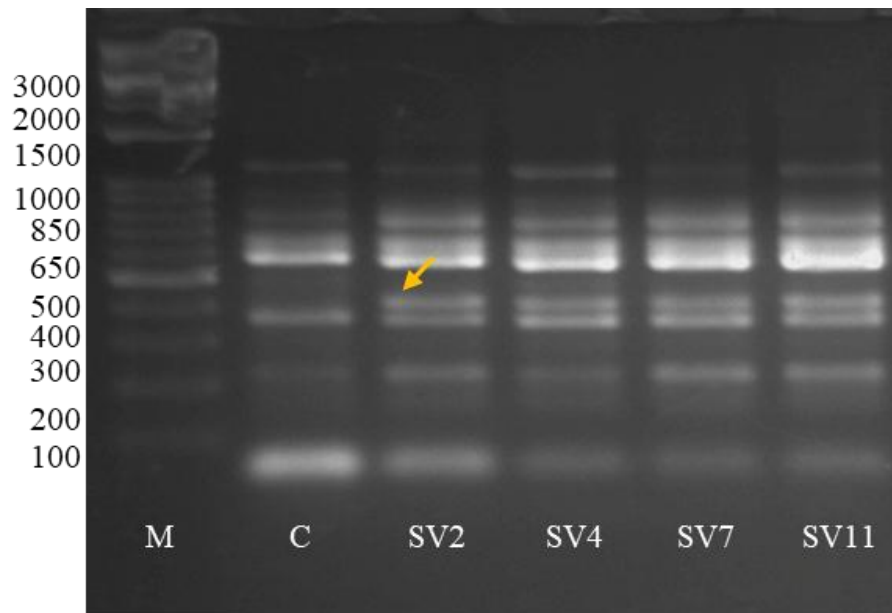


FIGURE 5. Boxplot of internal disease symptom index values (ID%) in Valery somaclones challenged with *Fusarium oxysporum* f. sp. *cubense* isolate CNPMF 229. (A) Somaclones generated in MS culture medium supplemented with 1 mg/L TDZ. (B) Somaclones generated in culture medium supplemented with 2 mg/L TDZ. HR (highly resistant), R (resistant), MR (moderately resistant), S (susceptible), HS (highly susceptible), C (control plant). SILK (highly susceptible control, Apple). Treatments marked with the same letters did not differ significantly from each other according to the Scott-Knott test ($p>0.05$).

3.3 Molecular Analysis

The genetic variability of somaclones from the Grand Naine and Valery cultivars obtained under the different treatments was evaluated using the IRAP, REMAP, and ISSR molecular markers. Overall, the amplification profiles generated had consistent and reproducible bands and exhibited a predominantly monomorphic pattern, which indicates the absence of detectable genetic variation in most of the markers used. This is desirable because these varieties are already accepted in the market due to their agronomic and organoleptic characteristics and resistance to Foc.

However, in the Grand Naine cultivar, polymorphic bands were observed in some somaclones. In treatment T1, a band of approximately 550 bp was detected in somaclones SV2, SV4, SV7, and SV11 using the REMAP marker (LTR726+8565) (Figure 6B).



REMAP- LTR726+8565

FIGURE 6. Two-percent agarose gel with the polymorphic electrophoretic profile of banana somaclones of the Grand Naine cultivar highly resistant to isolate CNPMF 229 of *Fusarium oxysporum* f. sp. *cubense*. Retrotransposon-Microsatellite Amplified Polymorphism (REMAP) markers, combination LTR726 + 8565. M = Ladder marker (Invitrogen®). C = Control plant (Grand Naine). Somaclones (SV2, SV4, SV7, SV11) generated in MS culture medium supplemented with 1 mg/L TDZ.

In treatment T2, additional polymorphic bands were observed in the Grand Naine cultivar. A band of approximately 500 bp was detected in somaclones SV21, SV23, SV25, SV27, SV28, and SV37 using IRAP markers (3'LTR + NIKITA) (Figure 7A). Another band of approximately 900 bp was amplified in somaclones SV21, SV23, SV25, SV27, SV28, and SV37 using a combination of IRAP primers (C0945 + NIKITA) (Figure 7B). In addition, a band of approximately 1600 bp was detected with the REMAP marker in somaclones SV21, SV23, SV25, SV27, SV28, and SV37 (LTR726 + 8347) (Figure 7B).

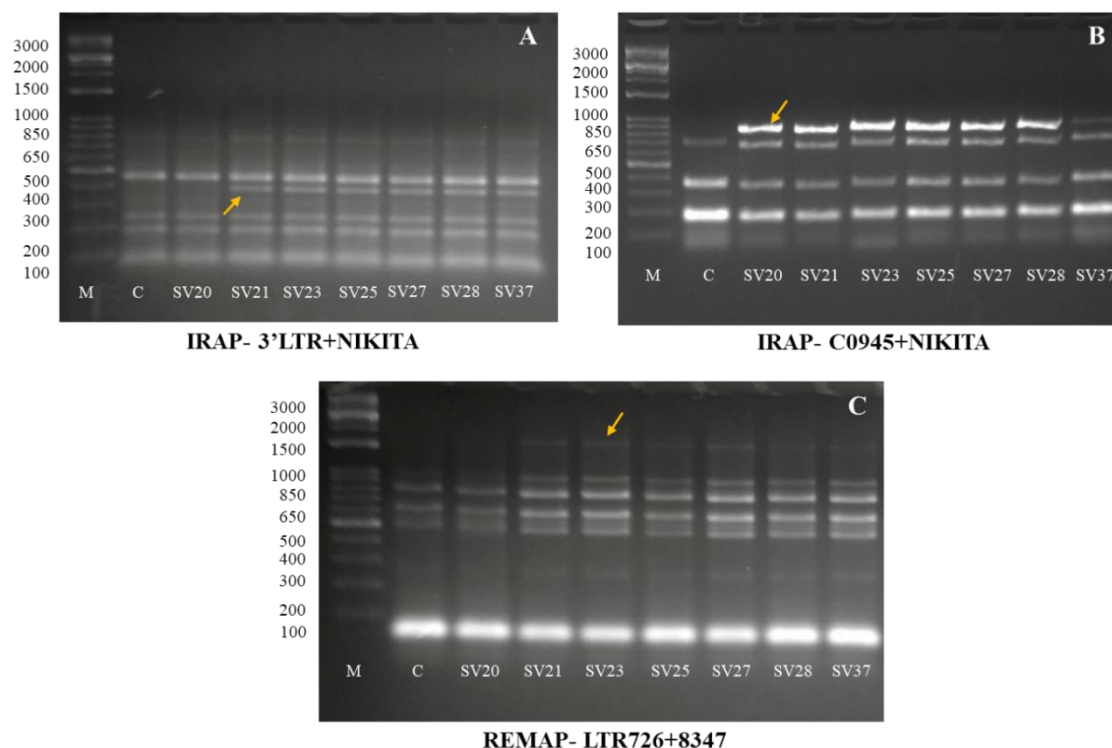


FIGURE 7. Two-percent agarose gel with the polymorphic electrophoretic profile of banana somaclones from the Grand Naine cultivar that are highly resistant to *Fusarium oxysporum* f. *sp. cubense* isolate CNPMF 229. (A) Inter-retrotransposon amplified polymorphism (IRAP) markers, combination 3'LTR + NIKITA; (B) IRAP markers, combination C0945 + NIKITA. (C) Retrotransposon-Microsatellite Amplified Polymorphism (REMAP) markers, combination LTR726 + 8347. M = Ladder marker (Invitrogen®). C = Control plant (Grand Naine). Somaclones (SV20, SV21, SV23, SV25, SV27, SV28, SV37) generated in MS culture medium supplemented with 2 mg/L TDZ.

3.4 Histological Analysis

The root clearing analysis followed by staining with Trypan blue showed the presence of fungal structures (hyphae) in the control plants of both genotypes evaluated. In the somaclones of the Grand Naine cultivar, hyphae were only detected in SV21, SV25, and SV37 from treatment T2, while fungal structures were not observed in SV2, SV4, SV7, and SV11 from treatment T1 and SV20, SV23, SV27, and SV28 from treatment T2. Similarly, in the Valey cultivar, somaclone SV1 in treatment T1 did not present Foc structures (Figure 8).

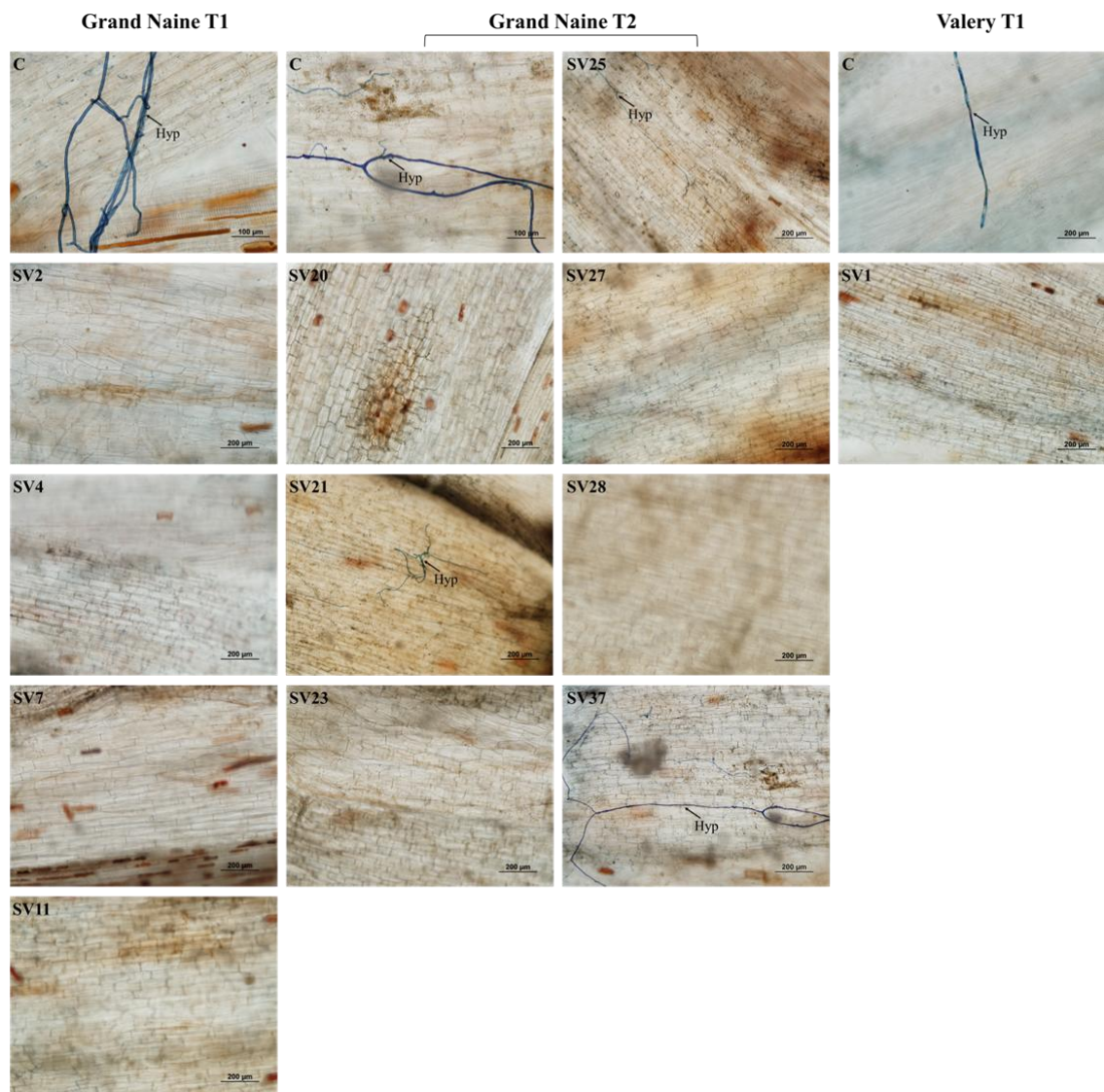


FIGURE 8. Clearing and staining of fungal structures with Trypan blue dye in the roots of somaclones and control banana plants of the Grand Naine and Valery cultivars 120 days after planting in a bed infested with isolate CNPMF 229 of *Fusarium oxysporum* f. sp. *cubense* (Foc). SV2, SV4, SV7, SV1: somaclones of 'Grand Naine' highly resistant to Foc generated in MS culture medium supplemented with 1 mg/L of TDZ (T1). SV20, SV21, SV23, SV25, SV27, SV28, SV37: somaclones of 'Grand Naine' highly resistant to Foc generated in MS culture medium supplemented with 2 mg/L of TDZ (T2). SV1: somaclone of 'Valery' highly resistant to Foc generated in MS culture medium supplemented with 2 mg/L TDZ (T1). The arrows indicate hyphae (Hyp).

4 Discussion

Somaclonal variation is a well-documented phenomenon characterized by genetic and epigenetic variability that can arise in plants grown in vitro (Ferreira et al., 2023). In this study, the growth regulator TDZ showed clear potential as an inducer of variability, especially in the

selection of somaclones resistant to Fusarium wilt in the Grand Naine and Valery banana cultivars. To this end, micropropagation was combined with different concentrations of TDZ and resistance to Foc was evaluated in two sequential stages, thereby allowing for a comprehensive analysis of the effect of somaclonal variation induced by this growth regulator on plant resistance.

The first evaluation of somaclones conducted 120 days after planting (Figure 1) showed promising results. Thirty-nine somaclonal variants of Grand Naine and 17 of Valery without symptoms of the disease were selected for a second stage of resistance validation (Figures 2 and 3). The reintroduction of these somaclones to tissue culture for a second resistance stability test represented a rigorous method for ensuring the reliability of the results.

The results showed that the TDZ concentration of 2 mg/L (T2) was the most effective in generating resistant somaclones in both genotypes. However, these findings differed from those of Ferreira et al. (2024), who reported greater efficiency of TDZ at 1 mg/L for the induction of resistant somaclones in the Prata Catarina (AAB) cultivar. This divergence may be related to genomic differences between the materials under study because growth regulators have varying effects depending on the genotype (Justine et al., 2022). In this context, Shirani et al. (2009) demonstrated that concentrations of TDZ above 2 μ M in banana and plantain genotypes (AAB and ABB) increased the occurrence of abnormal shoots and concluded that these genomes are more sensitive to high doses of TDZ than the AAA group. The literature confirms this effect in other crops. For example, El-Fattah et al. (2019) showed that different cultivars of tomatoes exhibit distinct phenotypic responses to identical doses of auxin and cytokinin. Similarly, Asadi and et al. (2021) reported that high concentrations of ANA in *Galanthus transcaucasicus* induced greater genetic variation while lower doses favored stability. Together, these findings suggest that cultivars of the Cavendish subgroup have greater physiological tolerance to high concentrations of TDZ, which allows in vitro stress to promote genetic and epigenetic changes without compromising the initial vigor of the plants. In contrast, a study on the Prata subgroup revealed that high doses may generate excessive oxidative and hormonal stress, resulting in loss of cell viability or random mutations without adaptive value (Ferreira et al., 2024). Thus, our results reinforce the need to optimize the protocols used for the induction of somaclonal variation, such as considering the concentration of the phytohormone for different groups and genotypes.

TDZ is involved in cell differentiation and mitotic activity and creates conditions conducive to somaclonal variation by inducing point mutations, chromosomal rearrangements, and

epigenetic modifications (Dewir et al., 2018). Although most of these changes occur randomly, studies such as those by Ferreira et al. (2020), Rebouças et al. 2021, and Ferreira et al. (2024) have demonstrated the potential of this growth regulator to induce somaclonal variants with desired characteristics, such as resistance to *Fusarium* wilt in banana plants.

The significant reduction in the DI by approximately 25% in Grand Naine and lower in Valery (Figures 2 and 3) suggests that TDZ activates defense mechanisms in plants that slow disease progression. The literature indicates that TDZ indirectly modulates hormonal pathways related to plant defense response. For example, Wang et al. (2025) showed that in strawberries, TDZ reduces the expression of key genes for cytokinin biosynthesis and promotes the expression of catabolic genes of the cytokinin oxidase/dehydrogenase (CKX) family. This mechanism is particularly relevant because pathogens of the genus *Fusarium* are capable of modulating the plant's hormonal balance to their benefit (Jameson, 2000; Qi et al., 2012; Han et al., 2019). Thus, CKX gene activity induced by TDZ inhibits the pathogen's ability to use the host's cytokinin to grow, thereby allowing the plant to reactivate its defense pathways.

The second evaluation in the greenhouse confirmed the resistance of 12 somaclones to Foc from both genotypes. The higher frequency of highly resistant plants in Grand Naine, which exhibited better performance with TDZ at 2 mg/L, confirms that the physiological stress imposed by higher concentrations of growth regulators induces favorable mutations (Figure 4B). However, according to the literature, other biological factors such as genotype (Kin et al., 1997), hormonal metabolism (El-Fattah et al., 2019), and plant defense responses (Dewir et al., 2018) also contribute to the efficiency of this strategy. This confirms that somaclone selection based on resistance tests is essential for identifying promising variants that can be incorporated in integrated *Fusarium* wilt management programs (Ferreira et al., 2024).

In our studies, highly resistant somaclones did not show considerable genetic differences compared to the control plant. This genetic stability may be a positive indicator for future tests based on the preservation of agronomic characteristics of interest and reduced risk of undesirable changes resulting from tissue culture-associated stress. Similar results were reported by Ferreira et al. (2024) when analyzing somaclones of the Prata Catarina cultivar and by Sampaio et al. (2024) when analyzing somaclones of the Grand Naine cultivar. Both confirmed genetic stability after analysis with the same DNA marker profile.

Despite the predominance of monomorphic profiles, the results presented herein showed the presence of some polymorphic bands in the somaclones of Grand Naine, which were detected using IRAP and REMAP markers. Although infrequent, this variability indicates the

occurrence of recombination events or retrotransposon insertion. Future sequencing of these polymorphic bands will be a crucial step for the molecular characterization of somaclones and their proper inclusion in *Musa* spp. databases. This approach will not only confirm the genetic authenticity of somaclonal variants but also determine whether the identified fragments are related to functionally relevant genes, especially those involved in the defense response. According to Sampaio et al. (2024), the presence of fragments homologous to calcium-binding proteins indicates a possible relationship between the detected polymorphisms and resistance mechanisms regulated by calcium-mediated signaling in Foc-resistant somaclones of the Grand Naine cultivar. Thus, further analysis of these sequences will contribute significantly to the identification of molecular markers for breeding programs, thereby accelerating the selection of resistant somaclones and providing important data on the genomic basis of somaclonal variation in *Musa* spp.

Understanding this variability is even more relevant when considering that the activation of transposable elements is widely recognized as a potential source of new genomic combinations or point mutations and occasionally gives rise to phenotypes with agronomically advantageous characteristics (Studer et al., 2011; Carpentier et al., 2019; Latzel et al., 2023). A classic example is the insertion of the BRACOPIA retrotransposon into the *BrFAE1* gene of *Brassica rapa*, an event that led to the emergence of the first cultivar with low erucic acid content—a milestone in the development of varieties intended for human consumption (Fukai et al., 2019). These findings reinforce that although somaclonal variation may pose a risk to genomic stability, it is also a valuable source of variability that can be exploited in genetic improvement programs (Teo et al., 2005; Butelli et al., 2012).

The low variability observed may also be related to limitations inherent to the markers that were used. ISSRs are based on the amplification of inter-microsatellite regions, and the detection of polymorphism depends on the mutation rate in simple repeats (Amiteye, 2021). Similarly, IRAP and REMAP are used to evaluate flanking regions of retrotransposons (Kalendar et al., 2021). The absence of polymorphisms in most combinations of IRAP, REMAP, and ISSR used in this study suggests that the number of rearrangements or insertions/deletions was not sufficient to be detected.

However, low polymorphism detection does not rule out the occurrence of more subtle genetic and epigenetic changes. For example, single nucleotide polymorphisms (SNPs) are more accurately characterized by next-generation sequencing technologies (Ahmad et al., 2020; Chen et al., 2023). In addition, in vitro culture can induce epigenetic modifications, especially

DNA methylation, with a direct impact on gene expression without altering the nucleotide sequence (Coronel et al., 2017). Complementary approaches, such as the use of epigenetic markers, methylation sensitive amplified polymorphism (MSAP) (Peraza-Echeverria et al., 2021), or higher resolution methodologies such as bisulfite sequencing (BS-seq) (Lin et al., 2019; Kou et al., 2022), can be applied to obtain a more comprehensive understanding of the variations induced in vitro in the selected somaclones.

In the analysis of root clearing, Foc structures were not observed in most somaclones. The presence of the pathogen was detected only in the somaclones of Grand Naine (SV21, SV25, and SV37) subjected to T2 treatment. These results contrast with those obtained in the control plants, which presented hyphae and developed symptoms of the disease.

The absence of symptoms in the somaclones, even in the presence of hyphae in the roots, suggests that although the initial defense barrier was not sufficient to prevent colonization by the pathogen, the plant was able to activate secondary mechanisms that limited the infection and prevented its progression to the vascular system. Similarly, Rocha et al. (2022) studied the histological responses of banana cultivars challenged with Foc and observed fungal structures in the roots of both susceptible and resistant genotypes. Moreover, Chen et al. (2019) observed Foc hyphae in the resistant cultivar 'p168' even in the absence of symptoms. These results led both authors to conclude that resistance does not inhibit pathogen penetration in the roots and confirmed the hypothesis that tolerance or resistance is not associated with preventing initial penetration but rather is related to activating internal mechanisms that prevent colonization and restrict infection to the root tissue, thus preventing damage to the vascular system.

This containment capacity may be the result of various defense responses, such as reinforcement of the cell wall by deposition of lignin and callose (Ellinger and Voigt, 2014; Chowdhury et al., 2014; Kumar et al., 2020; Kashyap et al., 2021), production of antimicrobial compounds such as phytoalexins (Boboshko et al., 2019; Kaur et al., 2022; Ninkuu et al., 2023), and activation of genes related to defense responses (Sun et al., 2019; Rocha et al., 2021; Oliveira et al., 2025). This diversity of responses among somaclones of the same cultivar shows the importance of somaclonal variation as a valuable source for genetic improvement. These findings also demonstrate the complexity of the interaction between somaclones and Foc and pave the way for new research focused on unraveling the molecular and biochemical mechanisms that confer resistance.

5 Conclusion

This study confirms that TDZ represents an efficient tool for inducing somaclonal variation in banana plants, especially for the selection of variants resistant to Fusarium wilt. TDZ (T2) at a concentration of 2 mg/L was most effective at inducing somaclones with complete resistance to Fusarium wilt in two assessment cycles.

The somaclones of the Grand Naine cultivar in T1 (SV2, SV4, SV7, and SV11) and T2 (SV20, SV21, SV23, SV25, SV27, SV28, and SV37/) and those of the Valery in T1 (VS1) were selected as highly resistant to Foc and thus represent the most promising potential sources of resistance for use in genetic improvement programs against Fusarium wilt.

The use of DNA markers based on retrotransposons, IRAP, and REMAP has proven to be effective for detecting subtle genomic changes, making them valuable tools for monitoring genetic variability in somaclones. As a subsequent step, we propose sequencing the identified polymorphic bands to characterize them in *Musa* spp. databases. This analysis will allow us to determine whether these bands correspond to known or candidate genes (*hits*), regulatory regions, or potentially useful alleles and new mutations associated with stress resistance. The integration of this information may provide relevant inputs for banana genetic improvement programs.

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Conflicts of Interest: The authors declare that they have no conflicts of interest.

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CAPÍTULO 2

**Análise molecular e histoquímica de somaclones da cultivar Grande Naine resistentes a
Fusarium oxysporum f. sp. *cubense* raça subtropical 4**

Artigo - Molecular and histochemical characterization of Grande Naine somaclones resistant to *Fusarium oxysporum* f. sp. *cubense* subtropical race 4, publicado na revista científica Euphytica, disponível em: <https://doi.org/10.1007/s10681-025-03645-x>



Molecular and histochemical characterization of Grande Naine somaclones resistant to *Fusarium oxysporum* f. sp. *cubense* subtropical race 4

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Abstract Banana farming is essential for the food and occupational security of numerous families who rely on subsistence agriculture. However, banana production has been severely affected by *Fusarium* wilt, caused by the pathogen *Fusarium oxysporum* f. sp. *cubense* (Foc), particularly the tropical race 4 (Foc TR4) and subtropical race 4 (Foc STR4). So far, the only reliable, effective way to manage the disease is by using resistant plant varieties, as no complete control methods are currently available. However,

developing these through conventional breeding remains challenging, particularly in combating TR4. Given this scenario, alternative approaches, such as somaclonal variation induction have shown promise in offering farmers resistant cultivars. This study aimed to molecularly and histochemically characterize somaclones of the Grand Naine cultivar resistant to Foc STR4. To evaluate genetic stability, the 14 somaclones were analyzed with IRAP markers (Inter-Retrotransposon Amplified Polymorphism), which revealed high stability compared to the control plant ('non-mutagenized Grand Naine'). Concurrently, defense responses were examined through qPCR and histochemical analyses. After inoculation

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with Foc STR4, somaclones E10, E13, E14, E15, and E16 showed complete resistance. The genotypes E10, E13, and E15 stand out due to their higher activation of defense genes, such as LOX, PIP2-3, PI206, and PTI6. Histochemical analyses revealed that somaclone E13 showed increased callose and phenolic compound deposition in the roots, with no fungal structures present. Overall, all somaclones showed superior resistance compared to the control, and their future commercial validation will be crucial for developing more productive and Fusarium wilt-resistant banana cultivars, significantly contributing to the sustainability of global banana production.

Keywords Genetic improvement · Fusarium wilt · *Musa spp* · Somaclonal variation

Introduction

Bananas and plantains (*Musa spp.*) are fruit crops primarily grown in Asia, Latin America, and Africa (FAOSTAT, 2025). In many countries across these regions, banana production serves as a staple food source for numerous families reliant on subsistence farming (Kimunye et al. 2021a, b). In 2023, bananas stood out as the world's most produced fruit, reaching an output of approximately 139 million tons, grown across 5.9 million hectares (FAOSTAT, 2025). Among commercial banana cultivars, those of the Cavendish subgroup (AAA) are the most widely grown and consumed banana varieties worldwide, accounting for nearly 50% of the global production and roughly 95% of the international export trade (García-Bastidas et al. 2022; Munhoz et al. 2024).

In 2022, Latin America accounted for 60% of the 21 million tons of bananas exported worldwide, with a significant portion coming from Cavendish banana monocultures grown on medium- and large-scale farms (Munhoz et al. 2024). Several cultivars of this subgroup, such as 'Grand Naine,' 'Williams,' and 'Valery,' are widely grown for international export. However, the inherent vulnerability of monocultures leaves these cultivars constantly exposed to threats, exacerbated by climate change, pests, and diseases. These challenges highlight the need for the effective management strategies to combat these threats and ensure sustainable production (Bebber 2022).

Pathogenic agents such as fungi, bacteria, nematodes, and viruses are the main challenges to banana production, with fungal infestations being the most critical. Among fungal pathogens, *Fusarium oxysporum* f. sp. *cubense* (Foc) has emerged as a global threat. This soil-dwelling fungus, responsible for Fusarium wilt, is particularly concerning due to its devastating impact on banana plantations worldwide, thus, requiring urgent control and management measures (Dita et al. 2018; Kimunye et al. 2021a, b).

Since the 1960s, after Race 1 of Fusarium wilt devastated numerous plantations of the 'Gros Michel' (AAA) cultivar, a dominant banana variety in global exports—Cavendish (AAA) cultivars emerged as the leading commercial variety, replacing 'Gros Michel' in the international market (Ploetz 2005; Ditas and Pérez 2014; Haddad et al. 2018). However, the emergence of a more aggressive and virulent pathogen variant, known as Foc Tropical Race 4 (TR4), which can infect Cavendish subgroup banana plants, threatens global banana production owing to its rapid proliferation across banana-growing regions (Dong et al. 2020; Hou et al. 2022). To date, Foc TR4 has been reported in 23 banana-producing countries, including recent cases in Latin American nations, such as Colombia, Peru, and Venezuela. This occurrence has heightened the threat to global banana production and put other countries in Central and South America on high alert (García-Bastidas et al. 2020; Acuña et al. 2022; Herrera et al. 2023; Munhoz et al. 2024).

Apart from replacing susceptible varieties with resistant ones, there are no fully effective control strategies for Fusarium wilt in banana plants (Blomme et al. 2024). Conventional breeding efforts to develop a Foc TR4-resistant variety, particularly within the Cavendish subgroup, have had limited success due to the high sterility and triploid nature of most cultivated bananas (Van Den Berg et al. 2007; Silva et al. 2022). Therefore, biotechnological strategies—including genetic engineering, gene editing, mutation induction, and somaclonal variation induction—generate new possibilities for the genetic improvement of banana plants. These approaches provide promising alternatives to overcome the limitations associated with classical breeding in developing resistant varieties (Sipen et al. 2011; Hou et al. 2022; Tripathi et al. 2024).

The banana breeding program run by the Brazilian Agricultural Research Corporation (Embrapa)

has made significant progress in developing varieties resistant to Foc. Among the adopted strategies, somaclonal variation induction has shown promising results. This technique involves inducing genetic variability by growing plant tissues *in vitro* in a medium supplemented with specific growth regulators, followed by successive subculturing. After this process, the somaclonal variants are rigorously selected based on pathogen resistance and evaluated under controlled conditions. In a recent study, Embrapa identified 14 somaclones of the Grand Naine cultivar resistant to Foc STR4, initially confirmed in greenhouse assays (Rebouças et al. 2021). These somaclones are currently under field evaluation; a necessary step to consolidate their resistance, representing a significant advancement in crop improvement. This result demonstrates the effectiveness of inducing somaclonal variation as a strategic tool for developing Foc-resistant species.

Although resistance to Foc STR4 in the 14 somaclones was already identified by Rebouças et al. (2021), a significant gap remains in understanding the genetic mechanisms conferring *Fusarium* wilt resistance. To date, no systematic studies have integrated analyses of genetic similarity and gene expression of these somaclones, limiting understanding of their genetic resistance basis. In this context, the present study aimed to evaluate the genetic variability of the somaclones using IRAP markers; analyze the expression of defense-related and post-formed compound deposition genes in response to Foc STR4 inoculation; and identify the most promising somaclones for breeding programs. This approach is crucial due to the need to deepen knowledge of resistance mechanisms and support the selection and validation of resistant genetic materials. Therefore, the results of this study are important for advancing banana breeding, directly contributing to the development of *Fusarium* wilt-resistant varieties.

Materials and methods

Plant material

Fourteen Somaclonal variants (referred to as somaclones) derived from the genotype 'Grand Naine' (AAA), previously selected as resistant to *Fusarium oxysporum* f. sp. *cubense* Foc STR4, were used,

namely: Embrapa 4 (E4), Embrapa 5 (E5), Embrapa 6 (E6), Embrapa 7 (E7), Embrapa 8 (E8), Embrapa 9 (E9), Embrapa 10 (E10), Embrapa 11 (E11), Embrapa 12 (E12), Embrapa 13 (E13), Embrapa 14 (E14), Embrapa 15 (E15), Embrapa 16 (E16), and Embrapa 17 (E17). As a control (C), a plant of the same genotype that did not undergo the somaclonal variation induction process was used. These somaclones were obtained by direct organogenesis from shoot apices of the Grand Naine cultivar. The explants were established *in vitro* and grown on MS medium (Murashige & Skoog, 1962) supplemented with Thidiazuron (TDZ), undergoing 10 consecutive subculture cycles for multiple shoot induction. This procedure followed the methodology described by Rebouças et al. (2021).

Analysis of genetic variability using IRAP markers.

Genomic DNA extraction

In a randomized block experiment with five blocks conducted in the experimental field, young leaves were collected from control plants and the 14 somaclones. Plants were six months old at the time of collection. Four plots were selected for each somaclone per block, totaling 20 replicates per somaclone. The inclusion of replicates aimed to assess genetic fidelity among clones of each somaclonal variant. After collection, leaves were immediately stored in an ultra-freezer at -80°C to preserve the material until analysis. DNA was extracted using the Cetyltrimethylammonium bromide protocol modified by Ferreira et al. (2019). DNA quality and quantity were assessed by 1% agarose gel electrophoresis stained with GelRed® (Biotium, Inc., Fremont, CA, USA).

PCR amplification of IRAP markers

For the genetic characterization of the 14 somaclones using the PCR-IRAP technique, seven LTR (Long Terminal Repeat) primer combinations from Teo et al. (2005) and Baumel et al. (2002) were used. (Table 1).

The amplification reactions were carried out in a final volume of 15 μL per sample, containing 4 μL of template DNA (10 ng), 0.75 μL of MgCl_2 (50 mM), 1.5 μL of $10 \times$ PCR buffer, 1.2 μL of dNTPs (2.5 mM), 1.5 μL of each primer (F and R) (10 mM), 0.3 μL of commercial Taq DNA polymerase (5 U

Primers	Sequence (5' -	References
5' LTR2	ATC ATT GCC TCT AGG GCA TAA TTC	Teo et al. (2005)
3' LTR	TGT TTC CCA TGC GAC GTT CCC CAA CA	Teo et al. (2005)
Nikita	5' -CGG ATT TGT TCA AGC CTA AAC C	Teo et al. (2005)
LTR6149	CTC GCT GCG CCA CTA CAT CAA CCG CGT TTA TT	Teo et al. (2005)
LTR6150	CTG GTT CGG CCC ATG TCT ATG TAT CCA CAC ATG TA	Teo et al. (2005)
CO947	TTG TTC ACC CAC CGT CTA CTT GC	Baumel et al. (2002)
CO945	GCA AGC TTC CGT TTC CGC	Baumel et al. (2002)
<i>Combinations of IRAP primers and their respective annealing temperatures (40)</i>		
Primer combination	CO947 3' LTR + LTR6150 + CO945 + LTR6150 3' LTR + LTR6150 3' LTR + Nikita	3' LTR + LTR6149 CO947 5' LTR2 46 °C 46 °C 46 °C 46 °C
At	R 42 °C	46 °C

In the greenhouse, the plants were inoculated when they reached approximately 20 cm in height. The experiment was conducted in 2-L pots containing a substrate based on fine granulated coconut fiber (Amafibra®) and pine bark (Tecnomax®), in which seedlings of each somaclone and control plants were transplanted. Inoculation with Foc (STR4) was performed using rice previously infested with a concentration of 10^6 CFU/g. A total of 40 g of inoculum was

distributed in four holes around each plant, ensuring uniform root exposure to the pathogen. This method is widely used for Foc inoculation in banana plants as it provides a consistent and homogeneous source of inoculum (Santana et al. 2024; Rocha et al. 2022). The experiment was designed using a completely randomized block design comprising the following treatments: somaclones vs. the Foc isolate; control plants vs. the Foc isolate; and uninoculated control plants. For each treatment, 20 plants were inoculated. Only autoclaved rice (without the pathogen) was added to the substrate in the non-inoculated control plants. Plant roots were collected at five different time points for subsequent analysis: 0, 12, 24, 48, and 72 h after inoculation (HAI). For each treatment, three biological replicates were collected at each time point.

Symptom assessment

At the end of the 90-day experimental period following inoculation, internal symptoms of Fusarium wilt were evaluated in 10 plants from each treatment. The assessment was conducted using the methods described by Dita and Pérez (2014) with modifications, replacing the one-to-five grading scale with zero-to-four. The internal symptom scores were converted to a disease intensity index (DI) using the formula described by McKinney (1923) based on symptom ratings (on a scale from zero to four). DI values were submitted to analysis of variance (ANOVA) to test the hypothesis of equality among treatments. Means were grouped using the Scott-Knott test at 5% probability ($p \leq 0.05$). All analyses were performed assuming a 95% confidence level. The data were plotted as boxplots to illustrate the DI results across the different treatments. The number of replicates was based on the number of plants assessed. Statistical analyses were performed using R software, version 4.4.1 (R core team, 2024).

Selection of primers

To elucidate the resistance of somaclones to Foc STR4, the relative expression of 12 pathogenesis-related genes described in the literature was evaluated. Primers previously used in studies evaluating the regulation of these genes in the interaction between *Musa* spp. and tropical/subtropical race 4 of Foc were selected. For gene expression normalization,

the reference genes tubulin and RPS2 were used, and both were validated for use in *Musa* spp. (Table 2).

RNA extraction and cDNA synthesis

For total RNA extraction, roots were collected from control plants and somaclones E10, E13, E14, E15, and E16—previously classified as highly resistant to isolate CNPMF 218—at intervals of 0, 12, 24, 48, and 72 HAI. At the time of collection, the samples were immediately immersed in liquid nitrogen to preserve genetic material integrity and subsequently stored in an ultra-freezer at -80°C . RNA extraction was performed according to the methodology described by Ferreira et al. (2019). The integrity and quantification of the extracted RNA were assessed using 1% agarose gel electrophoresis and spectrophotometry with a Nanodrop ND-2000 (Thermo Scientific, Waltham, MA, USA). RNA samples were treated with DNase (RNase-free TURBO, Ambion), and cDNA synthesis was performed using a High-Capacity RNA-to-cDNA Kit (Applied Biosystems, Waltham, MA, USA) following the manufacturer's instructions.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

qPCR analyses were performed using the ABI 7500 Fast Real-Time PCR System (Applied Biosystems). For each reaction, the following components were used: 1 μL of cDNA (100 ng/ μL), 0.3 μL of each primer (Table 2) at an initial concentration of 0.3 μM , 5 μL of SYBR Select Master Mix (Applied Biosystems), and 3.4 μL of nuclease-free water, for a final reaction volume of 10 μL . The amplification protocol consisted of an initial hold at 50°C for 2 min, followed by a denaturation step at 95°C for 2 min. Subsequently, 40 amplification cycles were performed with denaturation at 95°C for 15 s and annealing/extension at 60°C for 30 s, with fluorescence detection at the end of each extension cycle. The dissociation curve was determined after completing the amplification cycles. Each gene was analyzed with three technical replicates and three biological replicates, along with negative control (water), to ensure result accuracy. For the normalization of quantification cycle (Cq) values, the reference genes Tubulin and 25S were used, as described by Pfaffl et al. (2002).

Table 2 Primers used for gene expression analysis in the *Musa* spp. × *Fusarium oxysporum* f. sp. *cubense* interaction

Gene	Description	Sequence (5'-3')	pb	References
LOX	Lipoxygenases	F: ACGATGCAGACGGTATTGGAGT R: GGTACTGTCCGAAGTTGACG	94	Dong et al. (2019)
ATL	Auxin transporter-like protein 1	F: GGTTTCAGCTGCTCCTCCAAT R: AGAACAGCTGCAGGATCACC	161	Rocha et al. (2022)
CHI	Chitinase	F: TACTGGAAGTACAACCTACGGAGC R: CGTTCTGCTCGAGGTACTC	82	Dong et al. (2019)
PI206	Putative Disease resistance response protein 206	F: AGTACAACGGGAGCAGCTTC R: GATGAGCCTGCTGATGGTGT	128	Rocha et al. (2022)
PR1	Pathogenesis-related proteins 1	F: AGTTATGGACGAGCTACCCG R: GTAGCTGAAGTACTTCCCCTC	77	Dong et al. (2019)
RPPL1	Putative disease resistance RPP13-like protein 1	F: AGAAGCACTGGAGAAACATCG R: GGAACAGGGAACAGAAAACAAAG	141	Sun et al. (2019)
PIP2-3	Aquaporin	F: CTTCTGCTCCTCGTCTACACCG R: TCGCCAAGTGGACCATGAAA	122	Costa (2013)
PTI6	Gene related to pathogenesis	F: GTCAAGACGAGGAAGACGATC R: GACGAATCGGTAGCATCCAC	110	Sun et al. (2019)
RPM1	Belonging to NB-LRR genes	F: CTGGCATGCTTACTAGTCTCAG R: AGAATGGGAGGTAGAGGATGG	137	Sun et al. (2019)
NAK	Probable serine/threonine-protein kinase	F: AGCCAGAGCAGAGAAGGAAAG R: ACGGAAGTTTCTGGTGGCTG	159	Dong et al. (2020)
NPR1	Essential regulator of acquired systemic resistance	F: CGTATCACTTGCAATGGCTG R: CCTAAGGTGAACTCCGATGTG	150	Sun et al. (2019)
WRKY22	Transcription factor WRKY 22	F: CGTGACGTACGAAGGAGAGCA R: GGTCAACGCGAAGTCAACCA	95	Rocha et al. (2022)
TUB	Tubulina	F: TGTTGCATCCTGGTACTGCT R: GGCTTTCTTGCACTGGTACAC	102	Podevin et al. (2012)
RPS2	Ribosomal protein S	F: TAGGGATTCCGACGATTGTGTT R: TAGCGTCATCATTGGCTGGGA	84	Zhang et al. (2017)

Next, the Cq values used to determine the difference in gene expression between inoculated and control treatments at the different collection times were calculated using the exponential PCR reaction formula $QR = 2^{-\Delta\Delta C_t}$, as described by Livak and Schmittgen (2001). Results were expressed as mean ± standard deviation. Gene expression values were subjected to ANOVA to test the hypothesis of equality among somaclones. Means were grouped using the Scott-Knott test at 5% probability ($p \leq 0.05$). All analyses were conducted assuming a 95% confidence level. Statistical analyses were performed using R software, version 4.4.1 (2024).

Histological and histochemical analyses

Root fragments from somaclones E10, E13, E14, E15, E16, and the control plant were collected at intervals of 48 h, 15, 30, and 90 days after inoculation. The samples were immediately immersed in modified Karnovsky’s solution (Karnovsky 1965) (1% glutaraldehyde, 4% formaldehyde, 0.1 M CaCl₂, 0.2 M sodium cacodylate buffer, pH 7.2). After 48 h of fixation, the root fragments were dehydrated using an ascending ethanol series (30% to 100%) with 3-h intervals between each step. The fragments were infiltrated and embedded using the Histo-resin kit (hydrox-ethyl methacrylate, Leica, Heidelberg, Germany). After polymerization of the histo-resin, 8-µm-thick

histological sections were obtained using a Leitz 1516 microtome (Thermo Fisher Scientific, Waltham, MA, USA). The histological sections were mounted on slides and subjected to various staining methods. To detect phenolic compounds, the slides were stained with ferric chloride for 3 h, as described by Johansen (1940). Callose detection was performed using 0.05% aniline blue for 5 to 10 min, following the method of Hughes and McCully (1975). A blank slide was prepared as a negative control by replacing the dye with distilled water to ensure staining specificity. The sections were then analyzed and photographed using a B x S1 fluorescence microscope (Olympus Latin America, Miami, FL, USA).

The clearing and staining of roots and fungal structures were performed according to the method described by Phillips and Hayman (1970). The roots were immersed in a 10% potassium hydroxide (KOH) solution at room temperature for 48 h, followed by immersion in a 1% HCl solution for 5 min. Next, a 0.05% trypan blue solution (in lactic acid/glycerol/water at a 2:1:1 ratio) was applied for 1 h. After staining, the samples were mounted on slides, and the root fragments were photographed under an optical microscope (Olympus Latin America, Miami, FL, USA).

Results

Molecular analysis using IRAP markers

Twelve LTR primer combinations were used to investigate genetic differences between control plants and somaclones of the Grand Naine cultivar, previously selected for their resistance to Foc STR4. The PCR-IRAP technique, used for amplification, produced electrophoretic profiles with multiple bands. However, due to the high number of monomorphic bands, it was not possible to generate a dissimilarity matrix.

In somaclones E7 and E8, a polymorphic band of approximately 400 bp was detected and amplified using the 3'LTR + NIKITA primer combination. This fragment was exclusively present in the somaclones and absent in the control plant, indicating the occurrence of some "cis"-acting retrotransposon activation (Fig. 1: A and B).

Internal symptom assessment and disease index (DI)

The internal symptoms assessment indicated the presence of characteristic *Fusarium* wilt symptoms in the control plants, with severity scores ranging from one to three. Cross-sectional analysis of the rhizomes revealed a reddish-brown discoloration, with lesions originating at the tips and partially progressing toward the central cylinder (Fig. 2).

Overall, based on disease symptoms, the 14 soma-clones demonstrated resistance to the pathogen compared to the control plant. Although a detailed inspection revealed nearly imperceptible signs of infection in some specimens, somaclones E10, E13, E14, E15, and E16 showed no symptoms in the rhizome and were accordingly assigned a score of zero.

Therefore, these somaclones were classified as highly resistant to Foc STR4 and selected for subsequent gene expression analysis and histochemical studies to elucidate the defense mechanisms involved in the plant-pathogen interaction.

The aggressiveness of the isolate was assessed and quantified based on the internal symptom index, as shown in the boxplot (Fig. 2). The control plants showed an average DI of 40%. In comparison, somaclones E4, E5, and E12 showed an average DI of approximately 5%, while E7 and E17 recorded 8%; E6 and E9 showed 10%; and E8 and E11 had 13% DI. The somaclones E10, E13, E14, E15, and E16 showed no symptoms, indicating that Foc STR4 was unable to cause disease in these plants during the 90-day evaluation period (Fig. 2).

Gene expression analysis

To study the resistance of five somaclones of the Grand Naine cultivar to Foc STR4 at the molecular level, 12 genes (LOX, ATL, CHI, PI2o6, PR1, RPPL1, PIP2-3, PTI6, RPM1, NAK, NPR1, WRKY22) were selected. These genes have been previously associated with defense responses in the *Musa* spp. × Foc race STR4 and TR4 interaction. The samples were representative of the roots of the somaclones and the control plant at 0, 12, 24, 48, and 72 HAI. The analyzed genes showed distinct expression patterns in the somaclones compared to the control plant (Supplementary Table 1).

The lipoxygenase (LOX) gene showed upregulation in all analyzed somaclones (Fig. 3). At 12 HAI,

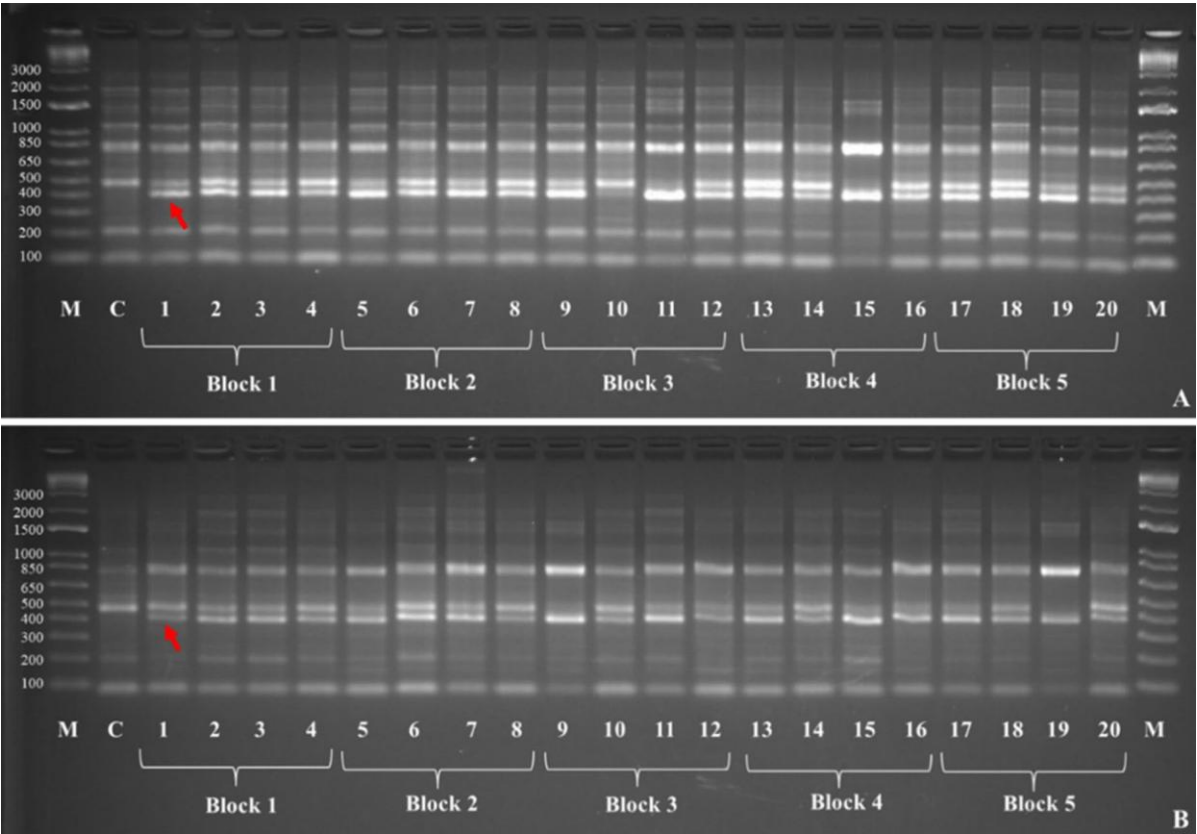


Fig. 1 2% agarose gel showing the polymorphic electrophoretic profile of somaclones from the Grand Naine banana cultivar resistant to *Fusarium oxysporum* f. sp. *cubense* STR4. Each gel represents 20 replicates of a single somaclone. **A**—Somaclone E7, inter-retrotransposon amplified polymorphism (IRAP) markers, primer combinations 3’LTR + NIKITA. **B**—Somaclone E8, IRAP markers, primer combinations 3’LTR + NIKITA. M = DNA Ladder Marker (Invitrogen®). C = control plant (Grand Naine); 1–20: number of somaclone replicates distributed in randomized blocks

only the somaclones E10 and E14 showed higher expression than the control plant. At the intermediate time points (24 and 48 HAI), all somaclones differed from the control, whereas at the final phase of evaluation, only somaclones E13, E15, and E16 differed from the control plant.

The gene encoding auxin transporter-like protein 1 (ATL) was downregulated only in somaclones E13 and E15 at 12 HAI, showing a statistically significant difference from the control plant (Fig. 3). At 24 and 48 HAI, ATL was upregulated in all somaclones, although somaclone E16 showed no significant difference from the control at 48 HAI. By the end of the evaluation period, this gene showed negative expression in all plants, including the control plant, which differed from somaclones E14 and E15.

The NPR1 gene, which encodes a key protein in systemic acquired resistance in plants, showed down-regulation in most somaclones across the evaluated time points. A statistically significant difference compared to the control plant was observed in somaclones E13, E15, and E16 at 12 HAI. At 24 HAI, only somaclone E15 showed a statistically significant difference compared to the control plant. In subsequent time points, only somaclone E14 did not differ statistically from the control plant at 72 HAI.

The gene responsible for synthesizing pathogenesis-related protein 1 (PR1) showed upregulation in somaclones E10, E13, and E16 across all evaluation time points. Among these, only the somaclone E13 maintained a statistically significant difference compared to the Control plant at all analyzed time points. On the other hand, somaclones E14 and E15 also

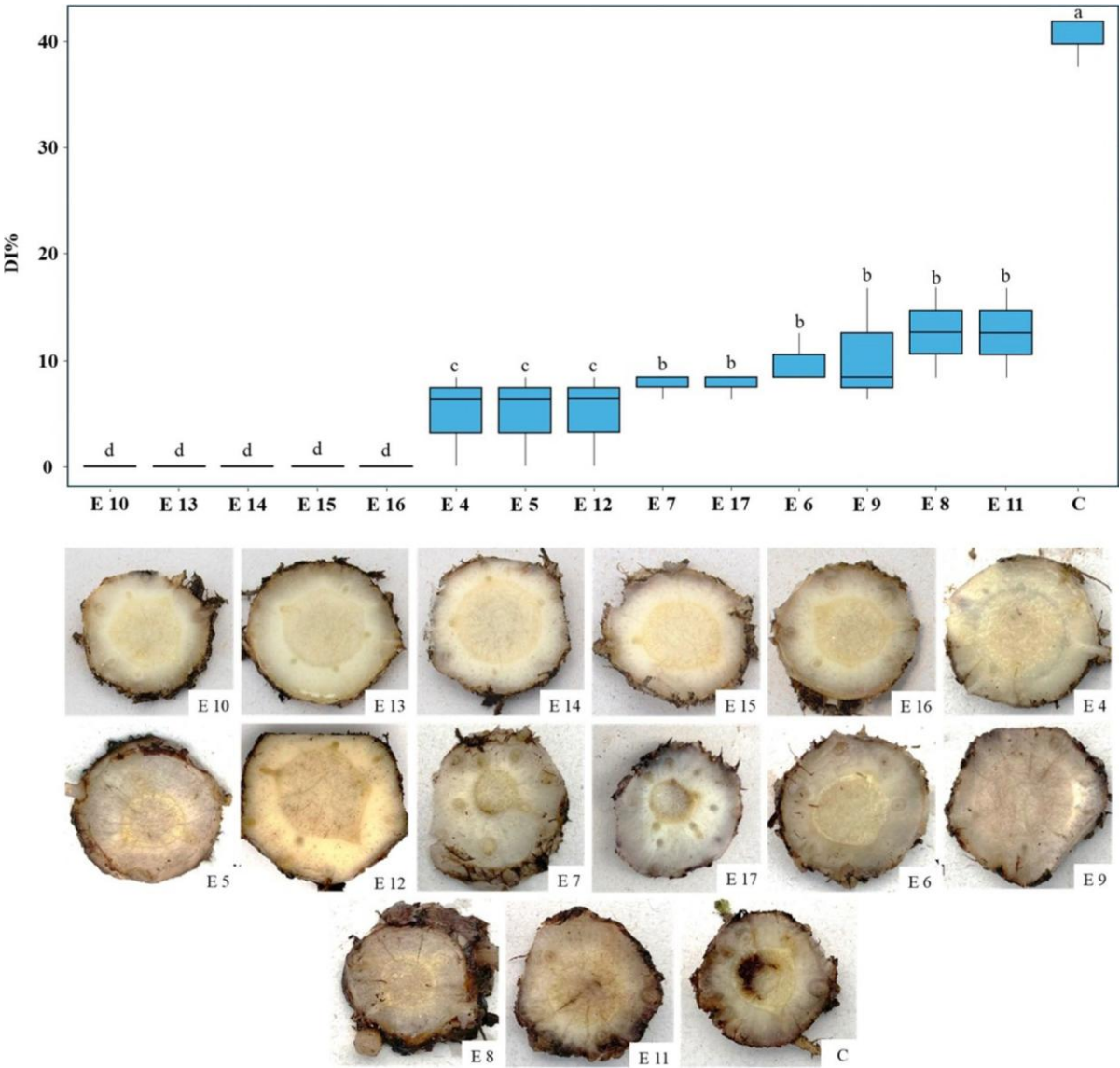


Fig. 2 Boxplot of internal disease symptom indices (DI%) and cross-sections of the rhizomes for *Fusarium oxysporum* f. sp. *cubense* ST4-resistant Grand Naine somaclones (n = 14). Treatments marked with the same letter do not differ statistically from each other according to the Scott-Knott test ($p > 0.05$). Somaclones E10, E13, E14, E15, and E16 showed no symp-

toms (highly resistant). Somaclones E4, E5, E6, E7, E8, E9, E11, and E12 showed only slight discoloration around the xylem (resistant). Control plants showed significant xylem discoloration (susceptible). Disease indices were calculated based on evaluating internal symptom scores from 10 plants of each somaclone, using the rating scale by Dita et al. 2014

differed from the control but showed downregulation of the PR1 gene (Fig. 3). It was observed that the chitinase (CHI) gene, which encodes enzymes involved in chitin degradation, was upregulated over the analyzed time periods in somaclones E10, E14, E15, and E16. Notably, somaclones E14 and E15 showed statistically higher

expression than the Control plant, specifically at 12 and 48 HAI (Fig. 3). In contrast, somaclone E13 showed downregulation of the CHI gene at all evaluated time points. The aquaporin gene (PIP2-3), which encodes proteins essential for water transport across cell membranes, showed significantly higher expression in all

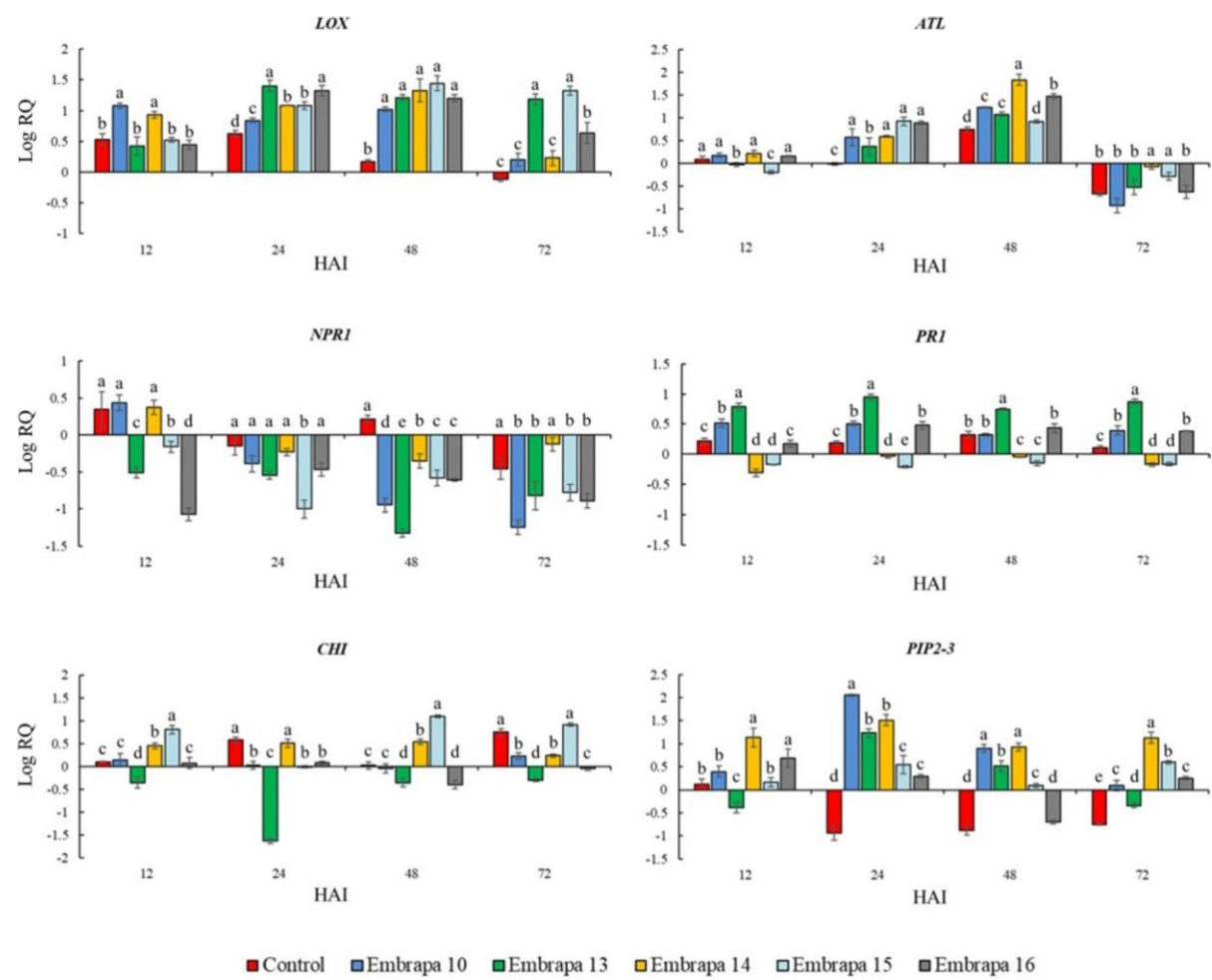


Fig. 3 Relative expression levels of defense-related genes in five banana somaclones derived from the Grand Naine cultivar at 12, 24, 48, and 72 h after inoculation (HAI) with *Fusarium oxysporum* f. sp. *cubense* subtropical race 4 (Foc ST4). The relative expression levels of the following genes were analyzed by qPCR: lipoxygenase (LOX), auxin transporter-like protein 1 (ALT), essential regulator of acquired systemic resistance

(NPR1), pathogenesis-related protein 1 (PR1), chitinase (CHI), and aquaporin (PIP2-3). The data represent the means $\pm$ standard deviations of three biological replicates and three technical replicates. The QR values correspond to genes with differential expression at 12, 24, 48, and 72 HAI compared to 0 HAI (non-inoculated plants)

somaclones compared to the control plant, particularly at 24 HAI. Among the analyzed somaclones, E14 stood out by showing statistically higher PIP2-3 gene expression than the control at all evaluated time points (Fig. 3). The PI206 gene, which encodes a putative disease resistance response-associated protein, showed positive expression in all somaclones, with statistically significant differences from the control plant, particularly at 24 HAI (Fig. 4). In subsequent time points, although PI206 expression remained

positive, only the somaclone E13 maintained expression levels higher than the control plant at 72 HAI. The RPM1 gene, which belongs to the NB-LRR class, was upregulated in somaclones E10, E13, and E15, showing statistically higher expression than the control plant exclusively at 24 HAI (Fig. 4). In contrast, somaclone E16 stood out due to the downregulation of RPM1 at all evaluated time points, differing significantly from both the control plant and the other somaclones.

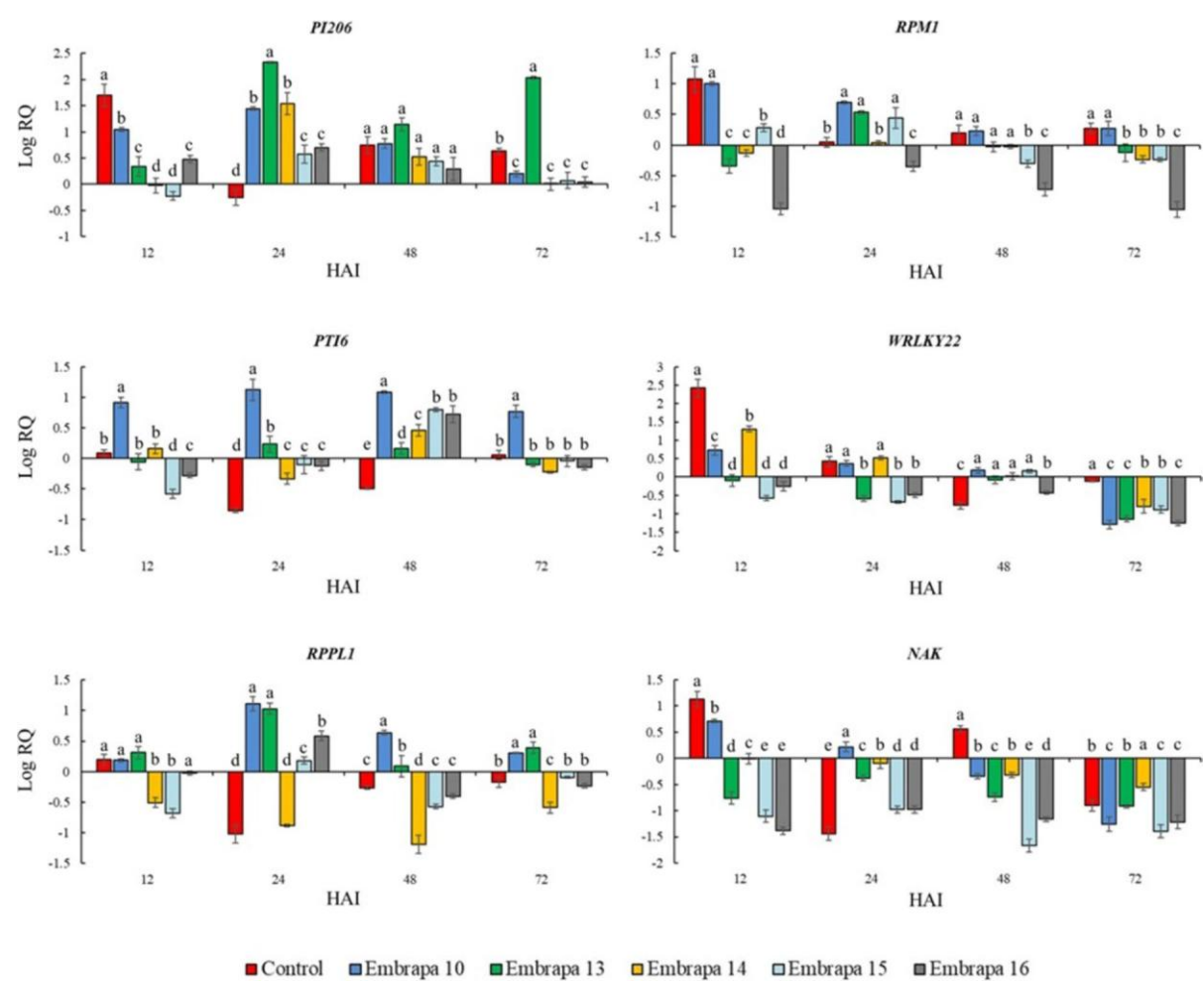


Fig. 4 Relative expression levels of defense-related genes in five somaclones derived from the Grand Naine cultivar at 12, 24, 48, and 72 h after inoculation (HAI) with *Fusarium oxysporum* f. sp. *cubense* subtropical race 4 (Foc STR4). The relative expression levels of the following genes were analyzed by RT-qPCR: Putative disease resistance response protein 206 (PI206), NB-LRR gene family member (RPM1), pathogenesis-

related genes (PTI6), transcription factor WRKY22, putative disease resistance RPP13-like protein 1 (RPPL1), and probable serine/threonine-protein kinase (NAK). The data represent the means $\pm$ standard deviations of three biological replicates and three technical replicates. The QR values correspond to genes with differential expression at 12, 24, 48, and 72 HAI compared to 0 HAI (non-inoculated plants)

The PTI6 gene, which plays a key role in regulating defense-related gene expression, was upregulated in all somaclones, with expression levels significantly higher than in the control plant at 48 HAI. Among the analyzed somaclones, only E10 maintained high levels of PTI6 expression at all evaluated time points. The transcription factor gene WRKY22, a member of the WRKY family that regulates the expression of defense genes against various microbial pathogens, showed higher and positive expression levels in somaclones E10, E14, and E15 compared to the control

plant at 48 HAI. At 72 HAI, all somaclones differed statistically from the control plant, even though they exhibited downregulation of this gene (Fig. 4). The RPPL1 gene, which encodes a putative protein involved in disease resistance response, was upregulated in several somaclones (Fig. 4). The somaclones E10, E13, E15, and E16 showed significantly higher expression than the control plant at 24 HAI. In subsequent periods, only the somaclones E10 and E13 maintained expression levels higher than the control plant.

The NAK gene, which encodes the serine/threonine protein kinase, was found to be downregulated in the somaclones, primarily at 48 and 72 HAI. Among the analyzed somaclones, only E10 showed positive expression levels higher than those of plant C at 24 HAI (Fig. 4).

Histochemical analyses

Evaluation of aniline blue-stained histological sections revealed the presence of callose, indicated by a blue-green coloration. It highlighted differences in the response of somaclones over time compared to the control plant. From 48 HAI onward, all analyzed somaclones showed increased callose production

compared to the Control plant, indicating early activation of defense mechanisms. As time progressed, callose production became more pronounced in the cortex, suggesting an expansion of the defense response to other regions of the root tissue—likely as a strategy to limit infection and protect adjacent tissues. The somaclone E13 stood out among the others, consistently exhibiting the highest callose levels at all evaluated time points (Fig. 5). This behavior suggests that somaclone E13 has a superior ability to mobilize callose reserves and strengthen structural barriers over time, which may be associated with greater pathogen resistance.

Histochemical analysis for phenolic compound detection in roots using the ferric chloride test

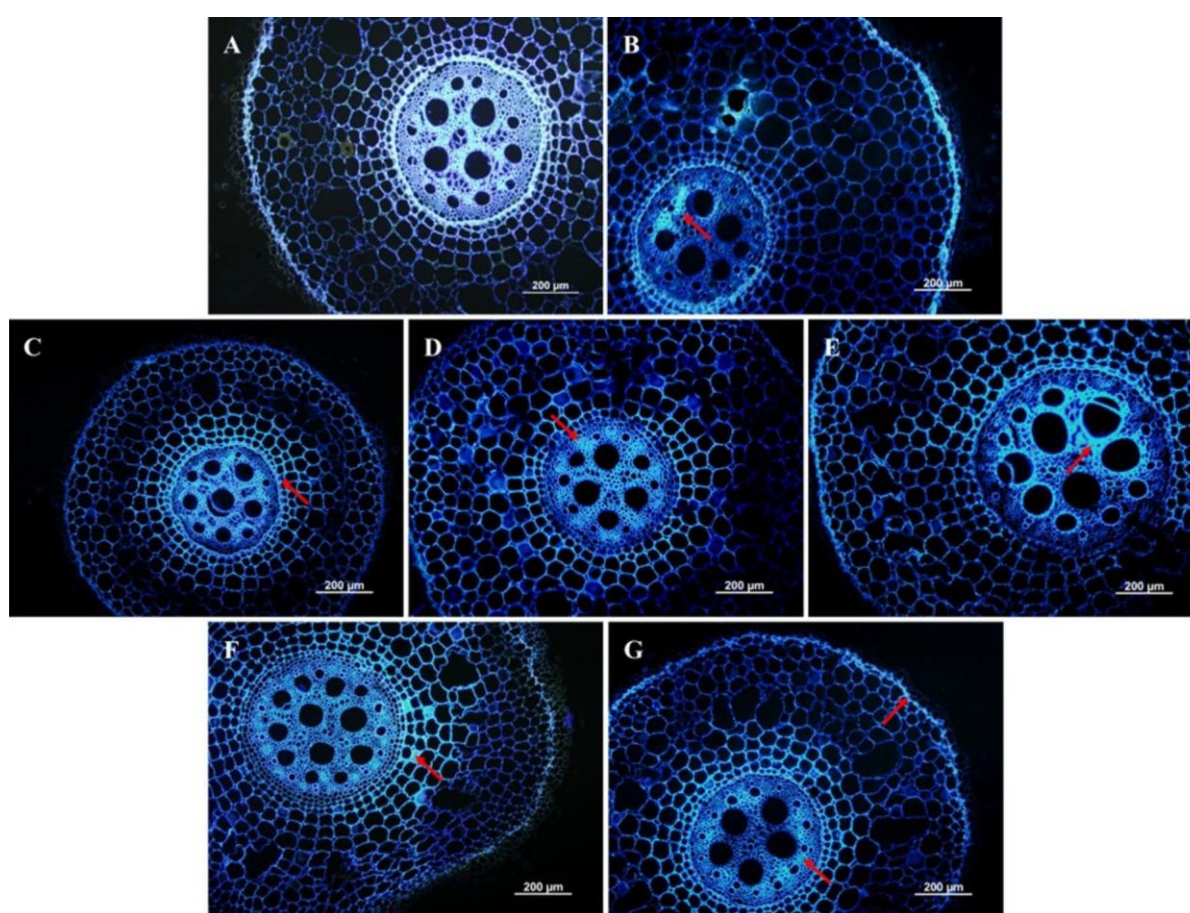


Fig. 5 Histochemical test with aniline blue to detect callose presence in roots of banana somaclones (cv. Grand Naine) and control plants (cv. Grand Naine) at 48 h after inoculation with *Fusarium oxysporum* f. sp. *cubense* subtropical race 4. A- Blank control (slide unstained with aniline blue). B- Con-

trol plant. C- Embrapa 10. D- Embrapa 13. E- Embrapa 14. F- Embrapa 15. G- Embrapa 16. The red arrows indicate blue-green fluorescence associated with callose concentration in the tissues

revealed the presence of these compounds in all evaluated plants, with the formation of small dark brown staining spots in root tissue observed from 48 HAI (Fig. 6). The results indicate that the somaclones produced a higher quantity of phenolic compounds compared to the control plant. Somaclones E13 and E16 showed higher phenolic compound production than the control plant from 48 HAI onward, with sustained production throughout the evaluation periods. The somaclones E10, E14, and E15 showed higher phenolic compound production compared to the control plant, with the highest yield observed from 15 DAI onward. The increase in these compounds over time demonstrates their role in lignification and the formation

of chemical barriers that can limit infection progression, enhancing the resistance of somaclones. A detailed analysis of root whitening and staining in Foc STR4 structures revealed a pattern of resistance to Foc infection among the studied somaclones. In the control plant, fungal structures of Foc STR4 were observed starting at 15 DAI, with initial visualization of chlamydospores and hyphae (Fig. 7). At 30 and 90 DAI, hyphae became visible, indicating infection progression. In contrast, somaclones E10 and E14 showed chlamydospores only at 90 DAI, suggesting initial resistance that delayed fungal colonization. Somaclones E15 and E16 also produced chlamydospores by 30 DAI, but not earlier, indicating resistance comparable to that of somaclones E10 and E14.

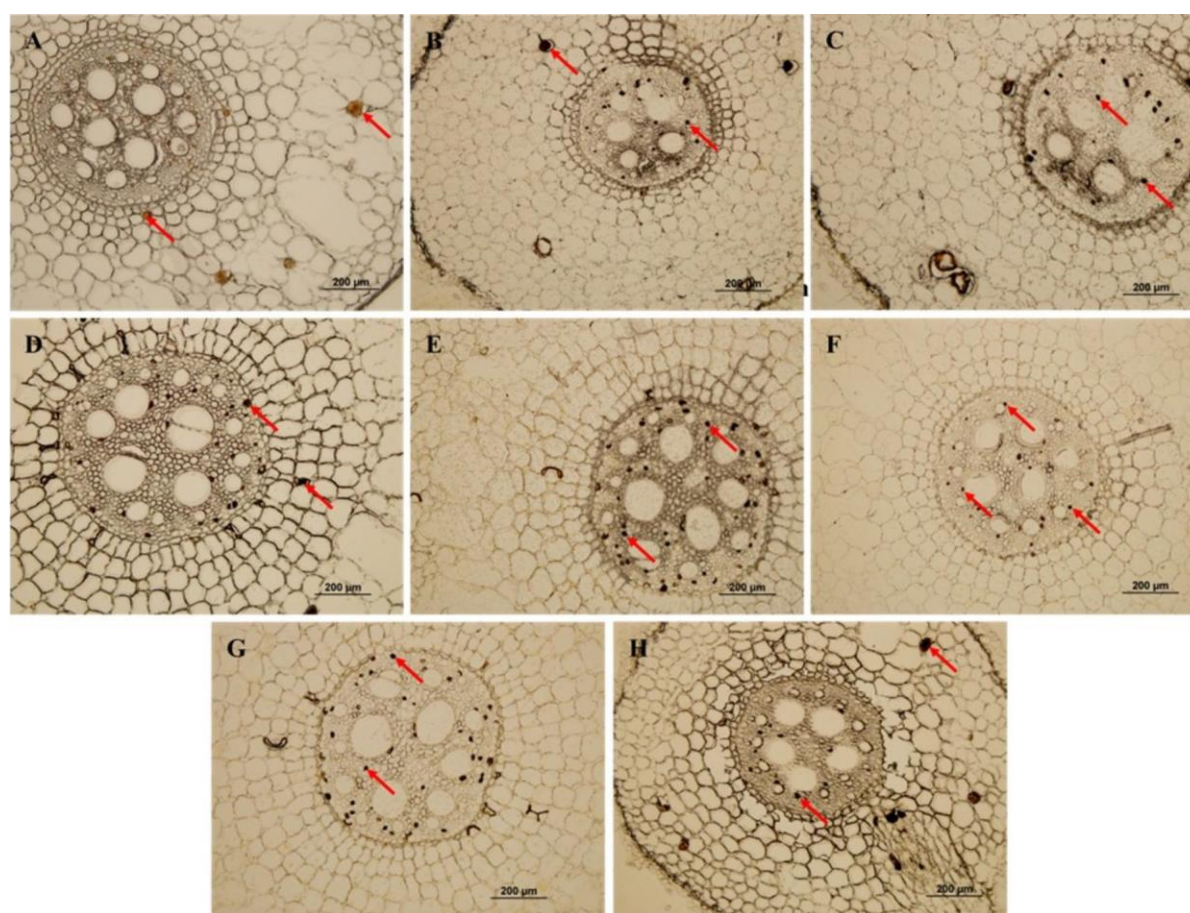


Fig. 6 Histochemical test using ferric chloride to detect phenolic compounds in roots of banana somaclones (cv. Grand Naine) and control plants (cv. Grand Naine) after inoculation with *Fusarium oxysporum* f. sp. *cubense* ST4. A- Blank test. B- Control (C) 48 HAI and 15 DAI. D- Embrapa 10, 15 DAI.

E- Embrapa 13, 48 HAI. F- Embrapa 14, 15 DAI. G- Embrapa 15, 15 DAI. H- Embrapa 16, 48 HAI. The red arrows indicate dark brown staining points associated with phenolic compound concentration in the root tissues

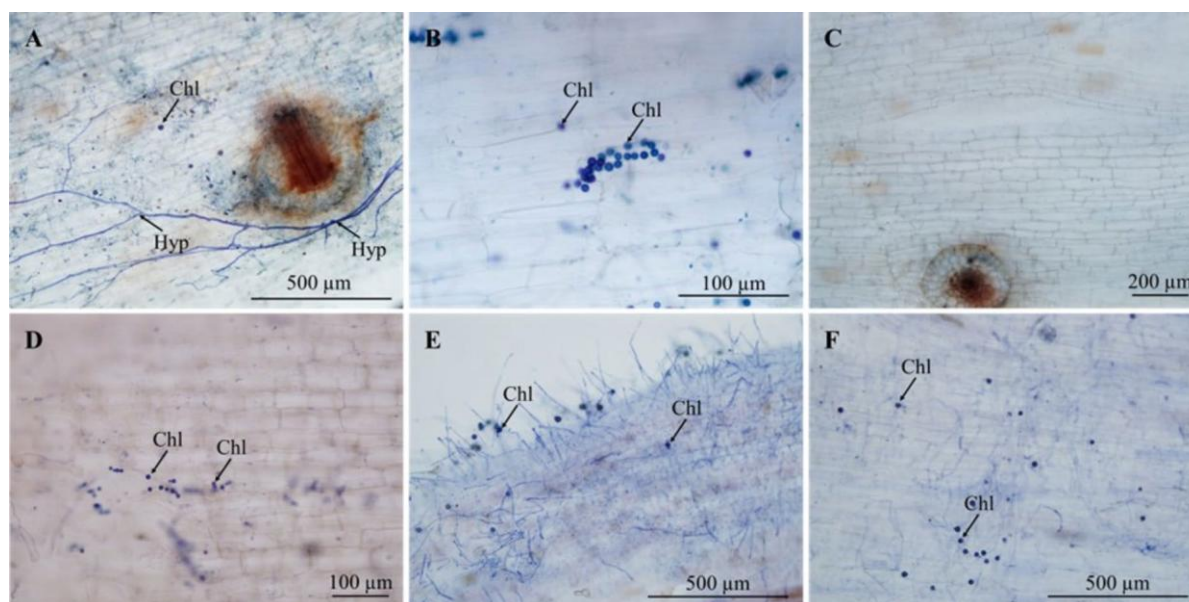


Fig. 7 Root bleaching and fungal structure staining with trypan blue in somaclone roots and control plants of the Grand Naine cultivar after inoculation with *Fusarium oxysporum* f. sp. *cubense* subtropical race 4. A- Control plant 30 DAI. B-

Embrapa 10, 90 DAI. C- Embrapa 13, 90 DAI. D- Embrapa 14, 90 DAI. E- Embrapa 15, 30 DAI. F- Embrapa 16, 30 DAI. The arrows indicate hyphae (Hyp) and chlamydospores (Chl)

However, it is important to highlight the performance of somaclone E13, which showed no fungal structures at any evaluated time point, suggesting either greater resistance or an effective barrier against pathogen penetration and colonization throughout the experimental period.

Discussion

In this study, molecular techniques and histochemical analyses were used to enhance our understanding of the genetic and physiological factors involved in the defense response of Grand Naine banana somaclones resistant to *Fusarium oxysporum* f. sp. *cubense* (Foc) race 4 Subtropical (Foc STR4). IRAP marker analysis was essential for assessing the genetic stability of somaclones compared to the control plant. Furthermore, the assessment of pathogenesis-related gene expression, combined with histochemical analyses, enabled the identification of molecular responses, and physiological and structural changes associated with the defense mechanisms activated during pathogen infection.

Retrotransposon-based DNA markers can enhance the detection of somaclonal variations, particularly when associated with stress-induced transposable element mobilization in the genome under in vitro conditions (Mirani et al. 2020; Kalendar et al. 2021). In the present study, the application of the PCR-IRAP technique to the 14 somaclones revealed a polymorphic band only in somaclones E7 and E8. These results indicate that the in vitro culture process was insufficient to induce detectable variations in most of the analyzed loci, suggesting high genetic stability in the evaluated somaclones compared to the control plant. Such genetic stability strongly suggests phenotypic conservation in the somaclones compared to the control plant (Fig. 1). Our findings are consistent with those of Ferreira et al. (2024), who, while analyzing resistant somaclones of the Prata Catarina cultivar using IRAP, REMAP, and ISSR markers, also reported high genetic similarity between the somaclones and the control plants from which they were derived.

Although IRAP markers did not reveal polymorphism in most somaclones, confirming their high genetic stability, symptom analysis revealed a clear distinction between susceptible control plants and

resistant somaclones (Fig. 1). This difference was corroborated by molecular and histochemical analyses, which highlighted the defense mechanisms activated in somaclones. These findings are consistent with the studies conducted by Rocha et al. (2022), who reported similar susceptibility profiles in the Grande Naine cultivar to Foc STR4.

Although nine somaclones showed initial signs of infection (scores ranging from 0 to 1), five of them—specifically E10, E13, E14, E15, and E16—showed no visible symptoms (Fig. 2-B). The absence of rhizome alterations in these somaclones suggests an effective defense response against Foc STR4 infection, given that the plants were under high selection pressure. This resistance was confirmed by gene expression analyses and histochemical tests, which demonstrated the activation of key genes involved in defense responses and the deposition of structural barriers, such as callose and phenolic compounds. These results are consistent with the findings of Rebouças et al. (2021), who also reported no symptoms in somaclones following exposure in inoculation tests with Foc STR4.

Although the 14 somaclonal variants had previously been identified as resistant to Foc STR4, a new evaluation was necessary to more selectively identify highly resistant somaclones characterized by the absence of internal symptoms. These somaclones served as the basis for gene expression and histochemical analyses, allowing deeper understanding of the defense mechanisms involved. This additional step not only confirmed the results of preliminary screenings but also ensured that only somaclones with complete resistance were investigated in greater details. This strategy strengthens data robustness and contributes significantly to breeding programs by enabling the identification of promising materials with high potential for durable resistance to Fusarium wilt.

In the quantitative analysis of the DI, where control plants showed an average DI of 40%, resistant somaclones showed significantly lower values, ranging from approximately 5% to 13%, indicating substantial resistance to the isolate (Fig. 2). In contrast, the highly resistant somaclones E10, E13, E14, E15, and E16 showed no infection symptoms (score zero). These results highlight the genetic variability among the 14 somaclones and suggest that the highly

resistant somaclones represent a promising source of genetic resistance against Foc STR4.

To investigate defense responses at the gene expression level in the five highly resistant somaclones, the expression of 12 genes involved in the defense response against Foc STR4 was analyzed using RT-qPCR. Among these are genes responsive to pathogen-associated molecular patterns (PAMPs), pathogenesis-related (PR) genes, genes involved in phytohormone-mediated signaling, transcription factors (TFs), resistance (R) genes, and a gene facilitating water transport across the membrane. In most somaclones, the expression of these genes was significantly higher than in the susceptible control, indicating their involvement in the resistance of these somaclones to Foc STR4 (Figs. 3 and 4).

The upregulation of the CHI gene in somaclones E14 and E15 exceeded that observed in the control plant at 12 and 48 HAI (Fig. 3). This result under-scores the importance of the CHI gene in defending these somaclones against Foc. It is known that chi-tinases are enzymes that degrade chitin present in fungal cell walls, resulting in the formation of oligomers. These oligomers are recognized by plant pattern recognition receptors (PRRs), triggering defense signaling pathways against pathogens (Kumar et al. 2018; Niu et al. 2018; Dong et al. 2020). The high expression levels of these genes may have contributed to inhibiting Foc proliferation and enhancing defense responses in these somaclones. Our findings are consistent with those of Fang et al. (2023), who observed a significant increase in chitinase levels in ‘Guijiao 9’ bananas in response to Foc TR4 infection.

Upon recognition of PAMPs, protein kinases mediate the transmission of extracellular signals into the cell, inducing appropriate cellular responses (Cakir & Kılıçkaya, 2015). In this study, the NAK gene was downregulated in most somaclones, particularly at 48 and 72 HAI (Fig. 4), suggesting that this gene does not play a central role in the defense response against Foc STR4. These results are consistent with Dong et al. (2020) where the serine/threonine-protein kinase gene in banana plants infected by Foc races

1 and 4 showed significant regulation in response to race 1 infection, while race 4 induced only minor changes. This suggests that the effectors secreted by Race 4 may inhibit the expression of this gene. Further studies involving other kinase receptors are

needed to elucidate the role of these proteins in the defense response of these somaclones against Foc.

The PI2O6 and RPM1 genes belong to the NBS-LRR family and play a crucial role in recognizing pathogenic effectors and activating effector-triggered immunity. In this study, PI2O6 showed upregulation in all somaclones, surpassing the control plant at 24 HAI (Fig. 4). Similarly, RPM1 showed high expression levels during the same period, but only in somaclones E10, E13, and E15 (Fig. 4). These results suggest that both genes significantly contribute to somaclone resistance by mediating the recognition of Foc STR4-specific effectors and activating plant immune responses. Similar findings were reported by Álvarez-López et al. (2022), who identified numerous NBS-LRR genes in the genome of *Musa acuminata* ssp. *malaccensis*, reinforcing the importance of these genes in pathogen defense and in the molecular recognition specificity of plant-pathogen interactions.

The balance between growth and defense in plants is regulated by a complex network of phytohormones (Cheng et al. 2019). In the present study, the expression patterns of phytohormone-related genes involved in plant defense responses—namely, jasmonic acid (JA), salicylic acid (SA), and auxin—showed significant variations in somaclones during Foc infection. The LOX gene, involved in JA synthesis, was upregulated in all somaclones. The high expression of this gene indicates an increased concentration of this phytohormone in the somaclones, suggesting that LOX acts as a key gene in the defense response against Foc (Fig. 3). This finding is consistent with Li et al. (2012), who found that high expression levels of LOX-like genes in resistant mutant plants were associated with elevated endogenous JA levels compared to the wild type.

Regarding the genes associated with SA accumulation, only PR1 was found to be upregulated in somaclones E10, E13, and E16. These results suggest that, in addition to JA, the significant accumulation of SA and its role in activating pathogenesis-related genes may have enhanced these somaclones' resistance to Foc STR4. This behavior is consistent with the findings of Van Den Berg et al. (2007), who reported the upregulation of *PR1* in the somaclone GCTCV-218 of "Giant Cavendish," resistant to Foc TR4, selected in Taiwan. On the other hand, the reduced expression of *ATL1*—a gene associated with auxin transport—in the somaclones observed at 72 HAI may have been a

plant strategy to prioritize defense over growth under pathogenic stress. Evidence suggests that pathogens can manipulate auxin biosynthesis in host plants to redirect resources for their own benefit, thereby weakening the plant's defense response (Domingo et al. 2009; Kidd et al. 2011; Qi et al. 2012; Cheng et al. 2019).

During exposure to stress, TFs play a key role in stress mitigation or other adaptive mechanisms by modulating gene expression patterns (Aamir et al. 2018). In our study, the genes PTI6 and WRKY22 showed distinct expression patterns compared to the control across the evaluated time points. The PTI6 gene showed upregulation in all analyzed somaclones, with expression levels significantly higher than the control, particularly at 48 HAI. The WRKY22 gene showed upregulation at 48 HAI only in somaclones E10, E14, and E15 (Fig. 4). Compared to previous studies, our results for PTI6 differ from those reported by Sum et al. (2019), who observed high regulation of this gene only in the late stages of Foc infection in the resistant cultivar Guijiao 9. These data suggest that early activation of PTI6 in the evaluated somaclones played a decisive role in initiating the defense mechanisms required to suppress the pathogen.

The RPPL1 gene encodes a protein similar to *RPP13*, an *Arabidopsis thaliana* gene involved in the response to pathogenic infections (Sun et al. 2019; Allen et al. 2008). In this study, RPPL1 showed significantly higher upregulation in somaclones E10, E13, E15, and E16 compared to the control plant at 24 HAI (Fig. 4). This result reinforces the resistance of these somaclones to Foc STR4, consistent with the findings of Sun et al. (2019), where the banana cultivar Guijiao 9 showed high expression of *RPPL1* throughout the experiment, conferring resistance to Foc TR4.

The obstruction of xylem vessels impairs water transport to the aerial parts of plants, making it one of the major contributing factors to Fusarium wilt. To minimize these symptoms, aquaporins—membrane channel proteins—can help by facilitating water transport and the selective flow of small solutes. Evidence suggests that aquaporin-encoding genes play a key regulatory role in plant-pathogen interactions (Li et al. 2020).

In this study, the PIP2-3 gene showed significantly higher expression in all somaclones compared to the control plant (Fig. 3). Our findings suggest that

PIP2-3 enhances water transport efficiency across cell membranes, promoting homeostasis under Foc infection-induced stress. These findings are consistent with Hemmati (2017), who reported increased aquaporin expression in Foc-infected “Cavendish” bananas, linking these proteins to water stress response and solute mobilization, which enhances plant defense.

In addition to revealing varying levels of expression in defense-related genes, this study also demonstrated an increase in the production of post-formed compounds, which act as structural barriers in the first line of defense against pathogens. Recent studies indicate that in the *Musa* spp. × Foc interaction, higher production of callose and phenolic compounds is closely associated with defense responses in resistant cultivars (Ming et al. 2021; Rocha et al. 2022).

The deposition of callose in the analyzed somaclones indicated an early activation of defense mechanisms compared to the control plant, with increased callose production as early as 48 HAI (Fig. 7). These data corroborate our histochemical staining analyses of fungal structures, indicating that callose deposition in root vascular tissues may have played a crucial role in inhibiting Foc colonization during early infection stages—particularly in the complete resistance observed in the E13 somaclone. Similar results were reported by Santana et al. (2024), who also observed higher callose deposition in resistant banana plants following interaction with Foc.

Analysis of roots stained with ferric chloride confirmed the presence of phenolic compounds in all evaluated plants, with higher production in somaclones compared to the control plant. Somaclones E13 and E16 stood out due to their high and increasing levels of phenolic compounds throughout the evaluation periods (Fig. 7). These data indicate that these somaclones show faster and more efficient activation of chemical defenses in response to Foc infection. Similar resistance mechanisms in *Musa* spp. have been widely documented (Ming et al. 2021; Soares et al. 2022), suggesting that high phenolic compound production is associated with lignification, which strengthens cell walls and enhances their resistance to pathogen-induced enzymatic degradation.

Analysis of Foc STR4 staining patterns in root tissues revealed significant differences in the somaclones’ response to infection. While the control plants showed high susceptibility, with pathogen structures visible as early as 15 DAI, most

somaclones showed chlamydospores only in the final stages of evaluation, indicating that early defenses delayed pathogen development. Visibly, only the somaclone E13 showed no fungal structures at any of the evaluated time points (Fig. 7), suggesting the use of highly effective physiological and biochemical barriers that prevented both pathogen penetration and colonization. Histochemical analyses supported this observation, revealing higher deposition of callose and phenolic compounds in this somaclone. These results demonstrate that the defense mechanisms of the somaclones were able to contain the infection’s progression, even in the presence of the pathogen. Similar results were reported by Chen et al. (2019), which also observed the presence of Foc hyphae in a resistant banana cultivar even in the absence of symptoms.

Conclusion

The 14 somaclonal variants of the Grand Naine banana cultivar, developed by Embrapa, were evaluated for genetic stability and resistance to Foc STR4. The IRAP markers used in this study were ineffective in detecting significant genetic variability among the somaclones, highlighting the need to employ other DNA markers to confirm this finding. Among the studied somaclones, E10, E13, E14, E15, and E16 were identified as potential resistance sources for banana genetic improvement, with E13 standing out. The genes LOX, PIP2-3, PI2o6, and PTI6 emerged as potential molecular markers for identifying and selecting Foc-resistant genotypes in a breeding program. Somaclones E10, E13, and E15 showed enhanced performance in regulating resistance genes and activating defense mechanisms. In particular, somaclone E13 showed higher callose deposition and phenolic compound accumulation, along with the absence of fungal structures in root tissues, positioning it as a promising candidate for resistance to Foc TR4. However, further studies are needed to validate the resistance of these somaclones under infested field conditions and ensure the preservation of desirable agronomic traits. This work will be conducted in collaboration with AgroSavia in Colombia, as the somaclones have been sent to that country and

are currently in quarantine. Following phytosanitary clearance, they will be challenged with Foc TR4.

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Declarations

Conflicts of interest The authors declare no conflict of interests.

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CONSIDERAÇÕES FINAIS

Os resultados desse trabalho reforçam o potencial da variação somaclonal induzida por TDZ para gerar plantas de bananeira com resistência completa à murcha de *Fusarium*. Contudo, várias frentes de investigação permanecem abertas e podem aprofundar significativamente o entendimento dos mecanismos envolvidos, bem como acelerar a aplicação prática destes somaclones.

Do ponto de vista genômico, o sequenciamento completo dos somaclones resistentes surge como um passo essencial para identificar mutações estruturais, variações no número de cópias (CNVs) e potenciais marcadores SNP associados à resistência. Estes dados permitirão ainda estabelecer correlações diretas entre eventos de variação somaclonal e fenótipos de defesa observados, criando bases sólidas para o desenvolvimento de ferramentas moleculares de seleção.

Complementarmente, análises proteômicas e metabolômicas deverão ser realizadas para caracterizar, de forma abrangente, a mobilização de proteínas e compostos bioquímicos envolvidos na resposta à Foc. A identificação de proteínas regulatórias, enzimas de defesa, compostos fenólicos e padrões de fosforilação permitirá compreender a resposta celular rápida dos somaclones e validar moléculas-chave que distinguem materiais resistentes de suscetíveis. Além disso, estudos transcriptômicos focados na interação entre esses somaclones e o patógeno poderão revelar os genes diferencialmente expressos associados à resistência, ampliando a compreensão dos mecanismos moleculares envolvidos e possibilitando a identificação de marcadores funcionais para programas de seleção assistida.

É fundamental, também, expandir o estudo agrônomo desses materiais, incluindo avaliações de produtividade, vigor, estrutura da planta e qualidade pós-colheita, além de análises sensoriais e físico-químicas dos frutos. Essas etapas garantirão que as variações somaclonais não comprometam atributos comerciais relevantes e que os somaclones possam ser considerados para uso em sistemas produtivos.

Para consolidar a durabilidade da resistência, futuros trabalhos devem incluir desafios com isolados mais agressivos, provenientes do banco da Embrapa, bem como com diferentes raças fisiológicas de Foc. Ensaio em diferentes locais em condições naturalmente infetadas, incluindo países onde a raça tropical 4 já está presente, como a Colômbia, serão essenciais para validar a estabilidade da resistência. Além disso, recomenda-se que, a partir do terceiro ciclo em campo, o material seja reintroduzido *in vitro* para avaliar a estabilidade das variações induzidas, seguido de novos ensaios em casa de vegetação.

Outro eixo importante consiste na otimização dos protocolos de indução de variação somaclonal, com a finalidade de aumentar a eficiência da obtenção de variantes, reduzir a frequência de mutações indesejáveis e permitir a aplicação da técnica em outros genótipos Cavendish e não-Cavendish. Ajustes finos na concentração de TDZ, alternância com outros fitoreguladores, tempo de exposição, condições de cultura e fases de regeneração poderão resultar em protocolos mais reprodutíveis e direcionados.

Por fim, dada a importância global da bananicultura, principalmente das cultivares Cavendish e o caráter internacional da ameaça representada pela TR4, sugere-se a integração desses somaclones em uma rede de avaliação internacional, envolvendo Colômbia, Equador e Costa Rica, países parceiros do programa de melhoramento da bananeira da Embrapa. Esta abordagem colaborativa permitirá validar a eficácia dos materiais em diferentes ambientes, sistemas produtivos e pressões de inóculo.