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MILEIDE DOS SANTOS FERREIRA

DESENVOLVIMENTO E CARACTERIZAÇÃO DE VARIANTES SOMACLONAIS DE BANANEIRA RESISTENTES À MURCHA DE FUSARIUM

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

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Coorientadora: Dra. Anelita de Jesus Rocha
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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 membros signatários abaixo.

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Resumo

As bananas desempenham um papel crucial na alimentação de milhões de pessoas e são fonte de renda para milhares de famílias em vários países. No Brasil, o Estado de São Paulo se destaca como o maior produtor, seguido pela Bahia e Minas Gerais. As bananeiras do subgrupo Prata, como ‘Prata-Anã’, ‘Gorutuba’ e ‘Catarina’, são os principais genótipos cultivados no país, formam a base da produção brasileira, representando aproximadamente 70% da área cultivada, principalmente para o consumo doméstico. A murcha de Fusarium, causada pelo fungo *Fusarium oxysporum* f. sp. *cubense* (Foc), é reconhecida como uma das doenças mais devastadoras para a cultura da bananeira em todo o mundo. A Raça Tropical 4 (TR4) destrói as plantações de banana em quaisquer situações ambientais, já a Raça Subtropical (SR4) causa danos quando as plantas são submetidas a algum estresses em condições ambientais subtropicais. Com isso, programas de melhoramento genético da bananeira em todo o mundo têm utilizado diversas tecnologias para gerar novos genótipos resistentes. Diante deste contexto, o trabalho teve como objetivo inicial elaborar uma revisão sistemática sobre o papel da variação somaclonal no melhoramento genético vegetal. Assim, um protocolo foi criado e rigorosamente seguido para cumprir as diretrizes estabelecidas nos itens preferenciais de relatórios para revisões sistemáticas e meta-análises (PRISMA). A análise dos 219 artigos selecionados revelaram que o número de subcultivos e os reguladores vegetais são as principais fontes de variação somaclonal in vitro. A revisão sistemática reuniu ainda informações sobre alterações morfológicas nos somaclones gerados e ferramentas para sua identificação, enriquecendo o conhecimento sobre essa técnica. Este trabalho também teve como objetivo induzir variação somaclonal in vitro em bananeiras da cultivar Prata Catarina para selecionar somaclones resistentes ao Isolado 229 de Foc. Foram selecionados 13 somaclones resistentes entre os dois tratamentos testados. As avaliações histológicas e histoquímicas podem indicar que houve a ativação de mecanismos de resistência pós-formados. As análises moleculares indicaram não haver diferenças genéticas entre os somaclones e a planta comercial, indicando que as características agronômicas comerciais não foram afetadas. Em conclusão, este trabalho reúne informações sobre a técnica de variação somaclonal e suas contribuições, oferecendo novas fontes de resistência à murcha de Fusarium para o programa de melhoramento genético da bananeira.

Palavras-chave: *Musa* spp.; somaclones; variação somaclonal; melhoramento genético.

ABSTRACT

Bananas play a crucial role in the diet of millions of people and are a source of income for thousands of families in various countries. In Brazil, the state of São Paulo stands out as the largest producer, followed by Bahia and Minas Gerais. Banana plants of the Prata subgroup, such as ‘Prata-Anã,’ ‘Gorutuba,’ and ‘Catarina,’ are the main genotypes cultivated in the country, forming the basis of Brazilian production and representing approximately 70% of the cultivated area, mainly for domestic consumption. Fusarium wilt, caused by the fungus *Fusarium oxysporum* f. sp. *cubense* (Foc), is recognized as one of the most devastating diseases for banana cultivation worldwide. Tropical Race 4 (TR4) destroys banana plantations in all environmental conditions, while Subtropical Race (SR4) causes damage when plants are subjected to some stress in subtropical environmental conditions. Consequently, banana breeding programs worldwide have used various technologies to generate new resistant genotypes. In this context, the initial aim of this work was to conduct a systematic review on the role of somaclonal variation in plant breeding. A protocol was created and rigorously followed to meet the guidelines established in the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA). The analysis of the 219 selected articles revealed that the number of subcultures and plant growth regulators are the main sources of in vitro somaclonal variation. The systematic review also gathered information on morphological changes in the generated somaclones and tools for their identification, enriching the knowledge of this technique. This work also aimed to induce in vitro somaclonal variation in Prata Catarina banana plants to select somaclones resistant to Foc Isolate 229. Thirteen resistant somaclones were selected from the two tested treatments. Histological and histochemical evaluations may indicate the activation of post-formed resistance mechanisms. Molecular analyses indicated no genetic differences between the somaclones and the commercial plant, suggesting that commercial agronomic characteristics were not affected. In conclusion, this work gathers information on the technique of somaclonal variation and its contributions, offering new sources of resistance to Fusarium wilt for the banana breeding program.

Keywords: *Musa* spp.; somaclones; somaclonal variation; genetic improvement.

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INTRODUÇÃO GERAL

As bananas representam uma das frutas mais consumidas em todo o mundo e desempenham um papel fundamental na alimentação de milhões de pessoas (Pico et al., 2019; Kb et al., 2024). Em 2022, a liderança na produção global de bananas foi da Índia, com 34,5 milhões de toneladas, seguida pela China, que registrou 11,8 milhões de toneladas. O Brasil ocupou o quinto lugar, com uma produção de 7 milhões de toneladas (FAOSTAT, 2024).

Segundo dados do IBGE (2022), o valor da produção nacional em 2022 foi de 11,9 bilhões de reais. O Estado de São Paulo liderou como o maior produtor, com uma produção avaliada em 1,705 bilhões de reais, seguido pelo Estado da Bahia, com 1,388 bilhões, e Minas Gerais, com 1,179 bilhões. As bananeiras do subgrupo Cavendish são produzidas com maior frequência para exportação no Brasil, enquanto as do grupo Prata são cultivadas principalmente para consumo doméstico.

Apesar da grande escala de produção, a bananicultura enfrenta desafios decorrentes de doenças que limitam a sua produção (Cordeiro et al., 2016). Destaque para aquelas causadas por fungos, as quais resultam em perdas significativas tanto na qualidade, quanto na produção da fruta. Entre essas enfermidades estão a Sigatoka amarela (*Pseudocercospora musicola*, Leach), a Sigatoka negra (*Pseudocercospora fijiensis*, Morelet) e a Murcha de Fusarium (*Fusarium oxysporum* f. sp. *cubense*) (Ploetz, 2015; Pegg et al., 2019; Ploetz, 2021).

A murcha de Fusarium, causada por *Fusarium oxysporum* f. sp. *cubense* (Foc), é reconhecida como uma das doenças mais devastadoras que afeta a cultura da banana em todo o mundo (Maryani et al., 2019). Esse patógeno está amplamente disseminado e é encontrado em praticamente todas as regiões produtoras de banana. Fusarium é um fungo habitante do solo que forma estruturas de resistência, conhecidas como clamidósporos, o que possibilita sua sobrevivência por décadas (Pegg et al., 2019). Foc foi subdividido em raças distintas, sendo a raça 4, especialmente a raça Tropical 4 (TR4), altamente patogênica, que causa uma maior preocupação para a indústria da banana (Ploetz, 2015).

O Foc TR4 está disseminado em muitos países da Ásia, África e Oceania, mas até o momento não foi detectado no Brasil, sendo classificado como uma praga quarentenária pelo Ministério da Agricultura e Pecuária do país. Entretanto, a doença foi oficialmente identificada na América Latina, com casos registrados na Colômbia em agosto de 2019, no Peru em abril de 2021 e na Venezuela no início de 2023 (García-Bastidas et al., 2020; Acuña et al., 2021; Mejías Herrera et al., 2023; Martínez et al., 2024).

Diante disso, o desenvolvimento de variedades resistentes é considerado como o método mais eficaz para mitigar os danos causados pelo fungo. Programas de melhoramento genético da bananeira em todo mundo, vêm desenvolvendo diversas tecnologias para gerar novos genótipos de bananeira resistentes (Hwang e Ko, 2004; Molina, 2016; Ferreira et al., 2020; Amorim et al., 2021; Rebouças et al., 2021; Rocha et al., 2022). A aplicação de ferramentas biotecnológicas tem impulsionado os estudos genéticos da bananeira, destacando-se técnicas como engenharia genética, indução de mutações e variação somaclonal.

Dentre as ferramentas citadas, a indução de variações somaclonais tem sido reconhecida como uma importante estratégia para gerar variabilidade genética e como uma alternativa para o melhoramento genético da bananeira. Isso possibilita a seleção de somaclones com características desejadas. A seleção de somaclones em espécies de propagação vegetativa, como as bananeiras, desempenha um papel crucial na multiplicação em larga escala de genótipos superiores. Esse processo permite preservar sua identidade genética e proporciona aos produtores um acesso rápido a esses genótipos melhorados (Amorim et al., 2021).

A variação somaclonal surge ao submeter a planta a estresses em condições de cultivo in vitro, o que podem ocasionar distúrbios durante a divisão celular, resultando em variações genéticas ou epigenéticas (Imran et al., 2021). As mudanças de origem genética são herdadas por gerações subsequentes, enquanto as mudanças epigenéticas correspondem a variações transitórias, devido ao estresse fisiológico sofrido in vitro (Anil et al., 2018; Penna et al., 2019; Penna et al., 2023). O número de ciclos de subcultivo e a influência de reguladores vegetais contribuem para a formação de variantes somaclonais no cultivo in vitro (Amorim et al., 2021; Ferreira et al., 2023).

A indução de variação somaclonal em bananeira tem se mostrado eficaz na geração de somaclones resistentes a murcha de Fusarium. Um exemplo notável é o trabalho realizado pelo Instituto de Pesquisa em Banana de Taiwan (TBRI) que obtiveram resultados promissores ao induzir variação somaclonal em bananas do subgrupo Cavendish. Identificaram plantas resistentes a Foc TR4, como o somaclone GCTCV-218, o qual foi posteriormente registrado para cultivo comercial sob o nome de Formosona, além de duas outras variantes somaclonais denominadas GCTCV-53 e

GCTCV-119 (Hwang e Ko, 2004; Molina, 2016). A Empresa Brasileira de Pesquisa Agropecuária (Embrapa) obteve resultados promissores no cultivo de bananeiras ao desenvolver somaclones do subgrupo Cavendish por meio da indução de variação somaclonal (Rebouças et al., 2021).

A aplicação da técnica de indução de variação somaclonal se mostra útil em programas de melhoramento genético direcionados à obtenção de variedades resistentes, ou com melhores características agronômicas, como também o desenvolvimento de variedades ornamentais (Bulbarela-Marini et al., 2023; Baloch et al., 2023; Bredy, 2023; Parrish et al., 2023; Patel et al., 2023; Pop et al., 2023; Zhang et al., 2023; Hernández et al. 2024). Há uma importância em se buscar conhecimento sobre esse tema, incluindo o papel da variação somaclonal no melhoramento genético, informações sobre os reagentes utilizados para induzir variação somaclonal, o número de subcultivos envolvidos, as culturas que empregam essa técnica e outros dados pertinentes.

Portanto, o objetivo desta pesquisa foi: 1) Produzir uma revisão sistemática da literatura publicada nos últimos dezesseis anos sobre o papel da variação somaclonal no melhoramento genético de plantas; 2) Induzir variação somaclonal em bananeiras da cultivar Prata Catarina, com o intuito de desenvolver novos genótipos de bananeiras resistentes à murcha de Fusarium.

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Capítulo 1

The Role of Somaclonal Variation in Plant Genetic Improvement: A Systematic Review

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Review

The Role of Somaclonal Variation in Plant Genetic Improvement: A Systematic Review

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Abstract: The instability of in vitro cultures may cause genetic and epigenetic changes in crops called somaclonal variations. Sometimes, these changes produce beneficial effects; for example, they can be used in breeding programs to generate new cultivars with desirable characteristics. In this article, we present a systematic review designed to answer the following question: How does somaclonal variation contribute to plant genetic improvement? Five electronic databases were searched for articles based on pre-established inclusion and exclusion criteria and with a standardized search string. The somaclonal variation technique has been most frequently applied to ornamental plants, with 49 species cited in 48 articles, and to the main agricultural crops, including sugarcane, rice, banana, potato and wheat, in different countries worldwide. In 69 studies, a technique was applied to evaluate the genetic diversity generated between clones, and, in 63 studies, agronomic performance characteristics were evaluated. Other studies are related to resistance to pathogens, ornamental characteristics and resistance to abiotic stresses. The application of the plant growth regulators (PGRs) benzylaminopurine (BAP) and dichlorophenoxyacetic acid (2,4-D) was the most common method for generating somaclones, and randomly amplified polymorphic DNA (RAPD) molecular markers were the most commonly used markers for identification and characterization. Somaclonal variation has been used in genetic improvement programs for the most economically important crops in the world, generating genetic diversity and supporting the launch of new genotypes resistant to diseases, pests and abiotic stresses. However, much remains to be explored, such as the genetic and epigenetic mechanisms from which somaclonal variation is derived.

Keywords: somaclones; genetic improvement; tissue culture; somaclonal variation; DNA markers

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1. Introduction

Plant diseases caused by phytopathogens cause losses to the global economy of more than 220 billion dollars annually [1]. At least 70 billion dollars are lost due to invasive pests worldwide, not to mention the loss of biodiversity caused by pathogens. In addition, abiotic factors such as water deficit, salinity and temperature extremes cause approximately 30 billion dollars in losses to global agriculture. This reality threatens the food security of several countries and harms small farmers and individuals living in regions where food security has not yet been achieved [1].

Therefore, genetic improvement programs seek ways to reduce the impacts caused by diseases, pests and abiotic stresses on agricultural crops through the development of resistant or tolerant cultivars. In order to achieve this goal, different strategies are used. Plant cell and tissue culture are traditionally used for the production, conservation and improvement of plant resources from an asexual process where clonal multiplication is expected to generate genetically uniform plants [2,3]. However, Braun [4] made the first observation and report of variation originated in cell and tissue cultures defined as somaclonal variation [5]. This has been one of the biggest longstanding problems, i.e., obtaining the genetic fidelity of plants from tissue culture in vitro [6]. However, in 1981, Larkin and Scowcroft identified somaclonal variation as a potential for crop enhancement, and this was later documented by other researchers [3,7,8].

Since then, new somaclones from different cultures with characteristics useful for breeding, such as resistance to pathogens, tolerance to abiotic stresses and high productivity, have been launched [9–13]. Somaclonal variation, in which clones of genetically identical plants have different phenotypes after regeneration, was observed in most explants subjected to micropropagation. It is more evident when cells are propagated in culture for long periods of time and when explants/micropropagated plants suffer several subsequent subcultures. The first studies involved genetic and epigenetic variations, which led to the hypothesis that plant growth hormones, such as auxins and cytokinins, could be responsible for these genetic changes observed in plants [14–16].

Rai [17] discussed the source and genetic basis of somaclonal variation, its detection methods and the advantages of this tool for agriculture, with the main emphasis on some useful somaclonal variants released as cultivars. Other studies have reviewed the potential application of somaclonal variants in the improvement of horticultural crops [18] and described the current status of understanding the genetic and epigenetic changes that occur during tissue culture [19]. To summarize the current status of knowledge generated on somaclonal variation in plant breeding, this article presents a systematic review (SR) of studies conducted in the last 16 years. The approach presented here makes use of the SR tool, which provides a summary of all the relevant evidence available on the applications of this tool in plant breeding. The main countries that work on somaclonal variation, the somaclones of various cultures generated globally, the purposes of the generated somaclones, the methods for induction of somaclonal variation, the number of subcultures, PGRs most used in the induction of somaclonal variation and their doses, the explants preferentially used, the main phenotypic characteristics observed in the somaclones, the molecular markers frequently used in the studies to detect somaclonal variation and information on the gene expression of some somaclones generated are presented.

2. Materials and Methods

This review was constructed based on preferred reports for SR and meta-analyses (PRISMA) using the open access software State of the Art by SR (Start) v.3.3 Beta 03; the three main steps used were planning, execution and summarization.

In the planning stage, a protocol was built https://doi.org/10.5281/zenodo.7674327 (accessed on 12 February 2023) to monitor the entire review process. The following features were defined: title, objective, keywords, research questions, research sources, research period covered and criteria for the inclusion/exclusion of articles. The main research question guiding the SR was as follows: How does the somaclonal variation technique contribute to plant genetic improvement? Based on this question, the secondary questions, which are described in Table 1, were defined.

Table 1. List of questions about the use of somaclonal variation as a tool in the genetic improvement of agricultural crops to be answered by a systematic review of articles published in the last fifteen years.

Research Questions
Q1. In which cultures has the somaclonal variation technique been applied?
Q2. For what purposes is the somaclonal variation technique applied?
Q3. What PGRs and doses are most used to generate somaclonal variants?
Q4. How many subcultures were made to generate somaclones?
Q5. In which countries is the somaclonal variation technique most often applied?
Q6. Which somaclones have already been generated?
Q7. What are the most frequent changes observed in the phenotypic characteristics of somaclones?
Q8. What molecular tools are used to characterize somaclonal variants?

The execution stage consisted of three phases: research, selection and extraction. The electronic searches were performed using a search string defined with the following keywords: "plant breeding" AND "somaclonal" OR "somaclone variation". This search string was designed to cover the largest possible number of articles on the subject and was used to identify articles in five databases: Web of Science (http://apps.isiknowledge.com) (accessed on 15 February 2020), PubMed (http://www.ncbi.nlm.nih.gov/pubmed) (accessed on 15 February 2020), Springer (https://www.springer.com/br) (accessed on 15 February 2020), Portal of Journals CAPES (http://www.periodicos.capes.gov.br/) (accessed on 15 February 2020). and Google Scholar (https://scholar.google.com.br/schhp?hl=en&as_sdt=0,5) (accessed on 15 February 2020). Each database was searched for articles published over a period of 16 years. Some documents were considered relevant but were published after the selection stage, so they were added manually. The results were exported in the BIBTEX, MEDILINE or RIS formats compatible with Start software.

We used a protocol for the development of the SR, and the search terms were based on the four PICO inclusion components (i.e., population, intervention, comparison, outcome and study type) [20] (Table 2).

Table 2. Definition of the PICO terms for the research question addressed in this study of somaclonal variation over the last 16 years.

Description	Abbreviation	Components of the Question
Population	P	Agricultural crops that were studied or for which somaclones were generated.
Interest/intervention	I	Somaclonal variation for plant breeding.
Comparison	C	Studies of plant breeding methods used to generate somaclones with agronomic traits.
Outcome	O	Overview of the technique of somaclonal variation in plant breeding.
Type of study	S	Scientific articles.

Initially, in the selection phase, only the title, abstract and keywords were read, and the articles that contained the terms defined in the search string within these features were selected. In the extraction phase, the articles were read in full, and the articles were accepted according to the predefined inclusion (I) and exclusion (E) criteria: (I) articles that contain in the title, abstract or keywords the terms plant breeding and somaclonal or somaclonal variation; (E) articles published in languages other than English; (E) articles that deviate from the topic; (E) review articles; (E) theses, dissertations and manuals; (E) book chapters; (E) articles published in annals of events; and (E) articles on the evaluation of plant fidelity after in vitro multiplication.

In the summarization step, graphs, tables, word clouds and bibliometric maps were generated to compose an SR. The frequencies of articles were calculated for the questions described in Table 1. The graphs were generated in R software [21] with the ggplot2 and dplyr packages. The bibliometric analyses were performed using VOSviewer_1.6.17 software [22].

Risk of bias

To evaluate the risk of bias among the articles selected for this SR, we adapted the Cochrane risk of bias tool protocol [23]. Three authors (MSF, AJR and FSN) evaluated the quality of the methods used to select the included studies, and the questions used to assess the risk of bias were the same as those developed for the protocol (found in Table 1). The studies were classified according to the number of questions answered that contributed to the SR. Three classifications were adopted:

1. Low risk of bias (low)—articles that answered 100% of the proposed questions.
2. Moderate risk of bias (moderate)—articles that answered up to 60% of the questions.
3. High risk of bias (high)—articles that answered up to 30% of the questions.

In addition, all the PRISMA guidelines were carefully followed; the PRISMA checklist is available for download at https://doi.org/10.5281/zenodo.7674859 (accessed on 20 February 2020).

3. Results

3.1. Screening of Studies

Figure 1 represents the PRISMA flow diagram used to screen the articles analysed in this review. The Web of Science was the database that contributed most to this review, with 1192 articles (27%). PubMed Central contributed 1069 articles (25%), followed by Google Scholar with 1010 (23%), Springer with 997 (23%) and the CAPES journal portal with 75 (2%) articles. Eleven important articles were manually added to this review because they reported the generation and study of somaclones with resistance to diseases, abiotic stresses and agronomic and molecular aspects [12,13,15,24–31]. In total, 4351 articles were identified in the databases, of which 882 were duplicates and 3725 were eliminated in the selection process. In the extraction phase, 629 articles were read in full, and 410 were excluded because they did not meet the inclusion criteria. A total of 219 articles were selected for this SR. The manuscripts were stored in an open access digital library available at https://doi.org/10.5281/zenodo.7641768 (accessed on 22 February 2023).

3.2. Bibliometric Analysis

A bibliometric map was made from the titles of the accepted articles (n = 219) (Figure 2A). There was a predominance of the terms somaclonal variation, somaclonal variant and somaclone between 2010 and 2015, which indicates a trend of publications during this period. The term RAPD (Randomly Amplified Polymorphic DNA) was also predominant in studies published between 2005 and 2015, showing that this molecular technique was used in previous studies and that new approaches related to molecular markers are possibly being adopted nowadays (Figure 2A). A second bibliometric map revealed the five journals with the largest numbers of publications on the theme of somaclonal variation; Plant Cell and Tissue and Organ Culture had the most publications, followed by the African Journal of Science and Technology, In Vitro Cellular and Developmental Biology—Plant, Plant Cell Reports and Euphytica (Figure 2B).

3.3. Main Countries and Cultures Evaluated

Studies on somaclonal variation in plant breeding were found in 42 countries, but most are concentrated in India (43) (Figure 3). Other countries that published a relatively high number of articles on the subject were Pakistan (18), China (18), Egypt (14), Brazil (12), Iran (11), the United States (10), Poland (10) and South Korea (9). Countries with fewer than 10 published articles are represented in bright green in the map shown in Figure 3. Regarding the agricultural crops studied, 82 species were evaluated, separated by crop types and summarized in Table S1. The plant species that are among the 10 most important crops in terms of production, according to data from the Food and Agriculture Organization (FAO) of the United Nations, were not separated. The other species were classified by cultivation type: fruits (9 species and 26 articles); forage, grasses and cereals (16 species and 21 articles); vegetables, roots and tubers (7 species and 17 articles); medicinal (13 species and 15 articles), condiments and spices (4 species and 9 articles); and ornamental (24 species and 45 articles) (Table S1). The most studied species were sugarcane (30), rice (18), banana (13), potato (10) and wheat (11) (Table S1).

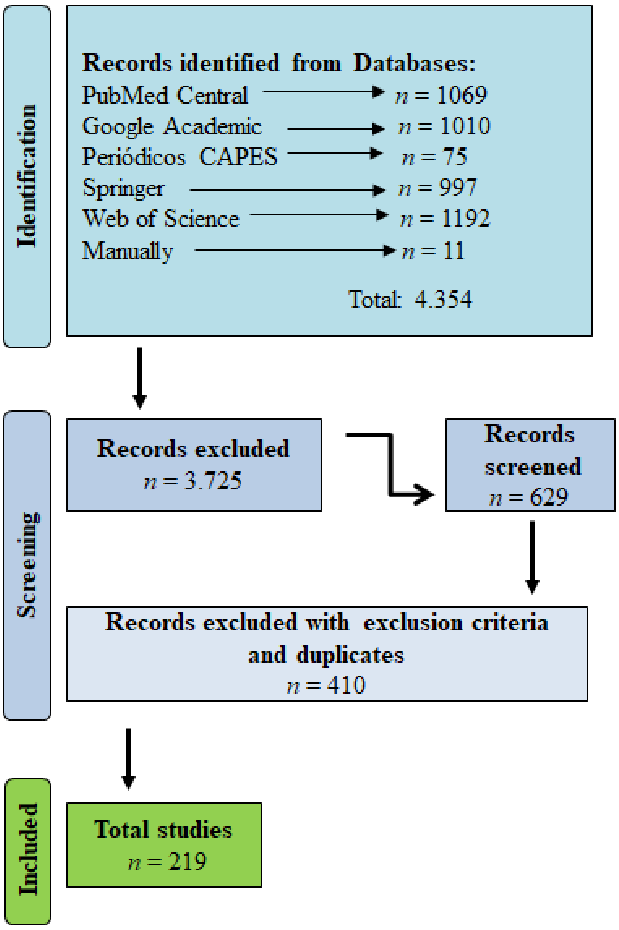

Figure 1. PRISMA flowchart. Process of selecting articles for inclusion or exclusion in a systematic review of the application of the somaclonal variation technique for plant genetic improvement; *n* = number of articles.

In India, the largest numbers of studies have been conducted on sugarcane (14), medicinal plants (8) and forage, grasses and cereals (7); in Pakistan, sugarcane (14) and potato (5); in China, rice (6) and ornamental plants (5); in Egypt, potato (3), vegetables, roots and tubers (3) and wheat (3); in Iran, fruits (4); in Brazil, ornamental plants (7) and fruits (2); in the United States and South Korea, ornamental plants (6, 4); and in Poland, vegetables, roots and tubers (Figure 3).

The largest number of somaclones has been generated for sugarcane (16), followed by ornamental plants (14), banana (9), medicinal plants (6), wheat (6) and rice (5) (Figure 4). Other crops generated a lower number of somaclones, such as millet (4), strawberries (4), pineapples (3), cactus (3) and potato (3) (Figure 4). A smaller number of somaclones was generated for other crops.

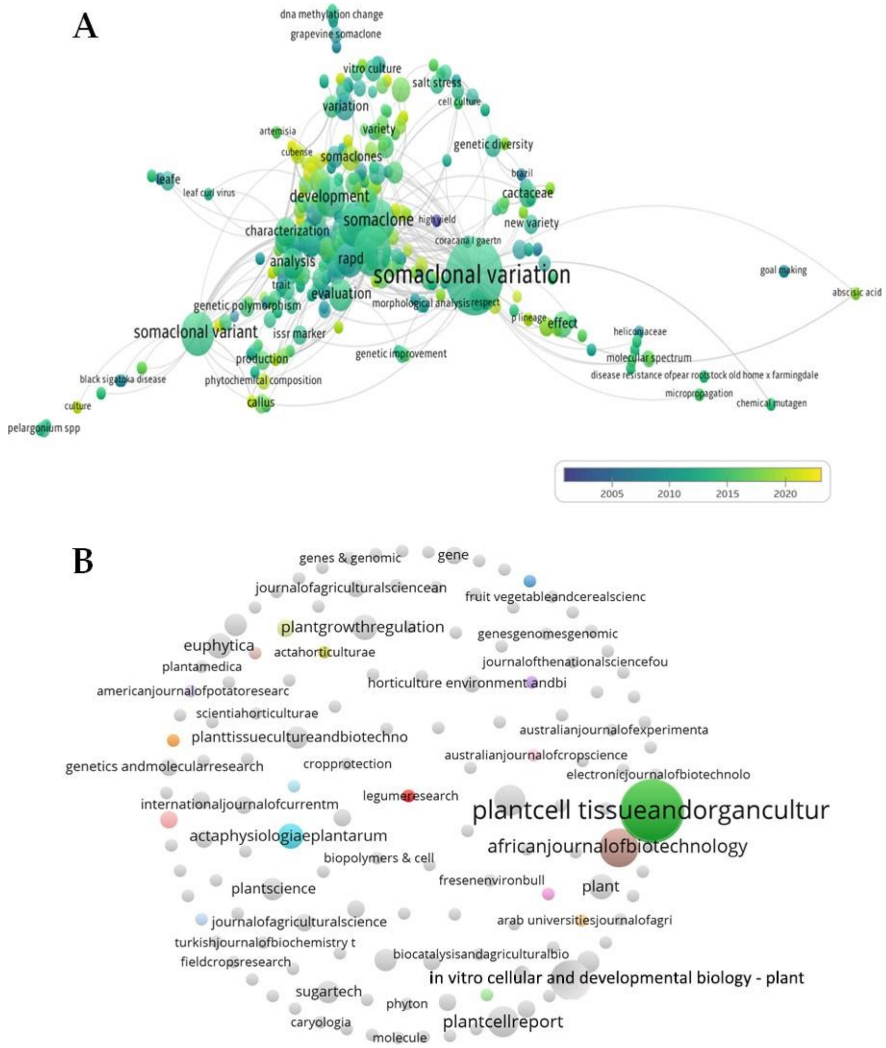

Figure 2. Biometric maps of manuscripts in the last 16 years regarding somaclonal variation in plant genetic breeding. Frequency of keywords (**A**); Frequency of Scientific Journals that published the most (**B**).

3.4. Methods for Inducing Somaclonal Variation

Regarding the method used to induce somaclonal variation, 154 articles mentioned only PGRs to induce variation. In 65 articles, previously generated somaclones were studied, and the method used for their generation was not reported (Figure 5). A higher number of studies was directed to evaluate the somaclones in the context of existing genetic diversity (69), followed by studies on agronomic traits for genetic improvement (63), pathogen-resistant somaclones (29), somaclones with ornamental characteristics (22), tolerance to salinity (17), tolerance to abiotic stress (10) and tolerance to water deficit (9) (Figure 5).

Of the articles that reported the use of PGRs, 68 reported benzylaminopurine (BAP), 62 dichlorophenocytic acid (2,4-D); 40 acetic α-naphthalene acid (NAA); 25 kinin (KIN/KT), 23 idolacetic acid (IAA); 15 reported indole-3-butyric acid (IBA); and 12 tiazuron (TDZ). Sixty-five articles did not mention the use of PGRs, as they evaluated only somaclones previously generated in other studies (Figure 6). The most used PGRs to generate somaclones with desirable agronomic characteristics in molecular studies of genetic diversity and pathogen resistance were BAP, 2,4-D and NAA, respectively (Figure 6). IAA was mainly used to promote variations related to resistance to pathogens; KIN, IBA and TDZ were used to induce variation in order to obtain the molecular characteristics of genetic and agronomic variability generated in somaclones (Figure 6).

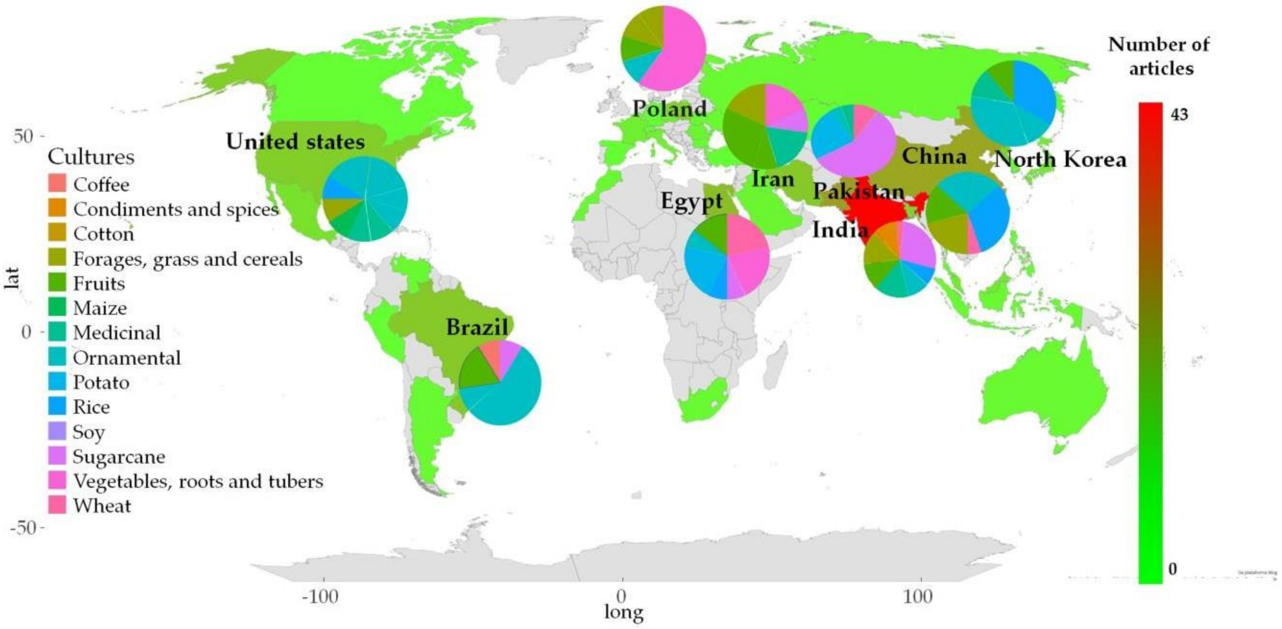

Figure 3. Number of articles published on somaclonal variation around the world in the last 16 years and main plant species studied. The countries shown in the light green colour have a lowest number of articles about somaclonal variation. The medium to intense green colours represent countries with approximately 21 studies on somaclonal variation, and the red colour represents countries with a higher number of studies on somaclonal variation.

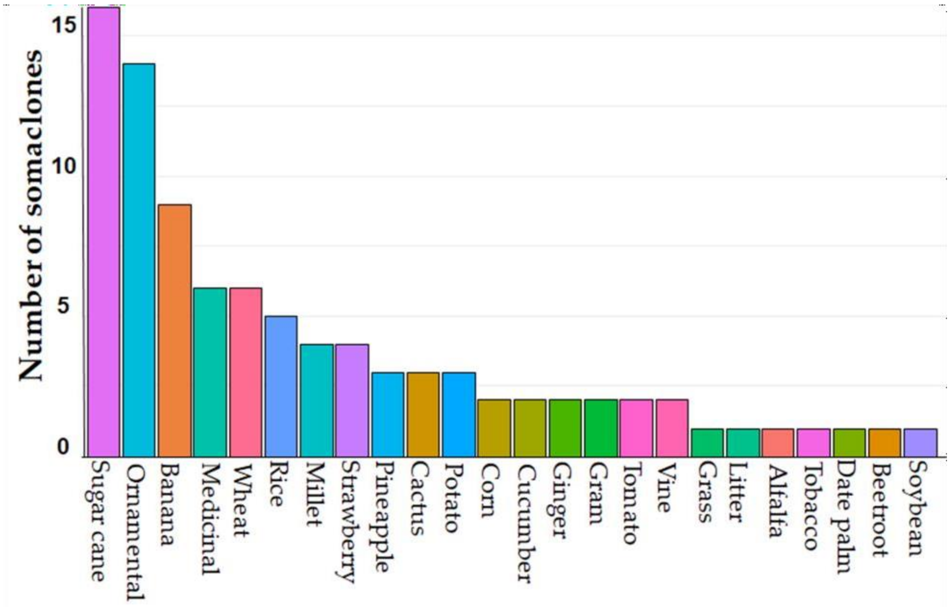

Figure 4. Number of generated somaclones separated by culture in the studies included in a systematic review of the application of somaclonal variation in plant genetic improvement.

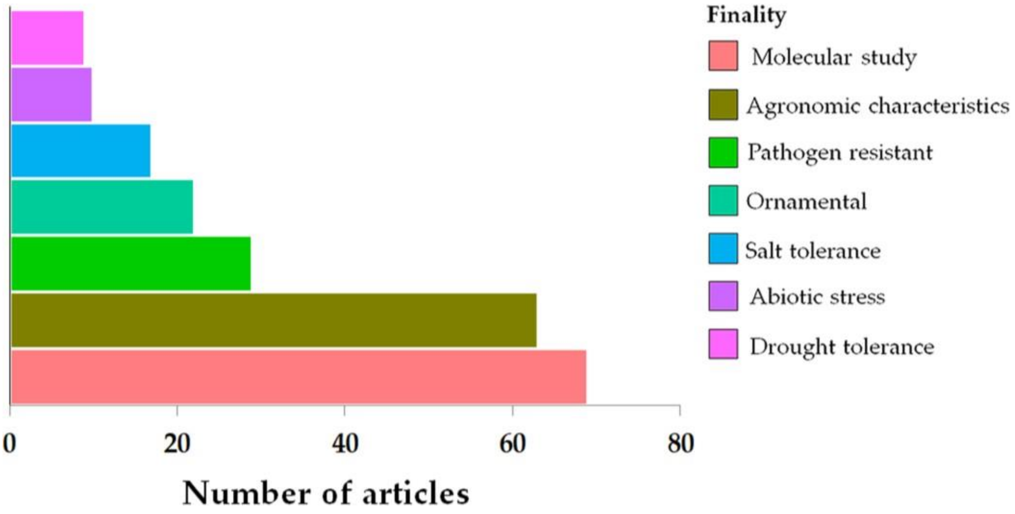

Figure 5. Number of articles separated by the reported purpose for generating somaclones. The data were generated for a systematic review of the application of somaclonal variation in plant genetic improvement.

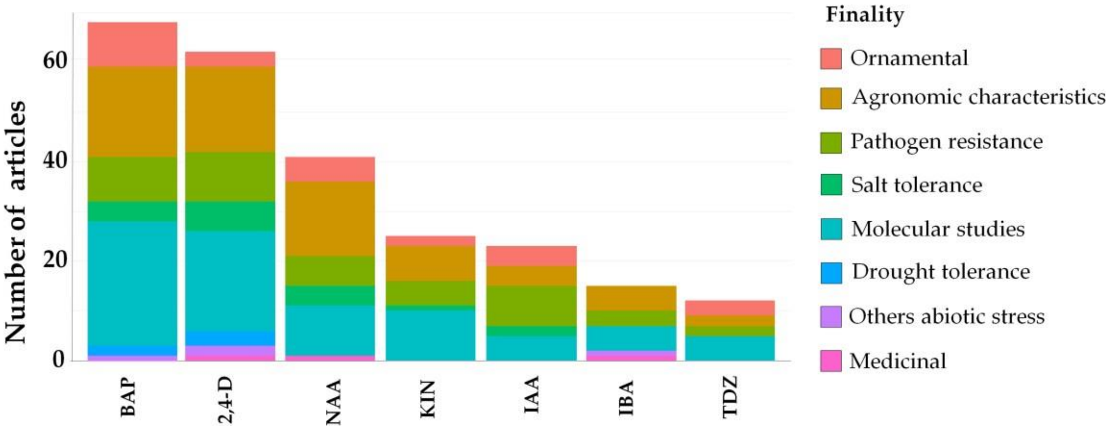

Figure 6. Most commonly used PGRs and the purpose of inducing somaclonal variation. benzylaminopurine (BAP); Dichlorophenoxyacetic acid (2,4-D); α-naphthalene acetic acid (NAA); kinetin (KIN/KT); idolacetic acid (IAA); indole-3-butyric acid (IBA); thidiazuron (TDZ). The data were obtained from the study of 219 articles included in an SR of the application of somaclonal variation in plant breeding.

There was high variation between the doses of the PGRs applied in the different manuscripts, varying from 0.01 mg/L to 16 mg/L (Figure 7). In general, the most reported doses of PGRs varied between the PGRs, whereas BAP presented the highest number of different doses applied per manuscript followed by 2,4-D and NAA (Figure 7). The most applied doses for the BAP were 1 mg/L (23), 2 mg/L (21), 0.05 mg/L (17) and 3 mg/L (9). For the 2,4-D, the most applied doses were 2 mg/L (26), 1 mg/L (18) and 3 mg/L (10) (Figure 8). The most applied doses for the NAA were 1 mg/L (13), 0.05 mg/L (11), 2 mg/L (9) and 0.1 mg/L (8). The KIN was mostly applied in doses of 0.05 mg/L (8), 1 mg/L (8) and 2 mg/L (7); IAA was preferably applied in doses of 2 mg/L (8) and 1 mg/L (5). The most applied doses for the TDZ and IBA were 1 mg/L (8, 6) and 2 mg/L (4, 5), respectively (Figure 7).

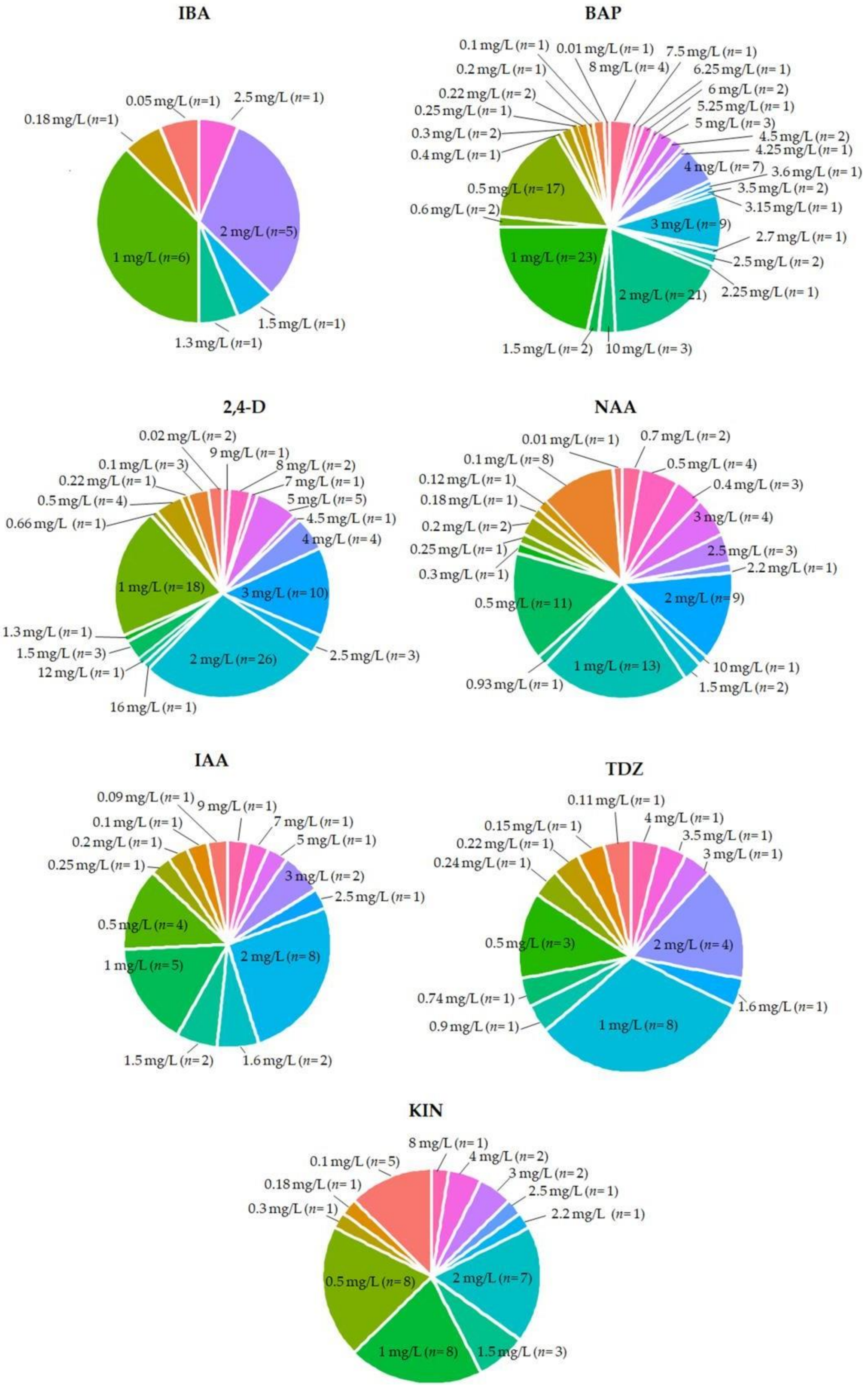

Figure 7. The most commonly used doses of PGRs to generate somaclonal variants in vitro. Dichlorophenoxyacetic acid (2,4-D); benzylaminopurine (BAP); idolacetic acid (IAA); indole-3-butyric acid (IBA); kinetin (KIN/KT); α-naphthalene acetic acid (NAA); thidiazuron (TDZ). The data were obtained from the study of 219 articles included in a systematic review on the application of somaclonal variation in plant breeding.

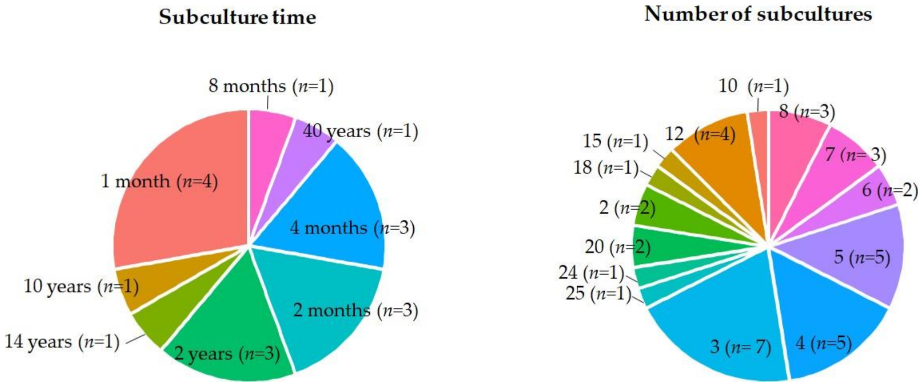

Figure 8. Pie charts summarizing the data of subculture time and number of subcultures in published articles in the last 16 years recognized in the systematic review: The role of somaclonal variation in plant genetic improvement: a systematic review.

Of the articles inserted in this SR, 17 referred to the time of subculture in months or years, ranging from one month to 40 years. In this case, five studies reported that the subcultures were carried out for one month and some subcultures for two months, four months and two years; both reported in three articles. The other subculture times were reported in only 1 article, such as 40, 14 and 10 years and 8 months (Figure 8). The studies that made clear the number of subcultures totaled 38; within these studies, the highest number recorded was 25 subcultures, and the lowest was only 2 subcultures (Figure 8). The number of subcultures recorded in most articles were three (7), four (5) and five (5).

3.5. Types of Explants

Among the sources of explants used, most articles mentioned leaves, except in studies of the species Vitis vinifera, Vanilla planifolia, Pisum sativum, Pennisetum glaucum and plants belonging to the family Poaceae and Orchidaceae. Seeds were the second most used source of explants, and this type of explant was most common among species belonging to the family Orchidaceae, Triticum species and other crops. In the articles inserted, the most reported cultures where somaclones were produced include Saccharum officinarum, species belonging to the Orchidaceae family and species belonging to the genus Musa. Leaves, seeds and rhizomes were also used as sources of explant (Figure 9).

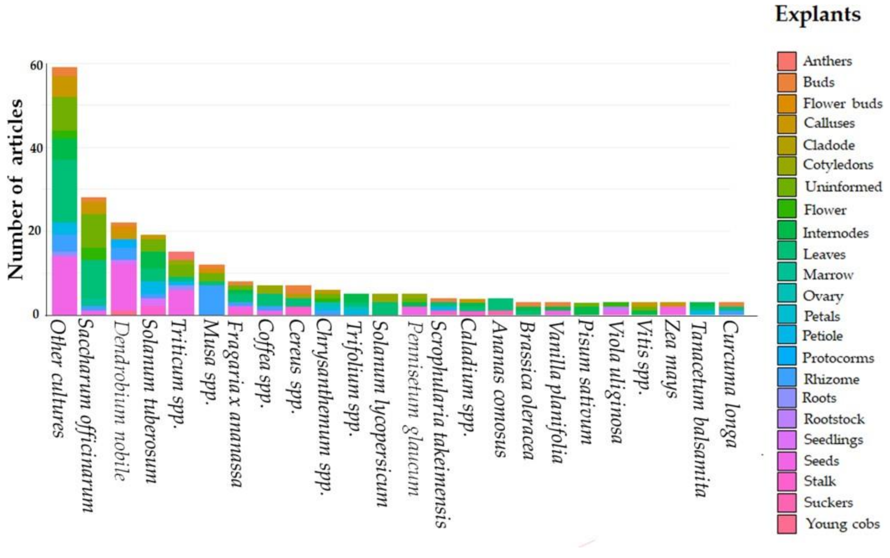

Figure 9. Most frequently used explants for induction of somaclonal variation per culture. The data were obtained from 219 articles included in the review of the application of somaclonal variation for plant breeding.

3.6. Phenotypic Modifications

Regarding the most frequent phenotypic modifications in somaclones, 69 studies described phenotypic modifications caused by genetic variation in several cultures (Table 3). Phenotypic changes were observed in plant structure, pigmentation, roots, stems, pseudostems, flowers, leaves, fruits and seeds. Several studies have described morphological changes in leaves, especially changes in colour and length, as detailed in Table 3. Regarding the plant structure, the articles that reported phenotypic changes referred to the presence of dwarf plants in different crops, such as pineapple, coffee and banana (Table 3).

Table 3. Morphological characteristics associated with the somaclonal variation event in different cultures.

Crop	Plant Part	Phenotypic Characteristic	Articles
Hedychiummuluense	Structure	Dwarf plant	[32]
Coffea arabica L.		Dwarf plant	[33]
Wheat spp.		Dwarf plant	[34]
Pineapple (*Ananas comosus* (L.) Merr.)		Dwarf plant	[35]
Pineapple (*Ananas comosus* (L.) Merr.)		Dwarf plant	[36]
Pineapple *Ananas comosus var.* MD2		Dwarf plant	[37]
Millet genotype 5141 B	Pigment	Albino plant	[38]
Wheat (*Triticum aestivum* L.)		Albino plant	[39]
Sweet potato (*Ipomoea batatas* (L.) Lam.)	Root	Reduction in number and compliance	[40]
Date palm (*Phoenix dactylifera* L.)		Increase in length	[41]
Wheat (*Triticum aestivum* L.)		Increase in length	[42]
Sugarcane (*Saccharum officinarum*) (VSI 434)	Stem	Colour variation	[43]
Sugarcane (*Saccharum spp.)* variety CoJ 64		Increase in diameter and length	[44]
Sugarcane (NIA-1198)		Greater length and number of internodes	[45]
Sugarcane (*Saccharum* spp.)		Biggest diameter	[46]
Sugarcane (*Saccharum* spp.)		Larger diameter and length	[47]
Sugarcane (*Saccharum* spp.)		Longer length	[48]
Sugarcane (*Saccharum* spp.)		Larger diameter and length	[49]
Sugarcane (*Saccharum* spp.) variety BL4		Increase in quantity, smaller diameter and greater length	[50]
Cymbopogon winterianus		Biggest diameter	[51]
Sugarcane (*Saccharum* spp.)		Longer length	[52]
Musa cv 'Williams', 'Ziv' e 'Grand Naine'	Pseudostem	Longer length	[53]
Musa cv. 'Grand Naine'		Colour variation	[54]
Musa cv 'FHIA-18´ (AAAB)		Appearance and colour	[55]
Coffee (*Coffea arabica* L.)	leaves	Larger number of leaves and larger leaf area	[56]
Dieffenbachia cv. 'Camouflage', 'Camille' and 'Star Brigh'		Leaf variegation, longer leaves and lanceolate leaves	[57]
Chrysanthemum *(Dendranthema grandiflora (Ramat.)* Kitam)		Variegated, marbled, pale green leaves	[58]
Tobacco (*Nicotiana tabacum*) variety 'Kanchan'		Larger number, length and width of the sheet	[59]
Scrophularia takesimensis		Leaf variegation	[60]
Orchid (*Dendrobium sonia*-28)		Narrow, pointed leaves	[61]

Table 3. *Cont.*

Crop	Plant Part	Phenotypic Characteristic	Articles
Japanese butterbur (*Petasites japonicus*)		Leaf blade size and leaf blade colour	[62]
Rice (*Oryza sativa* L.)		Rolling leaf	[63]
Japonica rice (*Oryza sativa* subsp. *japonica*)		Purple leaf sheath	[64]
Dieffenbachia cv. Camouflage		Variation in leaf colour	[65]
Sweet Potato (*Ipomoea batatas* (L.) Lam.)		Mature leaf shape and foliage colour Leaf petiole shorter and thicker Blade larger and lighter green colour	[66]
Rice (*Oryza sativa* L.)		Sheet width	[67]
Caladiums (*Caladium × hortulanum Birdsey*)		Variation in leaf colour	[68]
Epipremnum aureum 'Marble Queen'		Completely green, variegated and whitish leaves	[69]
Musa 'Prata Anã'		Absence of red spots on the leaves	[11]
Strawberry (Fragaria × ananassa)		Leaf area and longer leaf petioles	[70]
Augustine grass (*Stenotaphrum secundatum* (Walt.) Kuntze)		Short, narrow leaves	[71]
Sugarcane 'S97US297'		Leaf area	[72]
Dendrocalamus farinosus		Increased sheet length and width	[15]
Rice (*Oryza sativa* L.) 'Pokkali'		Leaf area and dry mass	[73]
Chrysanthemum *(Dendranthema grandiflora (Ramat.)* Kitam)	Flowers	Inflorescence colour and shape deviation	[74]
Phalaenopsis 'Spring Dancer'		Petal diameter	[75]
Doritaenopsis		Colour, complete fusion of lateral sepals with lip and reduced size	[76]
Phalaenopsis 'Wedding Promenade'		Flower width and petal thickness	[77]
Chrysanthemum *(Dendranthema grandiflora (Ramat.)* Kitam)		Inflorescence colour and altered inflorescence shape	[78]
Chrysanthemum *(Dendranthema grandiflora (Ramat.)* Kitam)		Flower colour, size and weight	[79]
Tomato (*Lycopersicon esculentum Mill.*)	Fruits and Seeds	Number of fruits	[80]
Strawberry *(Fragaria × ananassa)*		Number of fruits, fruit shape and difference in texture	[81]
Chili Pepper (*Capsicum Annuum* L.)		Number of fruits and total production of fresh and dried fruits	[82]
Grass pea (*Lathyrus sativus* L.)		Pod width and length, number of pods/plant, number of seeds/pod	[83]
Musa cv. 'Grand Naine'		Bunch length	[84]
Tomato (*Lycopersicon esculentum Mill.*)		Number of bunches, number of fruits/plant, fruit firmness and fruit weight	[85]
Wheat (*Triticum aestivum* L.)		Ear length and grain yield	[86]
Rice (*Oryza sativa* L.) cv PR113		Grains per panicle, grain weight and grain yield per plant	[87]
Sorghum (*Sorghum bicolor* L.)		Increase in seed size and grain yield	[88]
Millet (*Eleusine coracana*)		Grain yield per plant	[89]
Tomato (*Lycopersicon esculentum Mill.*)		Number of fruits	[90]

In relation to changes caused in pigmentation, the presence of albino phenotypes was documented only in millet and wheat crops. For modifications caused in the roots, the potato crop showed a reduction in number and conformity, and date palm and wheat crops showed an increase in root length (Table 3). Changes in the stems were reported mainly for sugarcane where phenotypes with colour variation, smaller diameter or increase in diameter and length were described, and the number of internodes increased (Table 3).

Phenotypic changes in the pseudostem were observed only for banana genotypes with changes in length increase and colour appearance variations. In relation to leaves, the alterations were reported mainly in medicinal plant species to increase substances used for therapeutic and ornamental purposes, where the presence of genotypes with variegation characteristics or alterations in colour and conformity are commercially desirable. Similarly, morphological changes in flowers have been documented only in ornamental plants. On the other hand, changes in fruits and seeds were reported in important food crops, mainly to increase the number of fruits in tomato and grain yields in rice, sorghum and corn (Table 3).

3.7. Molecular Studies

To detect somaclonal variations and analyse the genetic stability of plants grown in vitro, DNA-based molecular markers are the most commonly used approach. Many molecular markers were used in the studies included in this review, which varied according to culture and evaluation purpose (Table S2). As we have already shown in our bibliometric analysis, randomly amplified polymorphic DNA (RAPD) and Intersimple sequence repeat (ISSR) molecular markers were used in most studies by the year 2018, with a change in recent years to a greater number of studies with other markers, such as Methylation Sensitive Amplification Polymorphism (MSAP), Simple Sequence Repeat (SSR), Single Nucleotide Variants (SNV) and Amplified fragment length polymorphism (AFLP) (Figure 10). In the last year, only analyses applying single nucleotide polymorphism (SNPs) markers were reported.

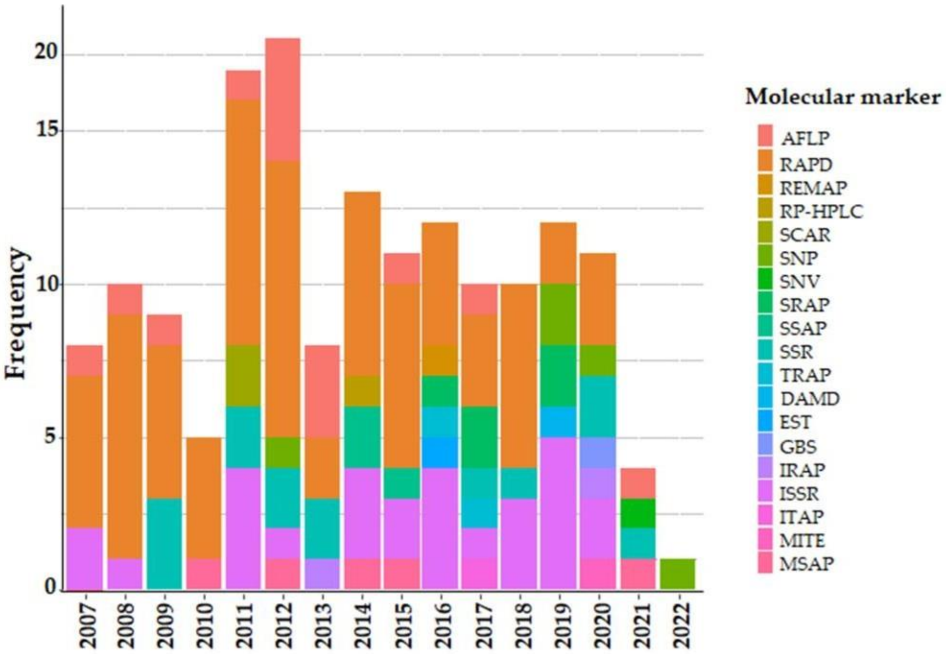

Figure 10. Frequency of molecular markers associated with strategies to identify genetic variation over the last 16 years. The data were obtained from articles included in the systematic review of the application of somaclonal variation in plant breeding.

As expected for the set of inserted articles, the objective of using each of the different molecular markers reported is to verify the mechanism related to somaclonal variation either by methylation in DNA or changes in the sequence of DNA base pairs. Some articles also evaluate, through markers, the presence of mutations (Table S2).

Among the 219 accepted articles, 12 evaluated the gene expression of the generated somaclones. Studies of expression of genes related to disease resistance, ornamental traits, protein expression and other molecular mechanisms are described in detail in Table S3.

A word cloud was made to identify the relevant genes analysed in somaclone studies, where the size of the name of each gene indicates the number of articles that describe the expression of the gene (Figure 11). The most frequent genes were PMADS4, Expansin and OP J-06, respectively. PMADS4 genes are considered higher-order protein complexes, responsible for changes in floral morphology in somaclonal variants. The Expansin gene is related to cell expansion; in the articles of this review, this gene was related to dwarfism events in somaclones. The Op J-06 genes are responsible for the Foc (Fusarium oxysporum f. sp. cubense) resistance response to banana somaclonal variants. Other genes were also noted in the word cloud, which indicates their expression in many studies of this review, such as the TDFs genes that are fragments derived from transcription and the RPK2 genes that are involved in signal transduction. These are in addition to NPR1 genes which function as master regulators of the plant hormone salicylic acid (SA) signalling and play an essential role in plant immunity (Figure 11).

Figure 11. Word cloud of the frequency of genes with differentiated expression shared in manuscripts regarding somaclonal variation in plant breeding.

3.8. *Risk of Bias*

The articles that answered 100% of the questions were classified as having a low risk of bias (180), and the articles that answered up to 60% of the questions were classified as having a moderate risk of bias (39) (Table S4). Manuscripts that answered up to 30% of the questions were not included, as they were considered as having a high risk of bias. The results indicate that the selected articles composing this SR are of high quality.

4. Discussion

4.1. *Screening of Studies*

This SR comprises articles that aimed to generate somaclonal variants or study somaclones generated or marketed in the last 16 years. Therefore, many articles were eliminated in the extraction stage (410) because they dealt only with genetic variability without breeding purposes, where somaclonal variation is labelled in germplasm banks or in seedlings for field planting as an undesirable characteristic; in these cases, the objective is to ensure the genetic fidelity of plants. On the other hand, we included in our SR a set of 219 articles that deal specifically with the use of the technique for obtaining somaclonal variants with desirable characteristics to plant breeding programs. Although our study includes an extensively large number of articles, which makes it difficult to extract and discuss in detail all the data, we try to list the main data obtained in summary form to derive conclusions and tendencies regarding the proposed subject.

Our bibliometric analysis confirmed that the term "somaclone" began to be more frequent in the last two decades, when studies on the induction of somaclonal variation began to be developed for genetic breeding purposes (Figure 2). At that time, several journals that are focused on publications in the areas of tissue culture and biotechnology began to publish articles with terms related to "somaclonal variation" (Figure 3). However, in previous years, the changes from in vitro cultivation described in different studies were tested to evaluate the genetic fidelity of plants in relation to the original plant and did not have the objective of generating somaclones to be applied in the genetic improvement of crops. Thus, the term "somaclones" becomes more frequent in recent years for this purpose [17,91,92]).

4.2. *Cultures Evaluated in Different Countries*

Among the countries that perform studies on somaclonal variation, India stands out as the country with the largest number of studies on this technique and is also the country that has generated the largest number of somaclones in the world, especially for sugarcane (Figure 4). India is the largest producer of sugarcane in the world [1], which may explain why there is a significant number of studies on somaclonal variation in this crop included in this SR.

Raza et al. [50] obtained the same results with somaclones of the BL4 cultivar. In turn, Doule et al. [47] and Nikam et al. [93] obtained somaclones with high Brix values that are useful for commercial cultivation. The sugarcane somaclonal variants Co94012 and VSI434 were developed in India and presented desirable characteristics, such as high yield, high sucrose content and moderate resistance to red rot. Somaclone VSI434 is the second sugarcane cultivar launched in India using somaclonal variation [43].

Ethanol production increased from 662 million litres in 1980 to 61 billion litres in 2018, and it is estimated that in 2022 the demand for ethanol will reach 97 billion litres worldwide. Currently, the United States leads the global ethanol market, followed by Brazil. Brazil is the main producer of sugarcane in the world, responsible for 40% of global production of this crop, which is the main raw material in the Brazilian ethanol industry. The development of sugarcane somaclones may contribute to increased ethanol production, increasing the production of biofuels worldwide [94–97].

A large variety of somaclones have been released for some plant species, especially ornamental plant crops; this sector has wide possibilities due to the great diversity that exists among ornamental species. The climate, altitude, culture of a region, etc., contribute to the genetic diversity among species of ornamental plants in different countries [32,98]. Many somaclones are generated from ornamental plants, especially Chrysanthemum and Cereus, the most common ornamental plants included in this SR [78,79,98]. The genetic variability that occurs in vitro, such as changes in colours, textures and plant size, contributes to the emergence of new phenotypic characteristics, enabling the launch of new ornamental plants and contributing significantly to this agribusiness.

Other crops with somaclones that have been generated for commercial purposes in the global food industry are rice, banana, potato and wheat [29,99]. La Candelaria and Yerua are two rice somaclones that were used as sources of alleles for the development of newstrains with tolerance to salinity [100]. Wheat crops have also generated somaclones with tolerance to this abiotic factor [101]. Other wheat somaclones were allele sources for the development of new somaclone strains with higher root growth under drought tolerancestress [42].

The generation of somaclonal variants allowed the selection and commercialization of some somaclones in certain cultures. In the banana crop, to obtain cultivars of the Cavendish subgroup tolerant to *Fusarium oxysporum* f. sp. *cubense*, tropical breed four (Foc-TR4), Sun et al. [102] identified somaclonal variants and selected nine resistant banana trees that survived in fields severely infested with Foc in China in 2010. Hwang and Ko [103] generated the cultivar 'Formosana' (GCTCV-218), a somaclone of Foc-TR4-tolerant banana, which is already in use by farmers and traders in some Asian countries.

4.3. *Methods for Inducing Somaclonal Variation*

Among the methods used for induction of somaclonal variation, methods that depend on PGRs were cited in 148 studies in the SR. The BAP and 2,4-D at doses of 0.5 mg/L, 1 mg/L and 2 mg/L were the most commonly used. The BAP is a cytokinin used for regulating the growth and development of plants in vitro [14]. The identification of genetic variation in micropropagated plants indicates that BAP has become a tool for breeding programs, since this regulator has been used to induce somaclones with desirable characteristics. The second most used PGR in callus culture processes was 2,4-D, since one of the functions of 2,4-D is to act in callogenesis, which is an important process for the indirect production of plants. Calli contain cells or groups of cells that have active cell division centres. According to Corpes et al. [104], the balance between auxins and cytokinins may directly influence the process of callus formation and development.

The use of these PGRs in high doses, combined with the number of subcultures, causes stress that leads to cellular instability, triggering genetic or epigenetic variations in plants in vitro. Genetic alterations are permanent, usually hereditary and non-reversible, such as changes in DNA base pairs, insertion, deletion or base substitution. Epigenetic changes are changes in the DNA methylation pattern and can be reversible, causing the loss of epigenetic characteristics generated in a plant [17,92,105]. Another factor of paramount importance for studies on the induction of somaclonal variation is the number of subcultures, which directly relates to the stress caused to the plant in vitro and induces genetic variation in plants. The use of PGRs, such as cytokinins and auxins, directly affects the genetic variation in plants subjected to subcultures, providing genetic variability and allowing the selection of traits of interest for breeding programs [14,18,92,106–109].

PGRs and the number of subcultures interfere with the generation of genetic variations in vitro and are of fundamental importance in the induction of somaclonal variation [110]. The combination of a high number of subcultures and a culture medium containing TDZ allowed the selection of somaclones resistant to Fusarium wilt (subtropical race 4, Foc STR4) in the cultivars 'Prata Anã' (*Musa,* AAB) [11] and 'Grand Naine' (*Musa,* AAA) [12]. According to the literature studied, the stem apices were the most popular explants for induction of somaclonal variation in banana. The explant most commonly used to induce somaclonal variation in sugarcane was young leaf meristem tissue [111]. This type of explant is preferable because the formation of embryogenic calli occurs in young leaves close to the meristem, inducing greater genetic variation [112,113]. Another widely used explant was seeds, especially in orchids. The successful use of seeds as explants in in vitro culture is due to the availability throughout the entire year of most crops that can be transformed via callus and have more growth of buds in direct regeneration [114,115].

4.4. *Phenotypic Modifications*

In nature, the appearance of genetic variation occurs more slowly and can occur between hundreds and thousands of years when compared to the induction of in vitro variation. Therefore, some genetic alterations observed in the field may come from micropropagated plants in which the use of PGRs and frequent subcultures occurs [16]. The occurrence of somaclonal variation in micropropagated plants has been studied for many years, and these variations occur in diverse cultures subjected to in vitro cultivation. Somaclones can be identified in a greenhouse, in the field and in vitro by observing changes in plant traits, such as leaf colour, texture, etiolation and other phenotypic changes (Table 2).

Epigenetic changes are responsible for phenotypic changes observed in somaclones, and these changes, such as loss of DNA methylation, may be reversible [15].

DNA methylation in the form of 5-methylcytosine (5mC) is an important epigenetic marker involved in gene expression and plays an important role in plant regulation and development [116]; in plants, it usually occurs in cytosine bases in all sequence contexts [92,117,118]. Although not recorded in our data, genetic and/or epigenetic changes that occur in vitro can also generate chimeras (mosaics). In chimeras, the variations affect the function of chloroplasts in different regions in the plant tissues of the same plant. This event occurs through variations in their plastomas, i.e., the region responsible for governing the expression of genes related to photosynthesis, with this change resulting in an albino phenotype [16]. These changes are responsible for presenting altered morphological characteristics in micropropagated plants.

In our study, we described the phenotypic changes in different parts of micropropagated plants in vitro to obtain somaclones (Table 3). In general, our data demonstrate that the adoption of in vitro micropropagation methods with the use of PGRs BAP, 2,4-D, NAA, TDZ, IAA and IBA at different doses together with successive cultivation has the potential to cause desirable modifications to the genetic improvement of various crops of agricultural and commercial importance.

Many results showed that supplementation with high concentration of 6-benzylaminopurine (4.0 mg/L BAP) alone or combined with indole-butyric acid (IBA) produces a higher percentage of dwarf variants [32–37]. Thus, plants with the dwarf phenotype have been reported for some crops; it serves as a marker for the presence of variations or as an important characteristic to facilitate cultural treatments and management in monocultured species, or as characteristics of ornamental interest [36,37]. In pineapple culture, useful mutants were identified with less spiny leaves that are easier to manage in the field and hence, represent another dwarf phenotype with ornamental value [35]. In wheat crop, a new strain of buckwheat, AS34, was developed by somatic variation and will be useful in wheat breeding programs, particularly because the modification of high commercial varieties reduces the risk of tilting; this is one of the most important agronomic characteristics of wheat [34].

The morphological alterations were seen more in plants of ornamental and medicinal interest. The SVT14 variants of Caladiums (*Caladium×hortulanum* Birdsey) presented rounder and thicker leaves and, in Chrysanthemum (*Dendranthema grandiflora*), changes were described in relation to the number of flowers, flower size, flower weight, leaf weight, stem weight or plant size, as well as a reduction in flowering induction time [68,78,79].

In the tobacco crop (*Nicotiana tabacum*), promising somaclones were developed with variations in the increase in length, width and number of leaves that can contribute to higher productivity of the crop [59]. Morphological changes in fruits and seeds were also found. Our results showed that tomato crop stood out with studies that obtained somaclones with changes in the number of fruits, i.e., an agronomic characteristic of great importance for this crop [80,85,90].

Our results showed promising results for obtaining improved cultivars in relation to grain yield, which is a target characteristic for the genetic improvement of large agricultural crops, such as corn, rice and wheat [86,87,89].

4.5. Molecular Studies

Some changes in the plant genome are not morphologically identified, and even visible changes require molecular evaluation. Thus, molecular markers are often used to identify these variations [3]. Based on polymerase chain reaction (PCR), several molecular markers, such as AFLPs, ISSRs and SSR markers, start codon-directed polymorphisms (ScoTs) and RAPDs, have been used to identify somaclonal variation [41]. The RAPD markers were the most commonly used to identify genetic variation in the studies included in this review [99,119]. According to our data, RAPD marker tests were widely applied to select these variations in micropropagated seedlings mainly up to the year 2018 (Figure 10). Although currently these markers are reported as very variable and are falling into disuse, the adoption of this technique for some time is justified because it is simpler and more economical, and by the ease of application in a less technical laboratory considering that the studies inserted in this SR are since 2007.

In addition, the use of RAPD markers depends on genetic markers located in parts of the DNA sequence, and large amounts of DNA are not required to locate the sequences. These markers are polymorphic and express genetic variations in band imprinting, thus making it possible to perform genetic mapping to indicate genetic diversity in parental genotypes; this is very useful for identifying variants among genotypes in germplasm banks with genetic characteristics that differ from clones of genotypes stored in banks [120,121]. However, we indicate that there may be a tendency to use improvements in the RAPD technique, such as Sequence Characterized Amplified Region (SCAR), DNA amplification fingerprint (DAF) and sequence-related amplified polymorphism (SRAP).

The ISSR marker is a low-cost and highly efficient method that detects very small genetic variations and is widely used in studies of plant genetic diversity and to determine genetic relationships. Similar to RAPD markers, ISSRs are dominant markers and do not require prior sequencing. One of the advantages of the AFLP technique, besides being a low-cost technique, is the detection of a larger number of loci and providing a wide coverage of the genome. AFLP markers are capable of detecting genetic variations such as chimeras and identification of mutants [122–124].

The IRAP and REMAP markers are based on retrotransposons. Retrotransposons move through an RNA molecule, are dispersed throughout the plant genome and can contain thousands of copies, thus contributing to size, structure, diversity and variation in the genome which may affect gene function. The IRAP and REMAP markers are, therefore, considered very efficient molecular markers to investigate genetic variability in plants [125]. Such markers were used to study genetic variation induced by tissue culture in date palms (*Phoenix dactylifera* L.) and alkaligrass (*Puccinellia chinampoensis* Ohwi) [126,127]. Other studies have demonstrated the efficacy of these markers to evaluate genetic diversity and stability in crops such as beans [128], Egyptian barley [129] and date palm [130].

Single nucleotide polymorphism (SNP) can be applied to characterize allelic variation, genome-wide mapping and as a tool for marker-assisted selection. In the last decade, the identification of SNPs plays an important role in molecular genetics providing a better understanding of genetic architecture and the identification of several economically important characteristics in various crops [131–134].

Some articles addressed the gene expression of the generated somaclones, providing information about the genes involved in the expression of morphological and genetic traits (Table S3). Analysis of the expression of genes involved in resistance to *Fusarium oxysporum* f. sp. *cubense* tropical race 4 (TR4) Guijiao 9, a somaclonal variant of banana belonging to the Cavendish subgroup, revealed that during the onset of infection by Foc TR4, resistant Guijiao 9 showed a higher number of differentially expressed genes (DEGs) than the susceptible Williams cultivar. Multiple resistance pathways were activated in Guijiao 9, and the DEG genes were involved in plant-pathogen interactions, signal transduction, secondary metabolism and other processes. This suggests that the pathogen response is regulated by multigene networks of DEG genes related to resistance [102].

In the study of Lee et al. [77], gene expression analysis was used to evaluate levels of endoreduplication in the variants of Phalaenopsis WP, an ornamental species. The study indicated that the high levels of endoreduplication in these variants are associated with changes in the normal growth of petals and leaves. In addition, high expression levels of the HPY2 gene are associated with endoreduplication only in some cases, indicating that additional genes are involved in the induction of polyploidy in Phalaenopsis WP variants. However, the PMADS4 gene studied was highly expressed in the petals of normal plants compared to those of somaclones, indicating its normal function in the development of floral parts. Hsu et al. [135] also studied gene expression in somaclones and found five sequences that showed higher expression levels in the wild plant than in Phalaenopsis Hsiang Fei cv. HF. These genes correspond to sequences encoding casein kinase, isocitrate dehydrogenase, cytochrome P450, EMF2 and an unknown protein. Two other sequences found in this study, whose roles were unknown, were expressed at a higher level in the somaclone plant than in the wild-type plant. The authors concluded that mosaic colour patterns and aberrant flower shapes may be caused by these genes in somaclonal variants of Phalaenopsis Hsiang Fei cv. HF. Further studies on the gene expression of somaclones are needed and may provide a more complete view of the genes involved in the changes that occur in somaclones. Understanding the mechanisms of somaclonal variation, as well as the expressed genes, may provide an alternative to generate somaclones of all cultures using previously described genes.

5. Conclusions

A total of 219 articles published between 2007 and 2022 were included in this review, encompassing a large number of studies in which somaclonal variants of various cultures were generated. The in vitro genetic diversity created in several plant species and agricultural crops has led to the emergence of characteristics related to resistance to biotic factors, improved agronomic performance and tolerance to abiotic stresses. Somaclonal variation has been used in genetic improvement programs of several crops worldwide, generating genetic diversity and providing the launch of new genotypes of important agricultural crops, such as sugarcane, wheat, rice, potato, banana and ornamental and medicinal plants, among others, with resistance to diseases, pests and abiotic stresses.

India, Pakistan, China, Egypt, Iran and Brazil have the largest numbers of studies on somaclonal variation in the world. Studies on sugarcane, ornamental plants and fruit plants have been the most common over the last 16 years. Studies involving the induction of somaclonal variation focused on the identification of molecular genetic variation, the selection of useful agronomic traits, resistance to pathogens, tolerance to salinity and tolerance to water deficit. Studies evaluating somaclones with tolerance to abiotic stresses, such as lead tolerance, toxic metal tolerance and copper tolerance, were also cited. This indicates that the induction of somaclonal variation has been explored in recent decades from several perspectives.

PGRs and frequent subcultures are the most commonly used techniques for the induction of somaclonal variation according to the results of this review. The PGRs BAP and 2,4-D with doses of 0.5 mg, 1 mg and 2 mg/L were the most commonly used. The use of subcultures and PGRs, and the concentrations of these PGRs to induce somaclonal variation, does not require very sophisticated techniques; this makes them accessible for studies of somaclonal variation in breeding programs. In addition, the launch of new cultivars derived from somaclonal variation is not a bureaucratic process and is considered inexpensive; it differs from the development of cultivars derived from other methods, such as genetically modified (GM) crops, which face major social and ethical obstacles.

It is observed that techniques for inducing somaclonal variation have been applied to a variety of crops. With the success of these techniques, many cultivars with agronomic characteristics useful for agriculture, such as nutrient quality, yield, disease resistance and tolerance to abiotic stress, should be included in different genetic improvement programs, and future studies may provide relevant information. Each year, new cultivars are launched, and many are being studied and evaluated for marketing purposes.

There is still a broad expectation that increasing the understanding of the mechanisms involved in somaclonal variation, the expression of genes of the generated somaclones and information about the biochemical and molecular pathways involved in the selection of somaclonal variants needs to be further explored. Future molecular research may help in the identification of somaclonal variants through polymorphic fragments involved in the process of somaclonal variation and selection of some genes associated with unique characteristics of somaclones. The expansion of knowledge on the genetic and epigenetic mechanisms of somaclonal variation will increase its use in crop breeding.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13030730/s1. Table S1: Plant species studied in articles on the application of the somaclonal variation technique in plant breeding in the last 16 years. Table S2: Molecular markers associated with strategies to identify genetic variation in various crops. The data were obtained from articles published in the last sixteen years on the application of somaclonal variation in plant breeding. Table S3: Genes associated with strategies to identify molecular changes in different crops. The data were obtained from articles published in the last 16 years on the application of somaclonal variation in plant breeding. Table S4: Assessment of the bias risk of the 219 articles included in the SR of the application of somaclonal variation for plant breeding over the last 16 years [135–269].

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Capítulo 2

Selection and characterization of somaclonal variants of Prata banana (AAB) resistant to Fusarium wilt

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Selection and characterization of somaclonal variants of Prata banana (AAB) resistant to Fusarium wilt

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Abstract: Fusarium wilt, caused by the fungus *Fusarium oxysporum* f. sp. *cubense* (Foc), is one of the most devastating diseases affecting banana cultivation worldwide. Although Foc tropical race 4 (TR4) has not yet been identified in Brazilian production areas, the damage caused by races 1 and subtropical 4 is the main cause of production losses, especially affecting cultivars of the Prata subgroup. Thus, the induction of somaclonal variation is a promising strategy in biotechnology to generate genetic variability and develop resistant varieties. The aim of this study was to induce somaclonal variation in the Prata Catarina cultivar (AAB genome) using successive subcultures in Murashige and Skoog (MS) medium enriched with the plant regulator Thidiazuron (TDZ) at two concentrations: 1 and 2 mg/L. After evaluating the symptoms, we selected 13 resistant somaclones that were not infected by the fungus. Histochemical and histological analyses of the somaclones indicated possible defense mechanisms that prevented colonization and/or infection by Foc, such as intense production of phenolic compounds, presence of cellulose and callose in the roots. Some somaclones showed no pathogen structures in the xylem-conducting vessels, indicating possible pre-penetration resistance. Furthermore, molecular studies indicated that the genetic alterations in the somaclones may have induced resistance to Foc without compromising the agronomic characteristics of the commercial genotype.

Keywords: *Musa spp.*; somaclones; genetic improvement

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1. Introduction

Bananas are the most consumed fresh fruit worldwide, with an estimated annual production of 114 million tons [1]. Currently, banana production is spread across several tropical and subtropical regions, especially Asia, Latin America and the Caribbean, and Africa. The largest producers are India (34.5 million tons), China (11.8 million tons), Indonesia (9.2 million tons), and Nigeria (8.0 million tons) [2]. Bananas of the Cavendish subgroup are grown on a large scale for export, dominating the American and European markets; however, there are hundreds of other cultivars used worldwide, mainly for domestic consumption and local or regional markets, playing a crucial role in the diet of the populations of the Indo-Malaysian, Asian, East African, and Latin American and Caribbean regions [3].

In Latin America and the Caribbean, Brazil is the largest producer of bananas, with an annual production of 7 million tons in an area of approximately 460,000 hectares [3]. This results in a productivity of 15 tons per hectare, annually generating more than USD 2.5 billion [2]. This production is mostly conducted by small producers throughout the country, and cultivars of the Prata subgroup (AAB genome) are widely grown and preferred by consumers owing to their unique texture and flavor. Notably, 70% of the area of banana cultivation is occupied by cultivars from this subgroup, especially the Prata Catarina cultivar, namely, a natural mutant derived from the Prata-Anã cultivar, with higher productivity and fruit quality [4]. Thus, the Prata banana plays a vital role, both in the agricultural economy and Brazilian food security [4,5].

Brazilian banana production, as in other countries, is threatened by the spread of *Fusarium oxysporum* f. sp. *cubense* tropical race 4 (Foc TR4), considered the most aggressive strain of Foc, which causes Fusarium wilt and has spread to different production areas [6, 7, 8]. Foc TR4 has the ability to infect a wide range of banana cultivars, including those of the Prata subgroup, causing devastation to plantations in Asia, Africa, and Latin America [9, 10].

Despite the potential destructive impact of Foc TR4 in Brazilian cultivation areas, considering its absence in the country, race 1 and subtrapical race 4 (STR4) are currently the biggest limitations to fruit production in the country, especially in the irrigated perimeters in the northern parts of Minas Gerais and Bahia, Ribeira Valley (state of São Paulo), and northern part of Santa Catarina; thus, extensive areas cultivated with Prata bananas are unviable for cultivation owing to the high infestation by race 1 [12]. Therefore, producers have replaced Prata bananas with Cavendish bananas, but this option is risky, considering the preference of Brazilian consumers for Prata banana types. Additionally, STR4 has caused damage to banana production in the southeastern and southern regions of Brazil, which experience harsh winters, facilitating infection by Foc [11]. In this context, the best strategy to contain the damage caused by *Fusarium oxysporum* f. sp. *cubense* is the use of resistant cultivars in addition to other tools, such as biological control and proper soil management, which together can mitigate the effects of the disease [13-15]. Accordingly, banana breeding programs at research institutions in different regions of the world have focused their efforts on exploiting the plant's genetic resistance to the pathogen to obtain a means of long-term control of the disease. These programs use different breeding strategies, especially hybridization, transgenesis or gene editing, mutagenesis, and in-vitro induction of somaclonal variation [16].

Among the aforementioned breeding methods, the induction of somaclonal variation has been widely used as an efficient option for the genetic improvement of various crops. This technique involves growing plant cells in a culture medium supplemented with cytokinins, such as Thidiazuron (TDZ), followed by successive subcultures in vitro. These factors are decisive in generating spontaneous and selectable genetic variations, enabling traits of interest to be obtained for breeding [17]. The variations observed in plants can arise from a number of factors, including somatic mutations, epigenetics, and stresses during in-vitro cultivation. After inducing somaclonal variation, an accurate phenotypic evaluation of the somaclones must be conducted to identify and select those with desirable characteristics for commercial cultivation, especially disease resistance [18-20].

In field tests, some somaclonal variants of "Cavendish" have been obtained and shown to have some level of tolerance to Foc TR4 [21]. In another study, somaclonal variants of the "Grande Naine" banana plant were identified in a greenhouse [22]. The most famous banana somaclone that is widespread in areas contaminated by Foc TR4 is Formosana (GCTCV 218), developed by the Taiwan Biodiversity Research Institute (TBRI) in Taiwan [23]. In sugarcane, a somaclone with resistance to brown rust (*Puccinia melanocephala*) was identified [24]. In another study, a somaclone with promising agronomic characteristics related to grain yield in wheat was identified [25]. In studies on rice (*Oryza sativa* L. cv. Nipponbare), three somaclones were selected with resistance to the fungus *Magnaporthe oryzae*, which causes rice brusone [26]. These results validate the application potential of the induction of somaclonal variation for genetic improvement in various crops.

In this study, we generated the first somaclonal variants of the Prata subgroup banana cv. Prata Catarina through in-vitro cultivation supplemented with the plant regulator TDZ. In the greenhouse, we selected somaclones resistant to Fusarium wilt based on a bioassay in beds infested with a strain of Foc STR4. To assess the extent of genetic diversity present in the selected somaclones, we used the molecular markers inter-retrotransposon amplified polymorphism (IRAP), retotransposon-microsatellite amplified polymorphism (REMAP), and inter-simple sequence repeat (ISSR); to observe plant-pathogen interactions, we evaluated compounds related to plant defense responses by means of histological and histochemical analyses.

2. Materials and Methods

2.1 Plant material

Seedlings of the Prata Catarina (AAB) cultivar were used for multiplication and the induction of somaclonal variation. This cultivar is a natural mutation selected in plantation areas in Brazil, derived from the Prata-Anã cultivar.

The seedlings were subcultured in Murashige and Skoog (MS) medium [27], supplemented with indoleacetic acid (IAA) (1.6 mL/L) and adenine hemisulfate (80 mg/L), to which different concentrations of TDZ were added. The treatments comprised two doses of TDZ: treatment 1 (T1), where the MS medium was supplemented with 1 mg of TDZ per liter; and treatment 2 (T2), using 2 mg of TDZ per liter.

Five subcultures were employed for each treatment, with an interval ranging between 40 and 60 days, depending on the development of the plants. At the end of the subcultivation of the two treatments, 2,400 plants were subjected to resistance assessment in the greenhouse, with 1,200 plants for each dose of TDZ. Additionally, 240 commercial Prata Catarina seedlings were selected to serve as the positive control.

2.2. *Preparation of the Foc inoculum*

In this study, we used isolate CNPMF 229, selected from the biological collection of the Phytopathology Laboratory at Embrapa Mandioca e Fruticultura. The isolate was chosen owing to its virulence and ability to aggressively infect banana varieties, including the Cavendish subgroup; when inoculated under controlled conditions, the same characteristics were observed in isolate CNPMF 218, classified as STR4, both of which were collected in the same region, namely, in the state of Santa Catarina [16, 28]. The isolate was grown on potato dextrose agar medium at 25 °C, under a 12-h photoperiod. After colony growth, a suspension of conidia was prepared and approximately 20 mL was deposited on 1 kg of sterilized rice. The medium was then incubated at 25 °C with a 12-h photoperiod. After 20 days, the colony-forming units (CFUs) were quantified using a series of dilutions to assess the concentration and viability of the spores. The CFUs were counted using a Neubauer chamber, and the concentration used for soil infestation in the beds in the greenhouse was 10^6 conidia/g of rice or inoculum [29].

2.3. *Evaluation of resistance in the greenhouse*

After 60 days of acclimatization, the somaclone seedlings were transferred to a greenhouse and planted in beds measuring 10 x 1 m, with soil infested with isolate CNPMF 229. After 90 days of planting, the somaclones were assessed for resistance to Foc. To achieve this, cross-sections were made in the rhizomes of the seedlings and the internal symptoms of rhizome discoloration were assessed using the scale proposed by Dita et al. [30]; namely, 1: no symptoms; 2: rhizome with initial discoloration; 3: discoloration of the rhizome throughout the vascular system; 4: rhizome with necrosis in most internal tissues; and 5: completely necrotic rhizome.

Based on the scores, an analysis of variance was conducted on the disease index (DI) estimates at a 5% significance level. The data were depicted in a boxplot graph with the DI results of the treatments. To calculate the DI, the scores obtained in the evaluations of the internal symptoms of the disease were transformed (0 to 4). The number of replications was based on the number of evaluated plants, namely, 1,200 for each treatment. The analyses were conducted using the R software [31].

2.4. *Histological and histochemical analysis*

Root fragments of the somaclones classified as resistant to isolate CNPMF 229 were collected and immersed in Karnovsky's solution [32] for a period of 48 h. The fragments were then dehydrated using an increasing series of ethanol at 3-h intervals, ranging from 30 to 100%. Infiltration and embedding were conducted using the historesin embedding kit (hydroxyethyl methacrylate, Leica). After polymerization of the historesin, histological sections measuring 8 μm were obtained using a Leitz 1516 microtome. These sections were mounted on histological slides and stained with ferric chloride for 3 h to detect phenolic compounds [33], and calcofluor white (0.01%) to detect cellulose. To detect callose, the slides were stained with aniline blue (0.05%) for 5 to 10 min [34]. The histological sections were subsequently analyzed and photographed using a B x S1 fluorescence microscope (Olympus Latin America).

The analysis of root clarification and staining of fungal structures was conducted according to the method described by Phillips and Hayman [35]. 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 30 min. Trypan blue dye in a 0.05% solution (lactic acid:glycerol:water = 2:1:1) was applied for 1 h. After staining, the slides were prepared and fragments were microphotographed using an optical microscope (Olympus Latin America).

2.5. Molecular analysis

2.5.1. Material collection and DNA extraction

Samples of young leaves from the resistant somaclones in T1 and T2, as well as the control, were collected and taken to Embrapa's Molecular Biology laboratory for DNA extraction using the methodology proposed by Doyle & Doyle [36], adapted by a previous study [37]. The DNA was quantified and its quality was assessed on a 1% agarose gel stained with GelRed®, and subjected to an electrophoretic run at 80 V for 1 h; subsequently, it was visualized using a UV transluminator.

PCR amplification and analysis using IRAP, ISSR, and REMAP markers

The IRAP marker analysis was based on the method described by Kalendar et al. [38]. The 20-μL reaction mixture comprised 25 ng of DNA, 0.3 μM primer, 2.5 mM MgCl, 0.2 mM deoxynucleotide triphosphate (dNTPs), 10× Taq buffer, and 0.3 U Taq DNA polymerase. The amplifications were conducted in a Veriti 96-Well Thermal Cycler (0.2 mL), Life Technologies, with the following settings: one cycle at 94 °C for 3 min; 35 cycles at 94 °C for 30 s, 42 °C for 1 min, and 72 °C and 72 °C for 45 s; and one cycle at 72 °C for 5 min and 4 °C.

Amplification between simple sequence repeats (ISSR) was conducted using the method described by Sankar [39]. The 25-μL reaction mixture comprised 50 ng of template DNA, 0.2 μM primer, 2.5 mM MgCl, 20 mM dNTPs, 10× Taq buffer, and 0.2 U Taq DNA polymerase. The amplifications were conducted in a Veriti 96-Well Thermal Cycler (0.2 mL), Life Technologies, with the following setting: one cycle at 94 °C for 3 min; 39 cycles at 94 °C for 40 s, 48 °C for 40 s, and 72 °C for 1 min; one cycle at 72 °C for 5 min and 4 °C.

The long terminal repeat (LTR) reverse primer 7286 REMAP was combined with seven LTR-SSR primers (Table 1), according to Kalendar et al. [38]. REMAP amplifications were conducted with a final volume of 25 μL, containing 50 ng of DNA, 0.2 μM LTR primer, 0.3 μM ISSR primer, 2.5 mM MgCl 2, 2 mM dNTPs, 10× Taq buffer, and 0.2 U Taq DNA polymerase. The amplifications were conducted in a Veriti 96-Well Thermal Cycler (0.2 mL), Life Technologies, with the following settings: one cycle at 94 °C for 3 min; 30 cycles at 94 °C for 30 s, 58 °C for 1 min, and 72 °C for 45 s; one cycle at 72 °C for 5 min and 4 °C.

The amplification products were separated on a 2.0% agarose gel and subjected to an electrophoretic run at 80 V for 3 to 4 h. They were stained with GelRed® and visualized using a UV transluminator.

Table 1. List of markers used to discriminate somaclones of Prata banana (AAB).

Initiator Identification		Nucleotide Sequence **(5–3)**	Annealing Temperature (°C)
REMAP*			
	LTR reverse 7286	GGAA11CATAGCATGGATAA TAAACGATTATC	
	8081	$(GA)_9C$	**54°C**

	8082	$(CT)_9G$	**54°C**
	8385	$(CAC)_7G$	**58°C**
	8386	$(GTG)_7C$	**58°C**
	8387	$(CA)_{10}G$	**54°C**
	8564	$(CAC)_7T$	**58°C**
	8565	$GT(CAC)_7$	**58°C**
IRAP**			
	LTR6149 + TR6150	CTCGCTCGCCCACTACATCAACCGCGTTTA CTGGTTCGCCCCATCTCTATCTATCCACAC	**42°C**
	LTR6150+ 5′LTR2	CTGGTTCGCCCCATCTCTATCTATCCACAC ATCATTGCCTCTAGGGCATAATTC	**42°C**
	3′LTR + LTR6150	TGTTTCCCATGCGACGTTCCCCAACA CTGGTTCGCCCCATCTCTATCTATCCACAC	**42°C**
	5′LTR2 + Nikita	ATCATTGCCTCTAGGGCATAATTC CGCATTTGTTCAAGCCTAAACC	**42°C**
	3′LTR + Nikita	TGTTTCCCATGCGACGTTCCCCAACA CGCATTTGTTCAAGCCTAAACC	**46°C**
	Nikita + LTR6149	CGCATTTGTTCAAGCCTAAACC CTCGCTCGCCCACTACATCAACCGCGTTTA	**46°C**
	5′LTR2 + LTR6150	ATCATTGCCTCTAGGGCATAATTC CTGGTTCGCCCCATCTCTATCTATCCACAC	**46°C**
	Sukula + LTR6150	GATAGGGTCGCATCTTGGGCGTGAC CTGGTTCGCCCCATCTCTATCTATCCACAC	**46°C**
ISSR***			
	ISSR-7	$(AG)_9$	**48°C**
	ISSR-23	$(AG)_8AT$	**45°C**

REMAP (Retrotransposon-microsatellite amplified polymorphism); **IRAP (Inter-retrotransposon amplified polymorphism); ***ISSR (Inter Simple Sequence Repeat).

3. Results

3.1. Resistance assessment in the greenhouse

Thirteen somaclones were selected with no symptoms (score 0) when inoculated with isolate CNPMF 229; namely, seven from T1 (TDZ dose 1 mg/L) named S1 to S7, and six from T2 (TDZ dose 2 mg/L), named S8 to S13. The susceptible plants between the treatments differed in terms of the aggressiveness of the isolate. In T1, 273 plants showed rhizomes with initial discoloration (score 1); 573 plants showed discoloration of rhizomes throughout the vascular system (score 2); 297 plants showed rhizomes with necrosis in most internal tissues (score 3); and 50 plants showed completely necrotic rhizomes (score 4). In T2, 240, 514, and 351 plants were classified with symptoms associated with grades 1, 2, and 3, and 89 plants were totally necrotic (grade 4). These results indicate that TDZ induced less resistance in T2 as 29% of the plants received scores of 3 or 4, which indicate greater aggressiveness of the pathogen. In T1, most plants received scores of 1 or 2, enabling the classification of genotypes as highly resistant or resistant (Figure

1). Based on these results, and for the Prata Catarina cultivar, a TDZ dose of 1 mg/L is ideal for inducing resistance to Foc in future studies.

The DI percentage of the controls for the Prata Catarina cultivar reached over 90%. In T2, the average DI percentage of the somaclone population was 60%, whereas T1 showed the lowest DI percentage, at approximately 50% (Figure 1).

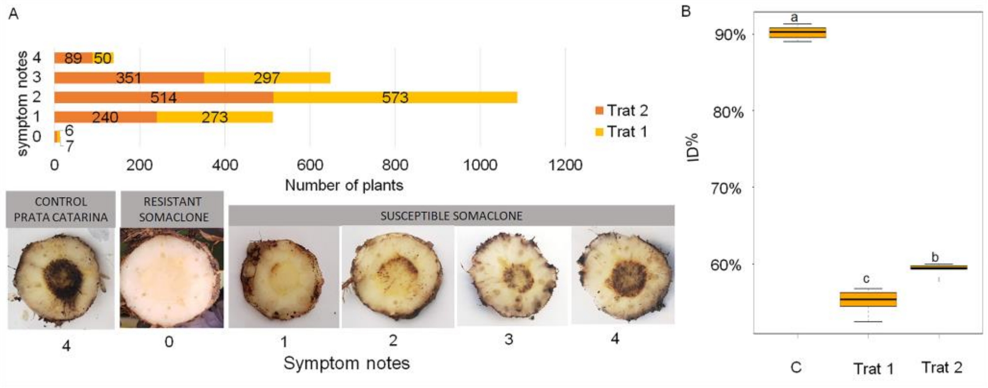

Figure 1. Internal symptoms of Fusarium wilt in the somaclones of Prata Catarina (AAB) banana plants evaluated in the greenhouse. (A) Bar graph with the number of plants with each grade of symptoms according to the grading scale, which varied from 1 to 4, and cross-section of the rhizome with the respective degrees of symptoms. (B) Boxplot of the internal disease symptom indices (DI%). Trat 1: treatment 1, with a TDZ dose of 1 mg/L; Trat 2: treatment 2, with a TDZ dose of 2 mg/L.

3.2. Histological and histochemical evaluation

To ascertain phenolic compounds, we detected small dots with a dark brown color in the rhizome tissue of all the somaclones and controls. The resistant somaclones S2, S3, and S7, associated with T1, showed a higher concentration of phenolic compounds compared with the control and other somaclones from the same treatment, as shown in Figure 2 (C, D, and H).

In T2, the resistant somaclones S9 and S11 (K and M) showed higher concentrations of phenolic compounds compared with the control and other resistant somaclones from the same treatment. This difference is shown in Figure 2.

We observed that among the two treatments, somaclones treated with a 1-mg/L dose of TDZ (T1) showed the most intense production of phenolic compounds.

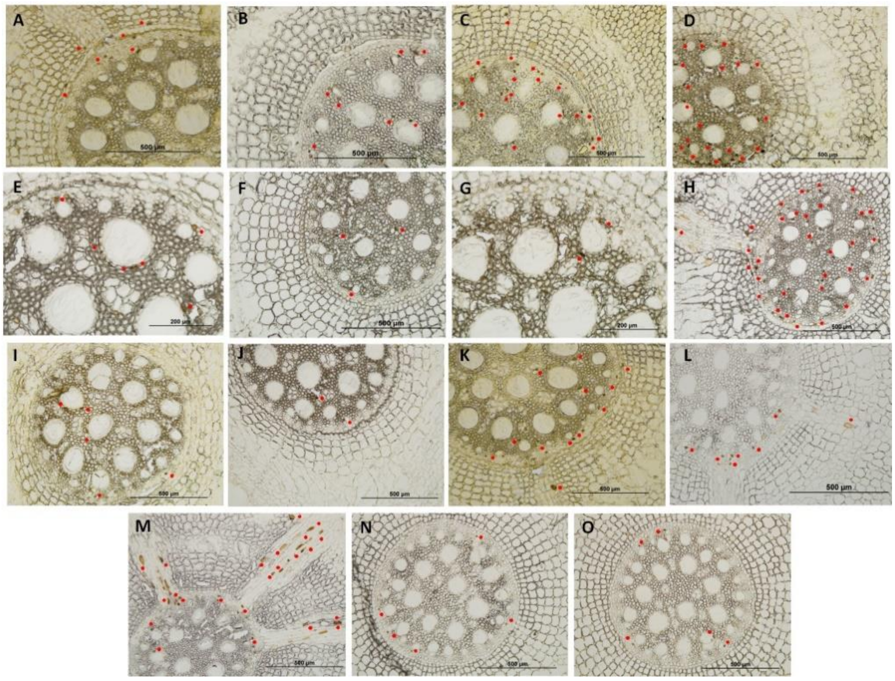

Figure 2. Cross-sectional micrographs of the roots of somaclones of the cultivar Prata Catarina, considered resistant to infection by Foc isolate CNPMF 229. The red dots indicate the presence of phenolic compounds. Controls of (A) T1 and (I) T2; (B) S1; (C) S2; (D) S3; (E) S4; (F) S5; (G) S6; and (H) S7, namely, resistant somaclones in T1; (J) S8; (K) S9; (L) S10; (M) S11; (N) S12; and (O) S13, namely, resistant somaclones in T2.

When assessing the presence of callose in the roots, somaclones S2 (C), S5 (F), and S7 (H), associated with T1, showed higher concentrations of this compound within the same treatment, indicated by the higher intensity of fluorescent light in the vascular tissue compared with the other somaclones and control. Considering T2, only somaclone S13 (O) showed a lower concentration of callose compared with the control. Notably, the somaclones derived from T2 showed higher concentrations of callose than those from T1 (Figure 3).

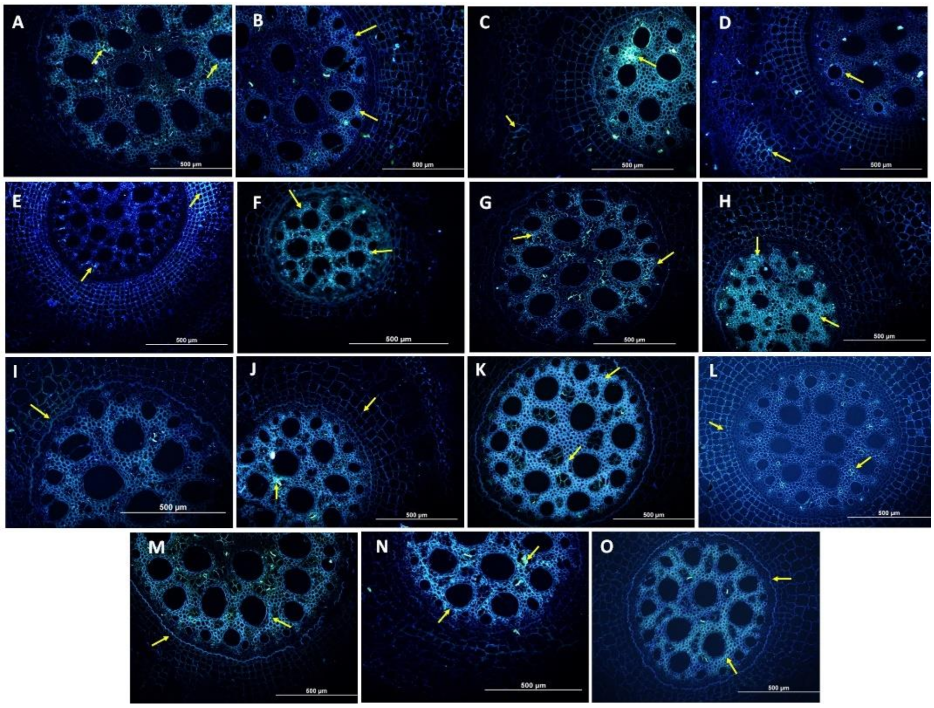

Figure 3. Fluorescence micrographs of cross-sections of the roots of somaclones of the cultivar Prata Catarina, considered resistant to infection by Foc isolate CNPMF 229. The yellow arrows indicate fluorescent regions with the presence of callose. Controls of (A) T1 and (I) T2; (B) S1; (C) S2; (D) S3; (E) S4; (F) S5; (G) S6; and (H) S7; these represent the resistant somaclones in T1; (J) S8; (K) S9; (L) S10; (M) S11; (N) S12; and (O) S13; these represent the resistant somaclones in T2.

The analysis of cellulose showed that somaclones S2 (C) and S4 (E), linked to T1, showed the highest amount of this compound, indicated by the bluish-white color in the root tissues, followed by somaclones S5 (F), S6 (G), and S7 (H) compared with the controls. Conversely, somaclones S1 (B) and S3 (D) indicated the lowest concentration of cellulose in this treatment, as shown in Figure 4.

In T2, somaclones S8 (J), S9 (K), and S13 (O) showed the highest concentration of cellulose. The other somaclones showed a lower or equal concentration of cellulose compared with the controls. T2 showed a greater number of somaclones with the presence of cellulose, as shown in Figure 4.

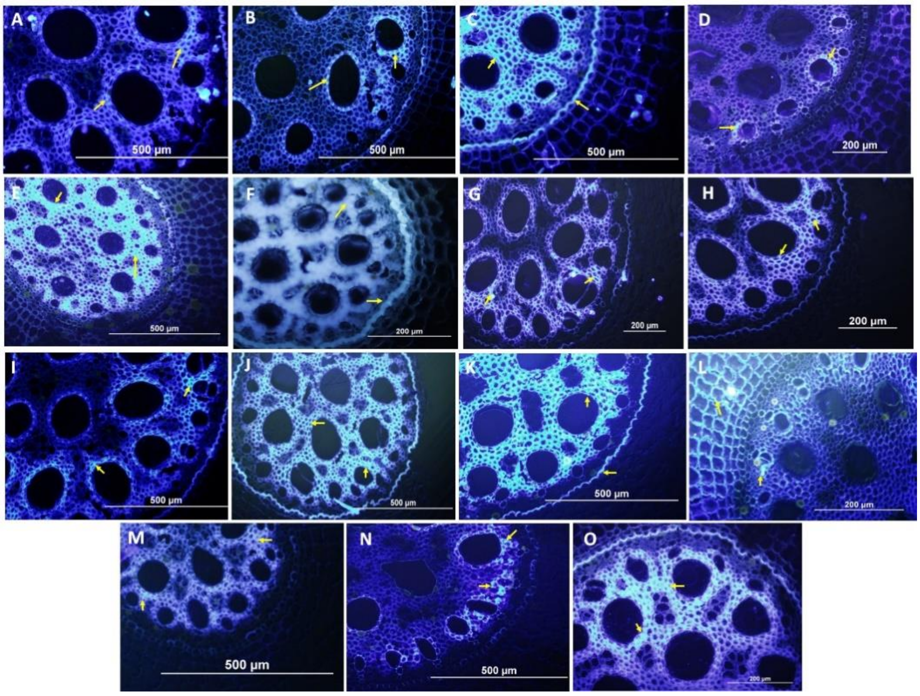

Figure 4. Fluorescence micrographs of cross-sections of the roots of somaclones of the cultivar Prata Catarina, considered resistant to infection by Foc isolate CNPMF 229. The yellow arrows indicate the presence of cellulose. Controls of (A) T1 and (I) T2; (B) S1; (C) S2; (D) S3; (E) S4; (F) S5; (G) S6; and (H) S7; these represent the resistant somaclones in T1; (J) S8; (K) S9; (L) S10; (M) S11; (N) S12; and (O) S13; these represent the resistant somaclones in T2.

In the evaluation of root whitening and staining, only the presence of hyphae was observed in the controls of the two treatments without the presence of chlamydospores, which were detected in the tissue of the vascular system of somaclones S3 (D), S5 (F), and S6 (H) in T1. In somaclones S1 (B), S2 (C), S4 (E), and S6 (G) of the same treatment, no pathogen structures were observed.

In T2, no pathogen structures were observed in the root tissue of somaclones S11 (M) and S13 (O). In somaclones S8 (J), S9 (K), S10 (L), and S11 (N) of the same treatment, the presence of chlamydospores was observed, as shown in Figure 5.

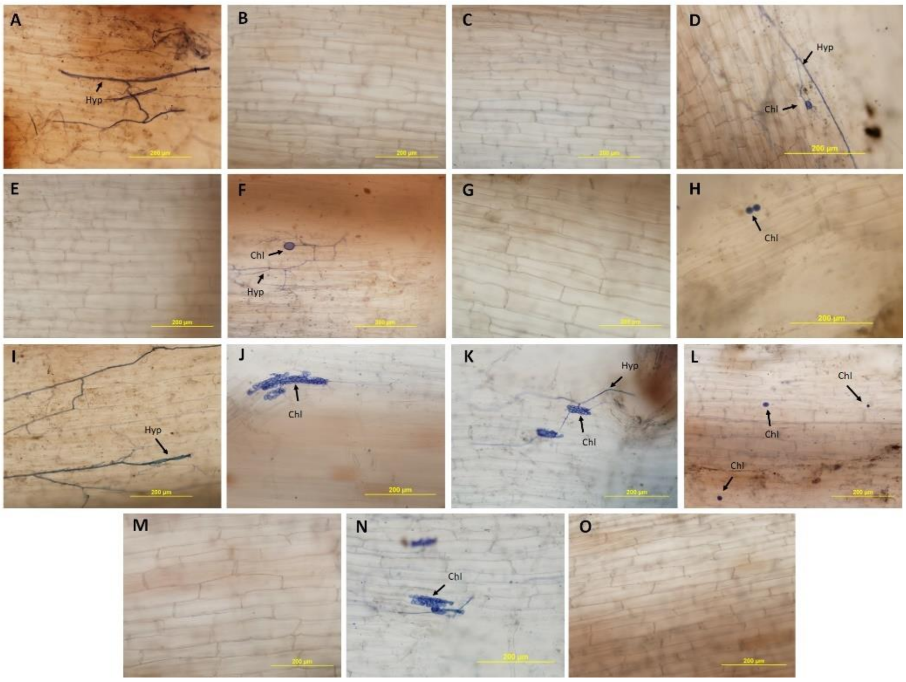

Figure 5. Micrographs of root fragments from somaclones of the cultivar Prata Catarina, considered resistant to infection by Foc isolate CNPMF 229. The arrows indicate chlamydospores (Chl) and fungal hyphae (Hyp). Controls of (A) T1 and (I) T2; (B) S1; (C) S2; (D) S3; (E) S4; (F) S5; (G) S6; and (H) S7; these represent the resistant somaclones in T1; (J) S8; (K) S9; (L) S10; (M) S11; (N) S12; and (O) S13; these represent the resistant somaclones in T2.

3.3. PCR amplification and marker analysis

The IRAP, REMAP, and ISSR markers could not identify significant genetic differences between the resistant somaclones and control (cultivar Prata Catarina), as indicated by the band patterns identified in the somaclones, which are identical to those in the control, as shown in Figure 6 (A, B, and C). Notably, the genetic alterations induced by the doses of TDZ in the somaclones only affected the level of resistance of the plants. Thus, we inferred that the selected resistant somaclones may have the same agronomic and sensory profile as the commercial Prata Catarina cultivar; this is an important fact as it increases the chances of adoption by producers and consumers. Notably, a complete agronomic and sensory characterization of the resistant somaclones will be the subject of the subsequent studies.

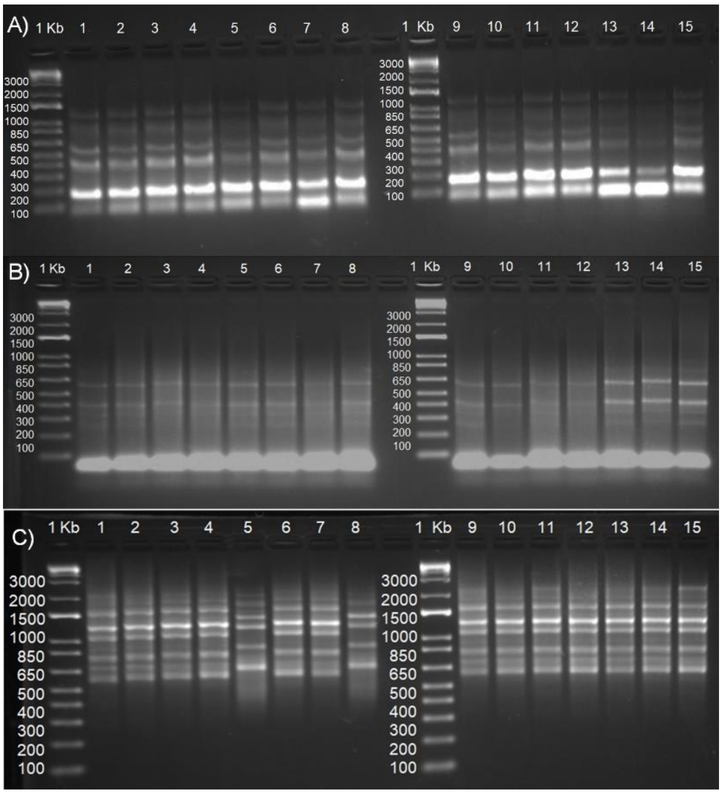

Figure 6. Molecular analysis to identify genetic changes in somaclones resistant to Foc isolate CNPMF 229. (A) Inter-retrotransposon amplified polymorphism (IRAP) markers Sukula + LTR6149 combination; (B) Retrotransposon-microsatellite amplified polymorphism (REMAP) markers REMAP: LTR reverse 7286 + 8387; (C) Inter -simple sequence repeat (ISSR) markers ISSR-7. 1kb Invitrogem® marker; Prata Catarina cultivar controls (1 and 9); 2: S1, 3: S2, 4: S3, 5: S4, 6: S5, 7: S6, and 8: S7; these correspond to the resistant somaclones in T1. 10: S8, 11: S9, 12: S10, 13: S11, 14: S12, and 15: S13; these correspond to the resistant somaclones in T2.

4. Discussion

4.1. Assessment of resistance in the greenhouse

In this study, we induced somaclonal variants derived from the Prata Catarina cultivar using the plant growth regulator TDZ at two concentrations, 1 and 2 mg/L. We evaluated 2,400 somaclones for their resistance to Fusarium wilt. Among these, 13 were resistant after phenotyping in greenhouse conditions using soil infested with the pathogen, seven were resistant with the 1-mg/L dose, and six were resistant with the 2-mg/L dose, thereby corresponding to a selection pressure of 0.5%.

In our study, we observed a greater number of highly resistant and resistant somaclones at a TDZ dose of 1 mg/L (280 somaclones or 23% of the total, corresponding to scores of 0 and 1) compared with those at a TDZ dose of 2 mg/L (246 somaclones or 20%, corresponding to scores of 0 and 1). In banana cultivation, TDZ is used as a plant regulator to induce somaclonal variation and generate Grand Naine (Cavendish) banana somaclones resistant to Foc STR4, as indicated by Rebouças et al. [22]. The authors obtained two resistant somaclones, corresponding to an average of 1% resistant somaclones. The TDZ dose of 1 mg/L used in the study by Rebouças et al. [22] reinforces the results obtained in this study.

In wheat (*Triticum aestivum* L.), the technique of somaclonal variation has been used to generate somaclones. Wang et al. [25] used 2,4-dichlorophenoxyacetic acid (2,4-D) at a dose of 2.0 mg/L to induce somaclonal variation. This synthetic auxin is responsible for plant growth and development. The authors subcultured the seeds for 4 weeks to induce callus and obtained a somaclone of dwarf wheat, called AS34; they observed that this new genotype showed positive effects on agronomic characteristics related to grain yield in F2 populations, especially associated with lower plant height.

Eeckhaut et al. [40] produced somaclones with ornamental characteristics using TDZ. In this study, the potential of protoplast regeneration to induce somaclonal variation in Chrysanthemum × morifolium, cultivar Arjuna, was evaluated. Fifty-four protoplast regenerants were produced and vegetatively propagated in a medium containing 0.1 mg/L of TDZ. Significant variations were observed between the regenerants, influencing the number, size, and weight of the flowers, weight of the leaves and stems, and overall size of the plants. A reduction in flowering induction time was also observed, up to 10 days earlier in some cases, as well as variations in flower types and colors. In our study, no morphological changes were observed in the somaclones, such as changes in leaf color or plant size, at least until three months of plant development and evaluation. A complete agronomic characterization of the somaclones will be conducted in subsequent stages of study.

Ferreira et al. [17] analyzed the role of somaclonal variation in plant breeding, observing that various plant regulators are used to induce somaclonal variation. Among these regulators, TDZ was remarkable, especially at doses of 1 and 2 mg/L. Additionally, TDZ was found to cause alterations in the phenotypic characteristics of various crops, such as changes in leaf and flower color, plant height, and resistance to pathogens, among others. Moreover, according to the authors, thousands of plants subjected to TDZ treatment are required to facilitate the selection of somaclones with desirable agronomic characteristics, especially genetic resistance to pathogens.

TDZ is a plant regulator that acts as a hormone, triggering various functions in plant tissues, including the increase in the formation of lateral buds and development of plants with a more desirable architecture for agricultural or ornamental production [41]. TDZ has various effects on fruit crops; for example, it can improve fruit size in kiwi (*Actinidia deliciosa* "Hayward"), pear (*Pyrus communis* L. cv "Spadona" and "Coscia"), and grapes (*Vitis vinifera* cv "Simone") [42-44], and increase yield in pears (*P. calleryana* cv "Hosui" and "Packham's Triumph") and cucumbers (*Cucumis sativa* L.) [45, 46]. It has high cytokinin activity in in-vitro cultures, promoting high rates of multiplication and shoot formation; it is effective in inducing callus and regenerating plants from plant tissues. TDZ is also commonly used in plant tissue cultures to promote shoot formation [47-49]; it has an influence on morphogenesis and rooting efficiency when used in concentrations above threshold levels and/or for prolonged periods [50, 51].

The concentrations of the plant regulator, together with the subcultures, cause genetic variations that can result in different phenotypes compared with the original matrices. Pop et al. [52] induced somaclonal variation in five grapevine cultivars (*Vitis vinifera*) using shoots as a source of explants in a culture medium containing 0.5 mg/L of 1-naphthylacetic acid (NAA) and 0.5 mg/L of TDZ after the 12th subcultivation. They obtained five somaclones of the cultivar "Merlot" and one somaclone of the varieties "Fetească Albă" and "Traminer Roz." They achieved significant results for the future of wine breeding programs. Similarly, Bidabadi et al. [53] investigated the effects of different concentrations of benzylaminopurine (BAP) and TDZ on somaclonal variation, based on genetic and phenotypic variability between micropropagated shoots subcultured six times in the banana cultivars "Berangan Intan," "Berangan," and "Rastali." The main results showed that with the highest concentrations of BAP (up to 9.9 mg/L) and TDZ (up to 1.6 mg/L), most shoots showed morphological changes, including undifferentiated shoots. In our study, TDZ doses of 1 and 2 mg/L, combined with five subcultures, generated efficient genetic alterations in terms of inducing resistance to Fusarium wilt.

Regardless of the studied culture, one determining factor for the induction of somaclonal variation in vitro is the number of subcultures to which the explant is submitted. Notably, from the fifth subcultivation onward, the explants can already undergo somaclonal variation [17]. In our study, we conducted five subcultivations and identified resistant somaclones. Similarly, Miyao et al. [26] employed five subcultures on lines regenerated from cell cultures of rice (*Oryza sativa* L. cv. Nipponbare) and obtained three lines with resistance to the fungus *Magnaporthe oryzae*, which causes rice brusone. These results confirm those of in-vitro variations from the fifth subcultivation cycle onward.

4.2. Analysis of resistance mechanisms by histological and histochemical evaluations

Somaclones S2, S3, and S7 (C, D, and H) in T1, and S9 and S11 in T2 showed the highest concentrations of phenolic compounds (Figure 2). Some resistance mechanisms may have been activated in the somaclones selected as resistant in this study, both pre- and post-formation [54]. Pre-formed mechanisms include: spines and trichomes, physical structures that hinder access to pathogens; the cuticle, a waxy layer that covers the epidermis and prevents the entry of pathogens; thickened cell walls, which reinforce the cell wall and hinder penetration by pathogens; and accumulation of phenolic compounds, namely, substances with antimicrobial and antioxidant properties that are capable of inhibiting the presence of pathogens. In our study, we observed the presence of phenolic compounds (Figure 2). Post-formed resistance mechanisms include: the hypersensitivity response or programmed cell death, wherein cells around the infection site die to contain the spread of the pathogen; and production of phytoalexins, antimicrobial compounds produced in response to infection by pathogens [54-56].

We quantified three compounds associated with resistance mechanisms: phenols, callose, and cellulose. Some plant defense mechanisms are constitutively present even in the absence of stress stimuli. For example, the presence of phenolic compounds, on the surface of leaves or in the cell wall, is a pre-formed mechanism. These compounds can deter herbivores and pathogens by acting as a physical or chemical barrier. Callose is a post-formed resistance mechanism as it reinforces the cell wall and blocks the penetration of pathogens once the plant has detected their presence. Similarly, the deposition of cellulose in the cell walls around an infection site is a post-formed mechanism that strengthens the cell structure and prevents the spread of pathogens [56].

Phenols are substances produced when the plant is infected by the pathogen and are accumulated in the vascular system to prevent the spread of infection. This strategy was identified by Rocha et al. [16], who studied the interaction between Musa sp vs. *Fusarium oxysporum* f. sp. *cubense*, with the aim of quantifying the virulence levels of different isolates when inoculated into resistant and susceptible banana cultivars. They observed a higher concentration of phenolic compounds in the roots of resistant plants. Similarly, Soares et al. [57] reported greater production and accumulation of phenolic compounds in cultivars resistant to *Pseudocercospora fijiensis*, based on histochemical analyses, which also enabled the identification of the presence of callose in the leaves of resistant genotypes in greater quantity compared with that in susceptible cultivars.

Phenolic compounds are associated with defense mechanisms and responses to adverse environmental conditions and are slightly involved in cell growth and development [58, 59]. However, there is an association between the production of phenols and cultivation of embryogenic callus as a reduction in the activity of cytochrome C and dehydrogenases, such as FADH2/NADH, and an increase in the concentration of phenolics are observed. Furthermore, Shirani et al. [60] observed that TDZ promoted greater production of phenolic compounds when used to promote the proliferation of shoots in a musaceae tissue culture. Based on the above studies, the resistant somaclones in this study may have shown a high concentration of phenolic compounds owing to the presence of TDZ, and not owing to the resistance response to Foc, which needs to be confirmed in further studies.

Without inducing somaclonal variation, Ncube et al. [61] investigated the effects of TDZ on the regeneration and production of phenolic compounds of *Merwilla plumbea* (Lindl.) plants during the transition from in-vitro to ex-vitro environments. They highlighted that exposing the plants to 0.45 μM of TDZ resulted in significantly higher levels of total phenolics in the plants regenerated in vitro, as well as greater antioxidant activity compared with the ex-vitro plants. Flow cytometry analysis indicated that the genomic stability of the regenerated plants was comparable with that of field-grown plants. The authors concluded that TDZ not only promoted efficient plant regeneration but also enhanced their antioxidant and phytochemical properties during adaptation to the ex-vitro environment. These results reinforce our findings, namely, the resistant somaclones maintained genetic stability compared with the commercial control, induced by the use of TDZ, and showed an increase in phenolic compounds ex vitro. However, we did not conduct in-vitro phenolic compound analyses, which prevents a direct comparison between in-vitro and ex-vitro results, which must be explored in future studies.

During fluorescence analysis, the presence of callose was observed in both treatments of the resistant somaclones. This observation suggests that the somaclones activated signaling pathways capable of detecting the presence of Foc in the roots through pathogen associated molecular patterns (PAMPs), which are specific molecules found in various pathogenic microorganisms, such as bacteria, viruses, fungi, and parasites. Conversely, pattern recognition receptors (PRRs) are receptors present in cells that recognize these patterns, initiating an immune response to fight the infection. These processes enable the production and deposition of callose at the pathogen’s infection sites, strengthening the cell wall and hindering pentration by the fungus. This phenomenon, known as PAMP-triggered immunity (PTI), includes certain responses, such as the accumulation of callose, which may have occurred in the resistant somaclones in this study, wherein the presence of callose was observed in greater concentration in somaclones S2 (C), S5 (F), S7 (H), and S9 (K). This indicated that through the action of PAMPs, the presence of Foc in the roots of the somaclones and activation of this post-formed resistance mechanism could be detected.

In resistant plants, callose formation is rapidly induced, deposited mainly at infection points to reinforce the cell walls and prevent penetration by the pathogen [62-65]. De Quadros et al. [66] investigated the defense mechanisms of the root and hypocotyl tissues of the common bean (*Phaseolus vulgaris* L.) against *Fusarium oxysporum* f. sp. *phaseoli* (Fop). Resistant and susceptible bean plants were inoculated by dipping their roots in a suspension of conidia. After inoculation, the authors examined the defense mechanisms of the root and hypocotyl in detail using microscopic techniques and biochemical assays. They observed that Fop colonized the epidermis and cortex inter- and intracellularly, reaching the xylem vessels more quickly in the susceptible genotype. Inoculation with Fop induced the accumulation of phenolic compounds and carbohydrates, as well as the deposition of callose inside the xylem vessels, especially in the resistant genotype.

The third component evaluated in our study, related to post-formed resistance mechanisms, was the presence of cellulose. When a plant is attacked by a pathogen, it activates defense responses to protect its tissues, and one of these responses involves the production of cellulose, namely, a crucial element of the cell wall. Cellulose strengthens cell walls, hindering the penetration and spread of the pathogen. Additionally, plants deposit additional layers of cellulose around the site of infection, creating physical barriers that isolate the pathogen and prevent its spread. The presence of pathogens also activates signaling pathways that increase cellulose production, regulating genes related to its biosynthesis. These mechanisms help plants to resist infection and defend themselves against pathogens [54, 63, 65].

In this study, we conducted fluorescence analysis to observe the presence of cellulose in the xylem-conducting vessels in the roots of the resistant somaclones. The somaclones from T2, especially S8 (J), S9 (K), and S13 (O), showed greater cellulose deposition compared with the control (Figure 4). Rocha et al. [16] also detected these compounds in banana cultivars after inoculation with Foc. During the interaction with Foc 229A isolate, which was also used in this study, cellulose was observed in the Prata-Anã and Grande Naine cultivars.

Phytopathogenic fungi produce enzymes that degrade the cell wall, such as cellulases, facilitating their invasion of host tissues. These enzymes break down cell wall components, such as wax and the cuticle, enbaling the penetration and spread of the pathogen [67, 68]. In this context, the resistant somaclones developed defense mechanisms with cellulose accumulation in the cell wall, which proved to be efficient against Foc. Thus, the pathogen was unable to produce enough enzymes to degrade the cellulose in the cell wall of the hosts owing to the high concentration of this compound in the vascular tissue of the resistant somaclones (Figure 4).

In the root clarification and staining technique, the absence of pathogen structures was observed in the resistant somaclones in both treatments with TDZ, indicating that these genotypes developed resistance mechanisms preventing the penetration of Foc, such as the action of phenolic compounds, callose, and cellulose. The presence of chlamydospores was detected in the tissue of the vascular system of somaclones S3, S5, and S6 in T1, and S8, S9, S10, and S12 in T2. The analyzed somaclones showed pathogen structures in the vascular system; however, successful infection by the pathogen was prevented. This resistance can be attributed to the aforementioned mechanisms or possible genetic alterations that have not yet been investigated.

These findings indicate that the pathogen was unable to successfully establish infection in somaclones of T1 and T2 owing to the effectiveness of the existing defense mechanisms. In somaclone S2, the three defense mechanisms analyzed in this study were identified: phenolic compounds, callose, and cellulose. In somaclone S7, phenolic compounds and callose were observed, whereas in somaclone S9, phenolic compounds and cellulose were detected. Somaclone S13 showed the presence of callose and cellulose. The other somaclones showed only one of the studied defense mechanisms. Notably, by presenting three different resistance mechanisms, somaclone S2 may be superior to the others selected in this study as the pathogen will need to overcome three different obstacles for successful infection.

Post-formed mechanisms of genetic resistance, such as phenolic compounds, callose, and cellulose, may have been activated by the resistant plants that contained structures of the pathogen in the root tissue, which did not enable the spread of infection (Figure 5). Xiao et al. [69], Warman et al. [70], and Rebouças et al. [22] observed that chlamydospores and microconidia germinate around the root tip and between the root hairs of banana genotypes, before penetrating the epidermal cells and moving through the intercellular elongation zone to start the infection process. Thus, the somaclones that did not contain Foc spores may have developed resistance mechanisms that did not enable the entry of the pathogen into the root tissues, which must be verified in future studies.

4.3. Analysis of the extent of genetic diversity in somaclones using molecular markers IRAP, REMAP, and ISSR

The molecular markers used in our study (IRAP, REMAP, and ISSR) were selected to cover different parts of the somaclone genome, enabling the detection of genetic variations between somaclones and between somaclones and their parents. IRAP markers help detect retrotransposons, which are DNA sequences that have the ability to move within an organism's genome, namely, they are mobile genetic elements that can cause mutations, activation, or deactivation of genes [71, 72]. ISSR markers are molecular tools used to analyze genetic polymorphisms. They amplify regions between microsatellites in DNA, detecting variations in the number of repeats. Owing to their high sensitivity and reproducibility, ISSR markers are widely applied in genetic diversity studies, phylogeny, genetic mapping, and plant breeding. Moreover, their versatility allows them to be used in different species without the need for prior knowledge of the genome, making them valuable for genetics and conservation research [73, 74]. REMAP markers combine elements of retrotransposons and microsatellites to identify polymorphisms in DNA. This technique is based on the amplification of regions between retrotransposons and microsatellites, enabling the detection of insertions and deletions that indicate genetic variations. REMAP markers are useful for genetic diversity studies, genetic mapping, and plant breeding.

Our results indicated that the resistant somaclones have high genetic similarity with the Prata Catarina cultivar, from which they were derived. The array of markers used indicated that the bands/allels patterns were mostly similar to the commercial cultivar. Thus, we inferred that the somaclones did not differ agronomically from Prata Catarina in terms of the agronomic characters, such as bunch weight, number of fruits, or the sensory profile. This information will be verified in future studies on the commercial potential of somaclones through field experiments.

Ferreira et al. [17] indicated that the IRAP and REMAP markers are considered efficient molecular markers for investigating teh genetic variability in various crops. These markers are based on retrotransposons that are dispersed throughout the plant genome and can contain thousands of copies, thus contributing to the size, structure, diversity, and variation of the genome, which is a factor that can affect gene function [75].

Muhammad et al. [76] used random amplified polymorphic DNA (RAPD) markers, which revealed greater polymorphism compared with IRAP markers when analyzing somaclones derived from the silk subgroup (AAB) banana cultivar called "Rasthali." They concluded that somaclonal variation appears to be derived from multiple indels scattered throughout the genome, as a response to stress induced by micropropagation. Therefore, for the comprehensive characterization of somaclonal variants, more than one DNA marker system must be employed to detect variations in various regions of the genome, as was used in our study.

Another molecular marker used to identify genetic variations, in addition to the aforementioned markers, is amplified fragment length polymorphism (AFLP). This molecular technique is widely used to detect genetic variations between different DNA samples, combining the digestion of DNA with restriction enzymes and selective amplification of DNA fragments using polymerase chain reaction (PCR), enabling the detailed analysis of somaclonal variation [17]. Munsamy et al. [24] investigated the increased frequency of somaclonal variants of sugarcane plants (*Saccharum* spp.) produced in vitro using the AFLP marker. They identified genetic variations in at least one sugarcane somaclone that was resistant to brown rust (*Puccinia melanocephala*).

Today, next-generation sequencing technology is increasingly being used to study somaclonal variation and genetic variability in plants. Approaches, including whole-genome sequencing, offer a more detailed view of genetic changes in somaclones [77]. Therefore, although the traditional methods discussed in this study are still widely used, whole-genome sequencing technologies have become more accessible and offer a more comprehensive and accurate perspective on somaclonal variation. These new technologies will be employed in our future studies on the selected resistant somaclones.

5. Conclusions

In this study, the application of the plant regulator TDZ proved to be effective in producing 13 somaclones derived from the cultivar Prata Catarina (subgroup Prata, AAB), with resistance to Fusarium wilt. The 1-mg/L dose was more efficient in obtaining somaclones in terms of the number of resistant plants. Molecular analysis revealed no genetic alterations in the somaclones, suggesting that their agronomic characteristics remained unchanged compared with those of the commercial genotype. Results from the histological and histochemical evaluations corroborate the observed resistance, indicating the presence of phenolic compounds, callose, and cellulose as possible contributing mechanisms to this resistance. These findings indicate the presence of post-formed resistance mechanisms in the resistant somaclones. Our results were promising, indicating that the induction of somaclonal variation is an effective and efficient approach for the development of banana cultivars resistant to this disease, which limits the production of Prata bananas in various producing regions of Brazil.

Supplementary Materials: Not applicable.

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CONCLUSÃO GERAL

A revisão sistemática sobre variação somaclonal que realizamos incluiu a análise de artigos publicados ao longo dos últimos 16 anos. Esta revisão marca a primeira abordagem sistemática sobre o tema. Nela, exploramos temas relevantes para os programas de melhoramento genético, visando o desenvolvimento de novas variedades de material vegetal com características agronômicas desejáveis em várias áreas, como resistência a patógenos, características agronômicas, ornamentais, medicinais, entre outros. Foram identificados 4.354 artigos, dos quais 219 foram selecionados de acordo com os critérios estabelecidos no protocolo e compõem esta revisão.

Os países com maior produção de publicações sobre o tema foram o Paquistão, China, Egito e Brasil. Diversas espécies foram discutidas, incluindo frutas, gramíneas, cereais, hortaliças, raízes, tubérculos e plantas ornamentais, evidenciando o amplo uso dessa técnica no melhoramento genético em diferentes países e para diversas espécies.

Os principais fatores de variação somaclonal in vitro incluem o número de subcultivos e os reguladores vegetais, sendo essas informações discutidas neste estudo, o que pode contribuir para trabalhos que visam induzir variação somaclonal em plantas. Além disso, são abordadas informações sobre alterações morfológicas dos somaclones gerados em varias culturas e as ferramentas utilizadas para identificá-los.

Foram obtidos resultados promissores na indução de variação somaclonal em bananeiras da cultivar Prata Catarina. Selecionamos e discutimos 13 somaclones resistentes. Além disso, os resultados das avaliações histológicas e histoquímicas comprovaram a ativação de mecanismos de resistência pós-formados. As análises moleculares sugeriram que as alterações genéticas induzidas pelas doses de TDZ nos somaclones afetaram apenas o nível de resistência das plantas. Por apresentar três diferentes mecanismos de resistência, o somaclone S2 pode ser superior aos outros selecionados neste trabalho, pois o patógeno precisará superar três obstáculos distintos para ter sucesso no processo de infecção. Inferimos que os somaclones resistentes selecionados podem apresentar o mesmo perfil agronômico e sensorial da cultivar comercial Prata Catarina. Esses resultados são significativos, e podem contribuir para mitigar os danos causados pelo Foc, nas áreas produtoras de banana e impulsionar avanços nos programas de melhoramento genético.