Molecular screening in the EPAGRI´S banana germplasm collection to identify sources of resistance to *Fusarium oxysporum* f. sp. *cubense* tropical race 4

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Abstract – The banana germplasm collection of EPAGRI - Estação Experimental de Itajaí, Santa Catarina, Brazil, holds 120 banana accessions from different origins, targeting at the conservation and the breeding. One of the main challenges of banana breeding is the selection of plants resistant to *Fusarium oxysporum* f. sp. *cubense*. This study aimed to identify, via molecular markers, the plants resistant to tropical race 4 (TR4), which is currently absent in Brazil. The results showed a wide presence of molecular marks associated with resistance to tropical race 4 in the genotypes of the germplasm collection. This information is important for the advancement of the EPAGRI banana breeding program.

Index terms: Fusarium wilt; SCAR markers; *Musa acuminata; Musa balbisiana.*

Seleção de fontes de resistência à raça 4 tropical de *Fusarium oxysporum* f. sp. *cubense* em uma coleção de germoplasma de bananeira através de marcadores moleculares

Resumo – A coleção de germoplasma de bananeira da EPAGRI, na Estação Experimental de Itajaí possui 120 acessos de diferentes origens, servindo os propósitos de conservação e melhoramento. Um dos maiores desafios do melhoramento genético de bananeira é a seleção de plantas resistentes ao *Fusarium oxysporum* f. sp. *cubense.* Este trabalho objetivou identificar, via marcadores moleculares, plantas resistentes a raça 4 tropical, atualmente ausente no Brasil. Os resultados mostraram uma ampla presença de marcas moleculares associadas à resistência da raça 4 tropical nos genótipos da coleção de germoplasma. Estas informações são importantes para o avanço do programa de melhoramento genético de bananeira da EPAGRI.

Termos para indexação: Murcha de fusarium; Marcadores SCAR; *Musa acuminata; Musa balbisiana.*

Introduction

The state of Santa Catarina, Brazil, stands out nationally in banana production, being the fourth largest national producer in 2018 with more than 700,000 tons produced (EPAGRI, 2020). Several pests and diseases negatively affect crop productivity, and the Panama disease caused by the soil fungus *Fusarium oxysporum* f. sp. *cubense* (Foc) is currently one of the main problems of banana production in Santa Catarina, causing economic losses to producers.

The most efficient way to control this disease is the use of genetic materials resistant to the four different physiological races of the fungus. The races 1, 2 and 4 affect banana species and their crosses. Race 3 affects only species of the genus *Heliconia* (PLOETZ, 2006).

Races 1, 2 and sub-tropical race 4 (STR4) are distributed in Brazil, however Tropical Race 4 (TR4) is not present in the national territory. Therefore, TR4 is important since it is more aggressive and attacks plants not attacked by races 1 and 2. Currently, TR4 is present in Asia, Oceania, Middle East, South America (specifically Colombia and Peru), and Africa, causing many economic losses due to the destruction of banana trees (GARCÍA-BASTIDAS et al., 2020; ORDONEZ et al., 2015; PLOETZ, 2015; THANGAVELU et al., 2020; SENASA, 2021).

All physiological races of Foc are subdivided in 24 vegetative compatibility groups (VCG). STR4 belonged to VCGs 0120, 0121, 0122, 0129, and 01211, whereas TR4 belong to VCG 01213/16 (ORDONEZ et al., 2015; THANGAVELU et al., 2020).

Germplasm collections are essential for humanity's food and energy security, since they preserve genotypes and genes of current and future importance, whether for direct use or for breeding programs (FU, 2017). When a germplasm collection becomes official, as well as with obligations of use, conservation, prospecting, and exchange of accesses, it can be transformed into an Active Germplasm Bank. The banana is among the 15 crops of greatest food importance for humanity and has 18 Actives Germplasm Banks spread around the world, which together reach around 14,000 accesses (VAN DEN HOUWE et al., 2020). Brazil has one of the largest banana Active Germplasm Bank in the world, maintained by the Brazilian Agricultural Research Corporation (Embrapa) in the state of Bahia, northeast Brazil. However, different Brazilian institutions (universities, state agricultural research companies, etc) maintain collections of *Musa* spp. throughout the country. In south Brazil,

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the germplasm collection of *Musa spp*. stands out, maintained by the EPAGRI - EEI. This collection was established in 1981, and holds about 120 accessions that come from collections made in the southern Brazil and introductions of genotypes from other institutions.

Molecular markers have been used for decades for the genetic characterization of crop and native species, mainly for the characterization of disease-resistant genotypes (VIEIRA et al., 2016). Thus, molecular markers available for *Musa spp*. can represent important advances in the selection of resistant plants from germplasm collections.

Sequence Characterized Amplified Region (SCAR) are specific polymerase chain reaction (PCR)-based molecular markers derived from Random Amplified Polymorphic DNA (RAPD) and other similar techniques. SCAR amplification by PCR uses a single specific primer pair to bound a genomic region of interest, such as disease resistance (MARIESCHI et al., 2016).

This study aimed to identify, via specific SCAR molecular markers, banana plants that show evidence of resistance to TR4 as a preventive genetic improvement action, since TR4 is not yet found in the national and Santa Catarina territory. Thus, it is important to previously identify sources of resistance to this pathogen. As a result, 101 genotypes from the banana germplasm collection at EPAGRI – EEI were evaluated concerning the two SCAR molecular markers linked to TR4 resistance.

Material and methods

Plant material

A total of 101 genotypes were sampled from the banana germplasm collection of EPAGRI - EEI (lat 26°57'17°S, long 48°45'51°W). This germplasm collection has a wide diversity of genotypes, genomic groups, and ploidys (Table 1). The reaction (resistance or susceptibility) of these genotypes to Foc race 1 development was recorded in naturally infested soil at EPAGRI - EEI.

DNA isolation and quality analysis

Total DNA from all genotypes was isolated from leaf samples based on the protocol described by Doyle & Doyle (1990), with modifications. The presence of contaminants, mainly proteins and phenolic compounds, in the total DNA samples was verified with the use of the spectrophotometer Bio Photometer Plus (Eppendorf, Hamburg, Germany). Total DNA samples without contaminants were considered with the ratios 260/280 and 260/230 values between 1.8 and 2.2, respectively.

Molecular markers amplification by PCR

The evaluation of SCAR markers ScaU1001 and ScaS0901 followed the methodology described by Wang et al. (2012). These SCAR markers were identified after the analysis of differentially amplified RAPD marks in the comparison of bulks between resistant and susceptible plants to Foc TR4. The isolated DNA was amplified via PCR with primers OPU1001F and OPU1001R for SCAR ScaU1001 and with primers OPS901F and OPS901R for SCAR ScaS0901. The SCAR markers amplified fragments of 1694bp and 1429bp, respectively. The Actin gene was used as an endogenous control of reactions. This primer region was designed based on the *Musa acuminata* genome and co-amplified in the PCR reactions using the ActF and ActR primers, producing a 416 bp fragment.

PCR reactions contained 20ng of DNA, 1X PCR buffer, 2.0mM of $MgCl_2$, 0.35mM of each dNTP, 0.2uM of each SCAR primer, 0.4uM of each Act primer, 1.5 U of Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA), in a final volume of 20uL. Reactions were conducted in a Veriti termocycler (Applied Biosystems, Carlsbad, CA, USA) with the following cycling: (1) 5 min at 95°C - denaturation step, (2) 30 cycles of 45 s at 95°C, 45 s at 60°C, and 2 min at 72°C, (3) 10 min at 72°C – final extension step. Reactions were evaluated in 1% agarose gels stained with ethidium bromide, and digitalized with Gel Doc XR1+ (Bio Rad Laboratories, Hercules, CA, EUA). The presence or absence of the expected bands were manually recorded for further analysis.

Results and discussion

Amplification results of the SCAR markers ScaS901 (Figure 1) and ScaU1001 (Figure 2) in the 101 genotypes of the EPAGRI - EEI banana germplasm collection showed a high frequency of bands related to TR4 resistance (Table 2). The molecular marker ScaU1001 amplified the resistance-linked band in 86 genotypes of the collection. The ScaS901 marker was amplified in 95 genotypes.

The two markers were amplified together in 85 of the 101 genotypes in the germplasm collection, representing 84.16% of the materials kept in the collection of the EPAGRI's banana breeding program. Five accessions were not enough to show amplification in either of the two markers. These materials are represented by ABB genomic composition.

The high frequency of positive genotypes, which showed amplification for the two markers linked to resistance to TR4, calls attention in a race considered to be highly destructive and with very few sources of resistance identified today. In addition, genotypes of groups highly susceptible to TR4, such as Cavendish and Prata, showed amplification in the two SCAR markers.

After validating the SCAR markers in only two genotypes known to be resistant and five genotypes susceptible to TR4, Wang et al. (2012) launched the hypothesis that each of these molecular markers is linked to one of the genes that confer resistance to Panama disease. This low number of genotypes used for the validation of the markers can culminate in the identification of false positives when the genotyping is extended to a germplasm collection with a broad genetic base, such as that of EPAGRI - EEI. According to Sutherland et al. (2013) and Li et al. (2015) the resistance to TR4 is quantitative and polygenic, therefore several genes act together in the referred pathosystem. Even if validated in a scientific publication, the two molecular markers of the SCAR type developed by Wang et al. (2012) cannot be taken as unique tools for the identification resistant genotypes.

Table 1. Accession names, genomic groups, and response to *Fusarium oxysporum* f. sp. *cubense* race 1 (R – Resistance, S – Susceptible, * - Unknown information)

*Tabela 1. Nomes dos acessos, grupos genômicos e resposta ao Fusarium oxysporum f. sp. cubense raça 1 (R – Resistência, S – Suscetibilidade, * - Informação desconhecida)*

Code	Accession name	Genome	Race 1 response	Code	Accession name	Genome	Race 1 response
1	FHIA-01	AAAB	R	52	DOMINICO HARTON	AAB	R
2	PRATA DO NORTE	AAB	S	53	FHIA-18 clone #3	AAAB	R
3	LEITE	AAA	R	54	JAPIRA	AAAB	R
4	PLATINA VELHA	*	S	55	PV-9401	AAAB	R
5	COLATINA OURO	AAAB	S	56	PACOVAN clone #3	AAB	S
6	OURO clone #1	AA	S	57	PACOVAN clone #4	AAB	S
7	OURO clone #2	AA	S	58	PACOVAN clone #5	AAB	S
8	MARANHÃO BRANCA	AAB	R	59	PACOVAN clone #6	AAB	S
9	TERRA	AAB	R	60	PRATA clone #1	AAB	S
10	TERRINHA	AAB	R	61	PRATA clone #2	AAB	S
11	FARTA VELHACO	AAB	R	62	BRANCA clone #1	AAB	S
12	PELIPITA	ABB	R	63	BRANCA clone #2	AAB	S
13	ABÓBORA	ABB	R	64	BRANCA clone #3	AAB	S
14	FIGO	ABB	R	65	PACOVAN clone #7	AAB	S
15	FIGO CINZA	ABB	R	66	BRS PRINCESA	AAAB	R
16	SÃO TOMÉ	AAA	S	67	BAGBAN158	*	*
17	COLONIAL	AAA	S	68	SEBO	ABB	R
18	ROXA	AAA	S	69	FIGUINHO	ABB	R
19	PADATH	AAB	S	70	FRENCH PLANTAIN	AAB	R
20	AZEDINHA	AAB	R	71	BAGBAN185	AAAA	R
21	PACOVAN #1	AAB	S	72	EX-34	AAB	R
22	VERDE	AAB	S	73	PRATA ANÃ clone #1	AAB	S
23	PACOVAN #2	AAB	S	74	BRANCA clone #4	AAB	S
24	PRATA PONTA APARADA	AAB	S	75	NANICÃO	AAA	R
25	PA-0322	AAAB	R	76	GRANDE NAIDE	AAA	R
26	FHIA-18 clone #1	AAAB	*	77	WILLIAMS	AAA	R
27	FHIA-02 clone #1	AAAA	S	78	BRS SCS BELLUNA	AAA	R
28	TERRA clone #2	AAB	R	79	SCS NANICÃO CORUPÁ	AAA	R
29	TERRA MARANHÃO clone #1	AAB	R	80	SCS PRATA CATARINA	AAB	R
30	TERRINHA clone #2	AAB	R	81	BAGBAN179	AAB	R
31	D´ANGOLA	AAB	R	82	PRATA EPAGRI 02	AAB	S
32	FHIA 21	AAB	R	83	BAGBAN187	AAB	S
33	TERRA ANÃ	AAB	R	84	SUPER ANÃ	AAB	S
34	BRS TROPICAL	AAAB	*	85	MODERNA	AAB	S
35	SH-3640	*	*	86	FERRO	AAA	R
36	SÃO FRANCISCO	*	S	87	ZELIC	AAA	R
37	ZULU	ABB	S	88	GALIL CAVENDISH	AAA	R
38	PRATA ZULU	ABB	S	89	IAC 2001	AAA	R
39	YANGAMBI	AAA	R	90	PRATA ANÃ clone #2	AAB	S
40	PA-4244	AAAB	R	91	PRATA ANÃ clone #3	AAB	S
41	PV-4285	AAAB	R	92	PRATA ANÃ clone #4	AAB	S
42	PV-4268	AAAB	R	93	PRATA ANÃ clone #5	AAB	S
43	PV-42.142	AAAB	R	94	PRATA ANÃ clone #6	AAB	S
44	ST-4208	AAAB	R	95	PRATA ANÃ clone #7	AAB	S
45	JV-0315	AAAB	*	96	PRATA BABITONGA	AAB	S
46	ST-1231	*	R	97	FHIA-02 clone #2	AAAA	S
47	PIONEIRA	AAAB	S	98	TERRA MARANHÃO clone #2	AAB	R
48	PV-0344	AAAB	*	99	FIGO ANÃ	ABB	R
49	FHIA-18 clone #2	AAAB	R	100	PRATA ANÃ clone #8	AAB	S
50	PA-9401	AAAB	R	101	BAGBAN197	AAA	R
51	ANGELA	*	*				

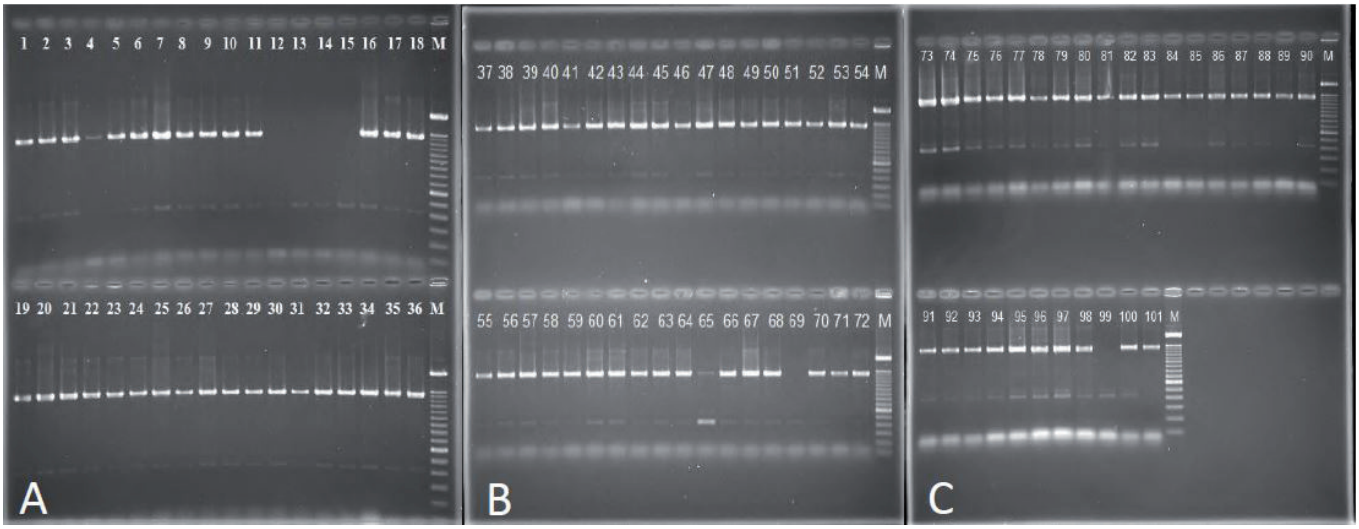

Figure 1. Multiplex PCR of the amplification of the SCAR marker ScaS901 (1429 bp) in combination with the endogenous Actin control (416 bp). A: genotypes 1 to 36; B: genotypes 37 to 72; C: genotypes 73 to 101. *Photos: Gustavo Henrique Ferrero Klabunde*
Figura 1. PCR multiplex da amplificação do marcador SCAR ScaS901 (1429 pb) em combinação com o controle endógeno Actina (416 pb). A: genótipos 1 a 36; B: genótipos 37 a 72; C: genótipos 73 a 101. Fotos: Gustavo Henrique Ferrero Klabunde

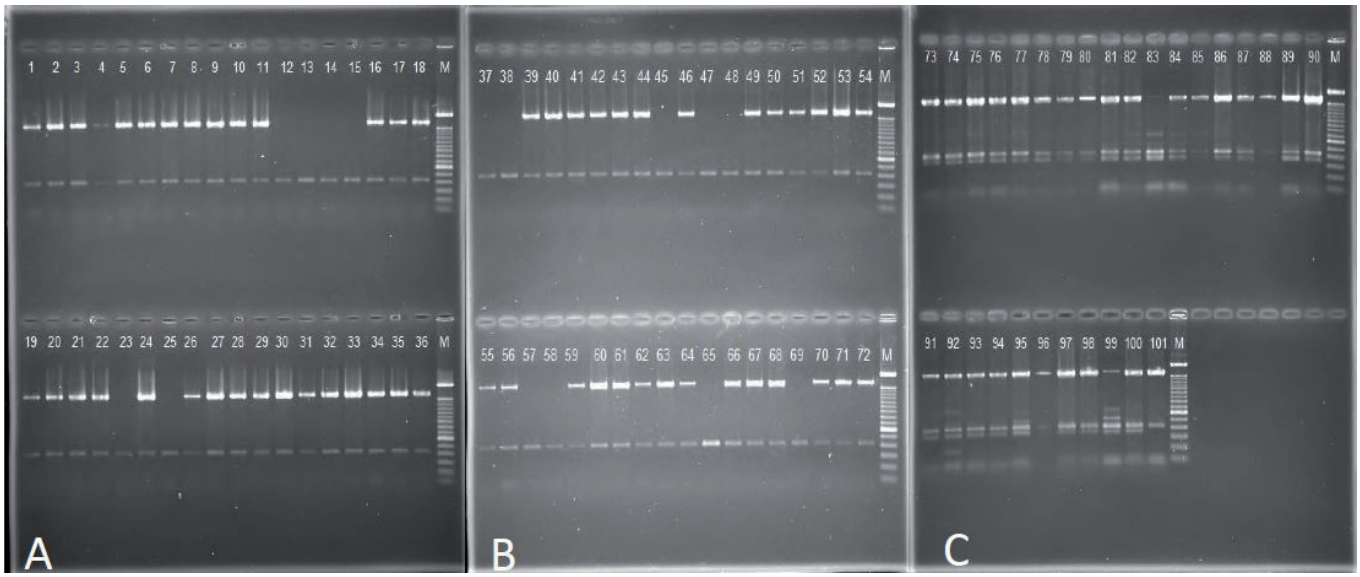

Figure 2. Multiplex PCR of SCAR marker amplification ScaU1001 (1684 bp) in combination with the endogenous Actin control (416 bp). a: genotypes 1 to 36; b: genotypes 37 to 72; c: genotypes 73 to 101. Photos: Gustavo Henrique Ferrero Klabunde
Figura 2. PCR multiplex da amplificação do marcador ScaU1001 (1684 bp) em combinação com o controle endógeno Actina (416 pb). A: genótipos 1 a 36; B: genótipos 37 a 72; C: genótipos 73 a 101.
Fotos: Gustavo Henrique Ferrero Klabunde

Resistance of potential accessions can only be confirmed after inoculation with Foc TR4 and disease evaluation in controlled environments where the TR4 is present. Due to the preference of the Brazilian consumer for fruits from the Cavendish (AAA) and Prata (AAB) subgroups, these accessions would have priority for resistance evaluation against TR4.

These markers can be linked to several genes responsible for the dynamics of the *Musa spp.* x TR4. The two markers did not show amplifications in the following genotypes: Pelipita, Abóbora, Figo, Figo cinza and Figuinho (ABB); suggesting that these materials are more susceptible because they do not present amplification in the two molecular markers. Further studies need to be conducted on these SCAR markers in order to quantify the real link between these marks and the possible associated resistance locus. The complete genome of *Musa acuminata* (D´HONT et al., 2012) can easily provide this information.

Table 2. Molecular profile of SCAR markers ScaS901 and ScaS1001 in all accessions. (P - Positive PCR amplification, N - Negative PCR amplification)

Tabela 2. Perfil molecular dos marcadores SCAR, ScaS901 e ScaS1001 nos acessos avaliados. (P – Amplificação via PCR positiva, N – Amplificação negativa via PCR)

Code	ScaS901	ScsU1001	Code	ScaS901	ScsU1001	Code	ScaS901	ScsU1001	Code	ScaS901	ScsU1001
1	P	P	27	P	P	53	P	P	79	P	P
2	P	P	28	P	P	54	P	P	80	P	P
3	P	P	29	P	P	55	P	P	81	P	P
4	P	P	30	P	P	56	P	P	82	P	P
5	P	P	31	P	P	57	P	N	83	P	P
6	P	P	32	P	P	58	P	N	84	P	P
7	P	P	33	P	P	59	P	P	85	P	P
8	P	P	34	P	P	60	P	P	86	P	P
9	P	P	35	P	P	61	P	P	87	P	P
10	P	P	36	P	P	62	P	P	88	P	P
11	P	P	37	P	N	63	P	P	89	P	P
12	N	N	38	P	N	64	P	P	90	P	P
13	N	N	39	P	P	65	P	N	91	P	P
14	N	N	40	P	P	66	P	P	92	P	P
15	N	N	41	P	P	67	P	P	93	P	P
16	P	P	42	P	P	68	P	P	94	P	P
17	P	P	43	P	P	69	N	N	95	P	P
18	P	P	44	P	P	70	P	P	96	P	P
19	P	P	45	P	N	71	P	P	97	P	P
20	P	P	46	P	P	72	P	P	98	P	P
21	P	P	47	P	N	73	P	P	99	N	P
22	P	P	48	P	N	74	P	P	100	P	P
23	P	N	49	P	P	75	P	P	101	P	P
24	P	P	50	P	P	76	P	P			
25	P	N	51	P	P	77	P	P			
26	P	P	52	P	P	78	P	P			

Conclusions

The high incidence of resistance related alleles in the EPAGRI – EEI germplasm collection should be treated with caution until further studies are conducted to elucidate the efficiency of these published markers.

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