Biotecnología Aplicada

Elfos scientiae
http://elfosscientiae.cigb.edu.cu/

RESEARCH

Molecular identification of promising *Nicotiana* spp. genotypes resistant to multiple diseases

✍Juan Luis Pérez-Rodríguez[1], Verónica Toledo[2], Humberto García[2], Marianela Díaz[2], Rafaela Gorski[3], Fernanda Tocate[3], Lucia Lopes[3], José Padua[3]

[1] UEB Estación Experimental Cabaiguán
Carretera Santa Lucía, Km 2, Cabaiguán, CP 62410, Sancti Spíritus, Cuba
[2] Instituto de Investigaciones del Tabaco
Carretera Tumbadero Km 8 ½, San Antonio de los Baños, CP 3500, Mayabeque, Cuba
[3] Global Leaf Science & Research. Ave. Gal. Plinio Tourinho, Río Negro, PC: 3200, Paraná, Brasil
✍espec.banco@eetcab.co.cu

ABSTRACT

At the international level, molecular marker-assisted selection has been used in tobacco (*Nicotiana tabacum* L.) breeding programs. However, its use in the genetic improvement of Cuban tobacco is limited. The objective of this research was to identify promising genotypes resistant to multiple diseases by means of DNA markers. Twenty-five accessions including Cuban cultivars and their parents were analyzed. As a result, the SCAR Mil27 marker showed the presence of the RBM1 region that provides partial resistance to *P. hyoscyami* in the Cuban dark tobacco cultivars, but not in the 'Criollo' and 'Corojo' cultivars, coincidentally reported as susceptible. The SCAR Php marker linked to the *Php* gene, which confers monogenic resistance to race 0 of *P. nicotianae*, was validated and its absence was proved in the evaluated cultivars. Of the Cuban cultivars only 'Criollo 98', 'Corojo 99', 'Corojo 2006' and 'Corojo 2012' were identified as carriers of the *N* gene, which confers dominant monogenic resistance to VMT. 'San Luis 22' was the only cultivar carrying the *Rk1* gene, which confers resistance to *Meloidogyne* sp. and the *va* gene, which confers monogenic resistance to PVY, was not identified in Cuban cultivars. This research constitutes the first molecular characterization of Cuban tobacco germplasm in terms of its resistance to the main diseases. Effective markers are now available for the selection of disease resistant genotypes, a fundamental step for their introduction in Cuban tobacco breeding programs.

Keywords: black shank, blue mold, nematodes, *Nicotiana tabacum* L., PVY, TMV, SCAR markers

RESUMEN

Identificación molecular de genotipos promisorios de *Nicotiana* spp. resistentes a múltiples enfermedades. A nivel internacional, se ha utilizado el mejoramiento asistido por marcadores moleculares en los programas de mejoramiento en tabaco (*Nicotiana tabacum* L.). No obstante, su uso en el mejoramiento genético del tabaco cubano es limitado. La presente investigación tuvo como objetivo identificar mediante marcadores de ADN genotipos promisorios resistentes a múltiples enfermedades. Se analizaron 25 accesiones entre las que se encontraban cultivares cubanos y sus progenitores. Como resultado, el marcador SCAR Mil27 mostró la presencia de la región RBM1 que brinda resistencia parcial a *P. hyoscyami* en los cultivares de tabaco tipo Negro cubano, no así en los cultivares 'Criollo' y 'Corojo', coincidentemente informados como susceptibles. Se validó el marcador SCAR Php ligado al gen *Php*, que confiere resistencia monogénica a la raza 0 de *P. nicotianae* y se comprobó su ausencia en los cultivares evaluados. De los cultivares cubanos solo 'Criollo 98', 'Corojo 99', 'Corojo 2006' y 'Corojo 2012' se identificaron como portadores del gen *N*, que confiere resistencia monógenica dominante a VMT. 'San Luis 22' fue el único cultivar portador del gen *Rk1*, que confiere resistencia a *Meloidogyne* sp. y no se identificó en los cultivares cubanos el gen va que confiere resistencia monogénica a PVY. Esta es la primera investigación sobre la caracterización molecular del germoplasma de tabaco cubano en cuanto a su resistencia a las principales enfermedades. Ahora se dispone de marcadores efectivos para la selección de genotipos resistentes a las enfermedades, un paso fundamental para su introducción en los programas cubanos de mejora genética del tabaco.

Palabras clave: pata prieta, moho azul, *Nicotiana tabacum* L., nemátodos, VMT, PVY, marcadores SCAR

How to cite (Vancouver style):
Pérez-Rodríguez JL, Toledo V, García H, Díaz M, Gorski R, Tocate F, *et al*. Molecular identification of promising *Nicotiana* spp. genotypes resistant to multiple diseases. Biotecnol Apl. 2021;38(2):2221-6.

Introduction

In the Tobacco Research Institute of Cuba (TRI), there have been obtained tobacco cultivars (*Nicotiana tabacum* L.) which respond to the quality standards that identify the Habano brands at international level [1]. Despite, under conditions of increasing inoculums due to favorable climatic environments for the development of diseases, current cultivars do not demonstrate the expected resistance [2].

Breeding programs conducted are based on phenotypic evaluation of the target traits [1]. Regarding disease resistance, selection is often performed in environments with low inoculum pressure, which has made effective selection difficult [3]. Moreover, the results of the phenotypic evaluation may not be reliable due to environmental effects [4, 5]. Since 1990's, the use of DNA molecular markers has provided important achievements in transferring favorable traits into commercial cultivars and hybrids.

Molecular markers are effective in the construction of high-density linkage maps and in the detection of Quantitative Trait Loci (QTL) and their mapping to specific genomic regions. Once a close

1. Espino M, Espino E. Catálogo de variedades comerciales de tabaco cubano. La Habana, Cuba: Instituto de Investigaciones del Tabaco, Grupo Empresarial de Tabaco de Cuba, Ministerio de la Agricultura; 2012.

2. González AC, Toledo V. Comportamiento de las accesiones del género *Nicotiana* informadas como fuentes de resistencia frente a aislamientos cubanos de *Phytophthora nicotianae* Breda de Haan. Rev Protección Veg. 2019;34(1):1-7.

link is established between a marker and a trait of interest, the marker can be used as the basis for indirect selection of the target gene or QTL by means of Marker Assisted Selection (MAS). MAS limits the role of phenotypic evaluation to the establishment of marker-trait linkages and provides a much easier and highly reliable means of indirect selection for traits that are affected by the environment and/or which phenotypic evaluation is tedious, slow or expensive [4, 6, 7]. This technology allows pyramiding different genes of resistance to multiple diseases in the same genome, achieving a wide resistance in the same cultivar [4, 8, 9].

Internationally, MAS has been used in tobacco improvement programs. Regarding disease resistance, the use of DNA markers to identify genotypes resistant to major diseases have been reported and are available to the scientific community [10, 11]. However, the use of MAS in the genetic improvement of Cuban tobacco has been very limited [12].

Therefore, the present research was aimed to characterize, using DNA molecular markers, the accessions of *Nicotiana* spp. in terms of resistance to main diseases. Such purpose constitutes an important step to introduce MAS in the genetic improvement of tobacco in Cuba.

Materials and methods

Plant material

The research was carried out at the Global Leaf Agronomy Development (GLAD), Rio Grande, Paraná state, Brazil, belonging to the British American Tobacco (BAT) company. A total of 25 *Nicotiana* accessions were analyzed. In the selection, Cuban cultivars were considered as well as their parents. In addition, controls of susceptibility and resistance to the main diseases that affect the crop in Cuba were included (Table 1).

The seeds were sown in expanded polystyrene trays, with 200 alveoli and Carolina Soil® substrate with Carolina Soil® substrate (70 % sphagnum peat, 20 % carbonized rice husks, 10 % perlite), based on the technology for the production of seedlings in floating trays described by García and Andino [22]. The trays were placed in protected culture conditions until their evaluation.

DNA extraction

In seedbed , the morphological characterization was performed on ten seedlings randomly selected for each replication (30 per treatment), following the methodology proposed by Torrecilla *et al*. [23]. For each seedling, leaf color, apex shape, leaf base shape and stem color were evaluated as qualitative morphological descriptors, at 40 d after seed sowing. Stem diameter, plant height, leaves number, length of main leaf, width of main leaf and plant fresh mass were determined as quantitative morphological descriptors, at 20, 30 and 40 d after seed sowing.

Genomic DNA was extracted from 6.0 mm diameter discs of leaves from six plants with 37 days of age using Biosprint 96 DNA Plant Kit (Qiagen), according to manufacturers' instructions. DNA quantification was performed in a UV/Visible NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) and DNA quality was measured by absorbance ratio (Abs_{260nm}-Abs_{320nm}) / (Abs_{280nm} – Abs_{320nm}). Samples with values in the range of 1.6-2.0 were considered to be good quality DNA. DNA isolates were stored at –20 ºC until use.

Table 1. *Nicotiana* spp. accessions used in the characterization by DNA markers of resistance to the main diseases affecting tobacco cultivation in Cuba

No.	Accession name	Observations
1	Criollo tradicional	Ancient commercial cultivar
2	Corojo tradicional	Ancient commercial cultivar. Control of susceptibility to *Peronospora hyoscyami* de Bary f.sp. tabacina (Adam) in Cuba [3]
3	Havana 142 Seed	Resistant to *Thielaviopsis basicola* (Berk. and Broome) Ferr. [13] and to *Pseudomonas syringae* pv. tabaci [14]
4	Oven 62	Resistant to *P. hyoscyami* from *Nicotiana debneyi* Domim [15]
5	Habana 92	Commercial cultivar [1]
6	Habana 2000	Commercial cultivar [1]
7	Criollo 98	Commercial cultivar [1]
8	Sancti Spiritus 96	Commercial cultivar [1]
9	Corojo 99	Commercial cultivar [1]
10	Corojo 2006	Commercial cultivar [1]
11	Corojo 2012	Commercial cultivar [1]
12	Bergerac C	Control of susceptibility to *Phytophthora nicotianae* Breda de Haan)[a]
13	San Luis 22	Commercial cultivar [1]
14	PH-89	Commercial cultivar[a]
15	Burley 21	It carries the *N* gene from *N. glutinosa*. Resistant to race 0 of *P. syringae* from *Nicotiana longiflora* Cabanilles [16]
16	TB-2519	Resistant to nematodes species *Meloidogyne incognita* (Kofoid and White) Chitwood, Tobacco Mosaic Virus (TMV) and to Potato Virus Y (PVY)[a]
17	DH-3433-17	Resistant to nematodes species *M. incognita*, TMV and to PVY[a]
18	Polalta	Resistant to Tomato spotted wilt virus (TSWV) from *N. alata* and to PVY [17]
19	Fumo da Corda	Resistant to *P. syringae*[a]
20	*Nicotiana rustica* Linneaus	Highly resistant to different Cuban isolates of *P. nicotianae* [2]. Possibly carries the *Wz* region. This region has also been reported to provide resistance to *P. syringae* races 0 and 1 [16]
21	*Nicotiana alata* Link y Otto	Resistance donor to TSWV [17]
22	*Nicotiana glutinosa* Linneaus	Donor of *N* gene that confers resistance to TMV [18]
23	*Nicotiana debneyi* Domim	Donor of *RBM1* region conferring resistance to *P. hyoscyami* [19]
24	*Nicotiana megalosiphon* Heurck y Mueller	It is reported as resistant to *P. nicotianae* [20] and to *P. hyoscyami* [21]
25	*Nicotiana excelsior* Black	Resistant to *P. hyoscyami* [15]

[a] Morphological characterization and disease resistance database. Genebank Global Leaf Science & Research, Brazil (unpublished data).

PCR reactions

The Sequence Characterized Amplified Region (SCAR) marker Mil275 was used to identify the presence of the region that confers resistance to *Peronospora hyoscyami* de Bary f.sp. tabacina (Adam) from *Nicotiana debneyi* Domim [23], named as RBM1 by Wu *et al*. [21]. The PCR reactions were performed in a final volume of 25 µL containing 1× Taq Pol reaction buffer (Invitrogen, Thermo Fisher Scientific, USA; 10×: 200 mM Tris-HCl pH 8.4, 500 mM KCl), 3% (m/v) PVP, 2.3 mM $MgCl_2$, 200 µM dNTPs, 0.4 µM of each primer, 1 U of Taq Polymerase

3. García H, Castro MdC, Oliva O, Enriquez G. 'BHmN'Variedad de tabaco (*Nicotiana tabacum* L.) resistente al moho azul (*Peronospora hyoscyami* de Bary f. Sp. *Tabacina*) desde estadios tempranos obtenida por nueva estrategia de selección con su androestéril. Cuba Tabaco 2010;11(2):17-23.

4. Singh B, Singh AK. Marker-assisted plant breeding: principles and practices. New Delhi: Springer India; 2015.

5. Laitinen RA, Nikoloski Z. Genetic basis of plasticity in plants. J Exp Bot. 2019;70(3):739-45.

6. Melese L. Marker assisted selection in comparison to conventional plant breeding: review article. Agric Res Technol. 2018;14(2):1-10.

(Invitrogen, Thermo Fisher Scientific, USA) and 30 ng of genomic DNA.

Amplification was carried out in a Applied Biosystems™ Veriti™ 96-Well Thermal Cycler (Thermo Fisher Scientific) as follows: 1 cycle at 94 ºC for 4 min followed by 25 cycles at 94 ºC for 30 s, at 58 ºC 45 s and 72 ºC for 1 min, with a final extension at 72 ºC for 5 min. The assay was performed three times and the expected band size for the fragment linked to this region was 205 bp. Cultivar 'Perevi' was used as a positive control (carrier of the region) and cultivar 'Coker 371-Gold' was used as a negative control (non-carrier of the region).

The SCAR Php marker was used to identify the presence of the *Php* gene [24]. The PCR reactions were performed in a final volume of 25 µL containing 1× Taq Pol reaction buffer (Invitrogen, Thermo Fisher Scientific, USA; 10×: 200 mM Tris-HCl pH 8.4, 500 mM KCl), 3% (m/v) PVP, 5.0 mM $MgCl_2$, 200 µM dNTPs, 0.3 µM of each primer, 1 U of Taq Polymerase (Invitrogen, Thermo Fisher Scientific, USA) and 30 ng of genomic DNA.

Amplification was carried out in a Applied Biosystems™ Veriti™ 96-Well Thermal Cycler (Thermo Fisher Scientific) as follows: 1 cycle at 94 ºC for 2 min followed by 29 cycles of 30 s at 94 ºC, 1 min at 69 ºC and 1 min at 72 ºC with a final extension at 72 ºC for 5 min. The assay was repeated three times and the expected band size for the fragment linked to the *Php* gene was 770 bp. The cultivar 'Coker 371-Gold' was used as a positive control and the cultivar 'K326' as a negative control.

The Qiagen Multiplex PCR Plus Kit (Qiagen, USA) with 2× PCR master mix (final concentration: 1×) was used to identify the presence of the *N* gene [25], *Rk1* gene [26] and the *va* gene [27]. The PCR reaction mixture consisted of 1.75 µL of QSolution, 6.25 µL of Master Mix, 1.0 µL of primer mix (TMV 5.0 µM, *M. icognita* 0.75 µM and PVY 0.75 µM) and 2.5 µL of genomic DNA in a final volume of 12.9 µL. Amplification was carried out in a Applied Biosystems™ Veriti™ 96-Well Thermal Cycler (Thermo Fisher Scientific) as follows: 15 min at 95 ºC (initial activation step), followed by 35 cycles at 94 ºC for 30 s, 53 ºC for 90 s, and 72 ºC for 2 min, with a final extension of 72 ºC for 10 min. The assay was repeated three times.

The number and expected length of the bands for the fragments linked to the resistant and susceptible genotypes of TMV were two fragments of 1800 and 1500 bp for the carrier and one fragment of 1500 bp for the non-carrier genotypes. Equivalently, for *M. incognita* race 1 y race 3, there was for the carrier one fragment of 360 bp, and two fragments of 360 and 320 bp for the non-carrier. Finally, for the Potato Virus Y (PVY), there were one fragment of 500 bp for the carrier and two fragments of 500 and 700 bp for the non-carrier. The cultivar 'Coker 371-Gold' was used as positive control and the cultivar 'TB 2519' as negative control.

All PCR reaction products were visualized in duplicate using 1.0 % agarose gel electrophoresis stained with 0.5× TBE Unisafe Dye® (Uniscience, Osasco, Brazil). DNA bands were separated at 150 V for 3 h in 1× Tris-Borato-EDTA buffer, and the size of the fragments was estimated from a standard molecular weight ladder (100 bp DNA ladder; Kasvi, Brazil).

Results and discussion

SCAR Mil275 marker

All the current Cuban dark tobacco cultivars amplified the 205 bp band originated by the SCAR marker Mil275 and linked to the *RBM1* region from *N. debneyi* [23]. The non-commercial cultivars 'Criollo' and 'Corojo', coincidentally informed as highly susceptible to the disease [1], did not produce the amplification product (Figure 1, Table 2).

Although all current cultivars of dark tobacco amplify the band to 205 bp, their resistance to *P. hyoscyami* infection has been reported to be variable [1]. Furthermore, it is noticiable that the cultivar 'San Luis 22', Virginia type, moderately resistant to this oomycete, did not show the 205 bp band characteristic of the resistant genotypes (Table 2). This was irrespective of its resistance coming from *N. debneyi* through the accession 'Bel 61-10' [28].

It is known that the interaction with *P. hyoscyami* suggests a polygenic resistance type, from the behavior of different genotypes of *N. tabacum* to the disease [15, 19]. Nevertheless, there is no information about the especific genes present in *RBM1* and their involvement in the phenotypic response to the pathogen.

Julio *et al*. [23] evaluated the resistance of 17 double haploid lines coming from the F1 BB16 (*N. degneyii* resistance) × TN86 (susceptible) to *P. hyoscyami* and determined a link between the Mil275 marker and

7. Tshikunde NM, Mashilo J, Shimelis H, Odindo A. Agronomic and physiological traits, and associated Quantitative Trait Loci (QTL) affecting yield response in wheat (*Triticum aestivum* L.): A review. Front Plant Sci. 2019;10:01428.

8. Liu R, Lu J, Zhou M, Zheng S, Liu Z, Zhang C, *et al*. Developing stripe rust resistant wheat (*Triticum aestivum* L.) lines with gene pyramiding strategy and marker-assisted selection. Genet Resour Crop Evol. 2020;67(2):381-91.

9. Kumar A, Sandhu N, Dixit S, Yadav S, Swamy B, Shamsudin NAAJR. Marker-assisted selection strategy to pyramid two or more QTLs for quantitative trait-grain yield under drought. Rice. 2018;11(1):35.

10. Bakaher N. Genetic markers in tobacco, usage for map development, diversity studies, and quantitative trait loci analysis. In: Ivanov N, Sierro N, Peitsch M (eds). The tobacco plant genome. Compendium of Plant Genomes. Cham: Springer Nature Switzerland AG; 2020. p. 43-9.

11. Yang S, Li D, Fisher AJ, Pramod S, Xu D. CORESTA Guide N 16; 2019.

12. Acanda Y, Rodríguez L, García H. La selección asistida por marcadores en el mejoramiento genético del tabaco. Cuba Tabaco. 2008;9(2):46-51.

13. Johnson J. Breeding tobacco for resistance to *Thielavia* root-rot, US Dept Agr Tech Bull. 1930;11:175.

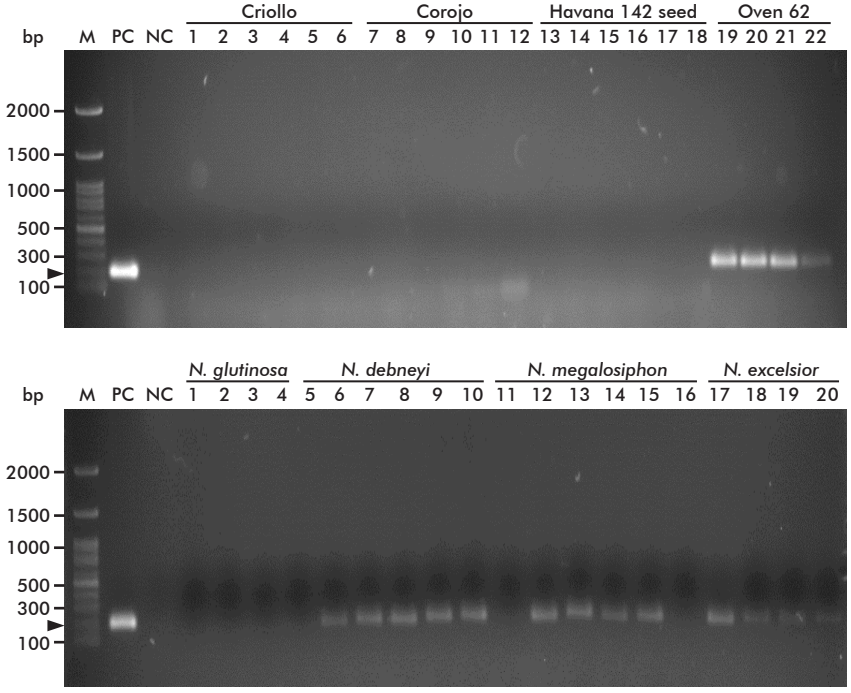

Figure 1. Agarose gel electrophoresis (1 %) showing amplification products generated by Sequence characterized amplified region (SCAR) Mil275 marker linked to RBM1 region associated to polygenic resistance to *Peronospora hyoscyami* of Bary f.sp. tobacco (Adam). Six different amplifications were visualized for each accession. Arrowheads indicate the expected DNA band at 205 bp. M: 100 bp DNA ladder, PC: positive control ('Perevi') and NC: negative control ('Burley 21'), bp: base pairs.

RBM1. Despite, it showed significant differences in the reaction of *P. hyoscyami* with double haploid lines that did not possess the marker Mil275 and suggested that additional genetic factors, not segregated with the marker, modulate the expression of resistance.

Although *N. debneyi* shows high resistance to the disease from early stages (seedling phase), this is not the case of cultivars [19]. It is possible that not carrying out phenotypic selection in the breeding programs from this phase, genes related to resistance of monogenic type (qualitative), present in the wild species and then absent in the cultivated tobacco, have not been carried over.

Particularly, the wild species *Nicotiana megalosiphon* Heurck and Mueller and *Nicotiana excelsior* Black amplified the band to 205 bp, linked to the RBM1 region (Figure 1, Table 2). There are no references to the SCAR Mil275 marker evaluation in wild species belonging to the genus *Nicotiana*. Milla *et al.* [15] genotyped the species *N. excelsior*, *Nicotiana velutina* Wheeler, *Nicotiana goodspeedii* Wheeler and *N. debneyi* using eight random amplified polymorphic DNA (RAPD) markers in coupling phase and two SCAR markers (SUBC180.251 and SOPR06.268), in both cases linked to the *RBM1* region of *N. debneyi*. They identified a high level of polymorphism among species, but only the presence of the RBM1 region in *N. degneyii*.

In our research, the species *N. debneyi* and *N. excelsior* were evaluated, coinciding with the study by Milla *et al*. [15], and *N. megalosiphon*. These accessions are endemic to Australia and belong to the Suaveolentes section according to the classification proposed by Knapp *et al*. [29]. Thus, the SCAR Mil275 marker may have amplified a common fragment, which does not imply the presence of the RBM1 region in the three species.

The SCAR Mil275 marker can be a valuable tool as part of the introduction of MAS in tobacco breeding programs in Cuba. Despite, its evaluation should be accompanied with other SCAR markers [15], so that, together, they provide a greater correlation with resistance to *P. hyoscyami*. Furthermore, the use of this marker should be avoided when the breeding programs involve wild species belonging to the Suaveolentes section.

SCAR Php marker

Internationally, both monogenic and polygenic resistance have been investigated in the development of cultivars resistant to *P. nicotianae* [30-32]. The introgressed *Php* gene of the specie *N. plumbaginifolia* confers complete resistance to race 0 of the pathogen, not to race 1 [33, 34]. In Cuba, there has only been reported the presence of race 0 in a generalized way [2]. In this research, the SCAR Php marker did not amplify the 770 bp fragment linked to the *Php* gene in the *N. tabacum* cultivars evaluated (Figure 2, Table 2). This result is consistent with the fact that the resistance shown by current cultivars increases from the seedling phase, where it is practically nil, to high levels at flowering time [3], which is characteristic of genotypes with partial resistance [35].

Only the *Nicotiana rustica* L. species showed the band at 770 bp linked to the *Php* gene in *N. tabacum* (Figure 2). There were no references of evaluating a population of *N. rustica* for the presence of the *Php* gene with this marker. Otherwise, the detection of diseased seedlings of this species in a population inoculated with *P. nicotianae* race 0 [36], is contradictory

Table 2. *Nicotiana* accessions screened for the presence (+) or absence (-) of DNA markers linked to resistance/susceptibility to *Peronospora hyoscyami* of Bary f.sp. tobacco (Adam), *Phytophthora nicotianae* Breda de Haan, Tobacco Mosaic Virus (TMV), *Meloidogyne incognita* (Kofoid and White) Chitwood races 1 and 3 and Potato Virus Y (PVY)

Accession name	SCAR Mil75	SCAR Php	Multiplex		
			TMV	*M. incognita*	PVY
Criollo tradicional	–	–	–	–	–
Corojo tradicional	–	–	–	–	–
Havana 142 Seed	–	–	–	–	+
Oven 62	+	–	–	–	–
Habana 92	+	–	–	–	–
Habana 2000	+	–	–	–	–
Criollo 98	+	–	+	–	–
Sancti Spiritus 96	+	–	–	–	–
Corojo 99	+	–	+	–	–
Corojo 2006	+	–	+	–	–
Corojo 2012	+	–	+	–	–
Bergerac C	+	–	+	–	+
San Luis 22	–	–	–	+	–
PH-89	–	–	–	+	–
Burley 21	–	–	+	–	–
TB-2519	–	–	+	+	+
DH-3433-17	–	–	+	+	+
Polalta	+	–	–	–	+
Fumo da Corda	–	–	–	–	–
N. rustica L.	–	+	ND	ND	ND
N. alata	+	–	ND	ND	ND
N. glutinosa L.	–	–	ND	ND	ND
N. debneyi	+	–	ND	ND	ND
N. megalosiphon	+	–	ND	ND	ND
N. excelsior	+	–	ND	ND	ND

SCAR Mil75, SCAR Php: SCAR Mil75 and SCAR Php markers linked to polygenic resistance of *N. tabacum* to *P. hyocyami* from *N. debneyi* (*RBM1* region) [1], and to *P. nicotianae* race 0 from *N. plumbaginifolia* (*Php* gene) [26], respectively.
TMV, M. incognita and PVY: Multiplex marker linked to monogenic resistance of N. tabacum to TMV (N gene), M. incognita, race 1 and race 3 (Rk1 gene) and PVY (va gene), respectively.
+, –: Presence and absence, respectively, of amplification products linked to resistant genotypes. ND: Not discerned between resistant and susceptible genotypes.

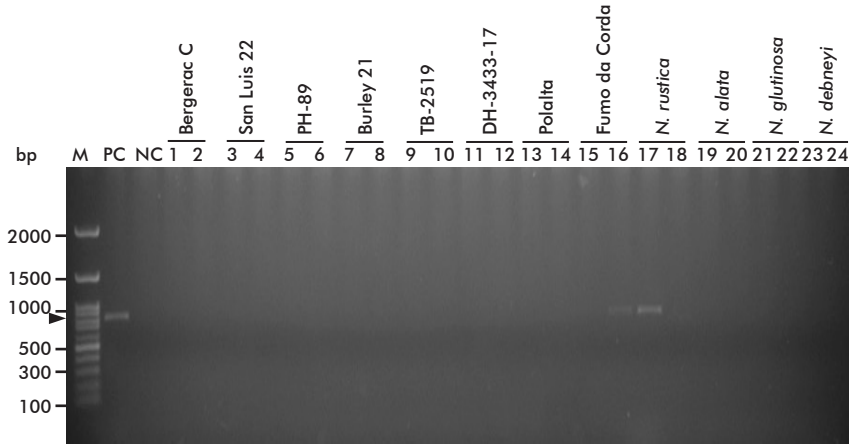

Figure 2. Agarose gel electrophoresis (1 %) showing amplification products generated by Sequence characterized amplified region (SCAR) Php marker linked to *Php* gene associated to monogenic resistance to *Phytophthora nicotianae* Breda de Haan. Two different amplifications were visualized for each accession. The arrowhead indicates the expected DNA band at 770 bp. M: 100 bp DNA ladder (Promega). PC: positive control ('Coker 371-Gold'). NC: negative control ('K326'). bp: base pairs.

14. Knoche K, Clayton M, Fulton R. Comparison of resistance in tobacco to *Pseudomonas syringae* pv. *tabaci* races 0 and 1 by infectivity titrations and bacterial multiplication. Phytopathology. 1987;77(9):1364-8.

with the immunity shown towards this race of the pathogen from genotypes carrying the *Php* gene [37, 38]. Therefore, the most accurate hypothesis could be related to the amplification of a common DNA fragment between *N. rustica* and *N. tabacum*, unrelated to resistance to *P. nicotianae*, similar to what happened with the SCAR Mil75 marker for the species *N. excelsior* and *N. megalosiphon* (Figure 2, Table 2).

However, the SCAR Php marker amplified the product linked to the *Php* gene in the 'Coker 371-Gold' control, previously informed as homozygous *Php*/*Php* [37, 39]. It proved its validity when Cuban cultivars were not identified as carriers, in agreement with that proposed for five of them by Martínez-Pacheco *et al*. [38]. Besides, the SCAR marker used here constitutes a more reproducible tool than the RAPD marker UBC30490 proposed by Johnson *et al*. [37] and used by Martínez-Pacheco *et al*. [38], therefore, more effective to be used in the MAS [40].

Dluge [41] found that when introgressing in chromosome seven of *N. tabacum* aimed to improve the resistance to *P. hyoscyami,* the RBM1 region derived from *N. debneyi* partially replaces the QTL *Phn7.1*, this region been associated to the resistance to *P. nicotianae* present in Cuban cultivars. Considering that both diseases are the ones that cause more losses to the cultivation in Cuba [1, 42], the obtention of resistant cultivars to both *P. hyoscyami* and *P. nicotianae* becomes a challenge for tobacco breeders [3]. The incorporation of the *Php* gene in the breeding programs of Cuban tobacco will allow obtaining cultivars with high resistance to both diseases.

Pyramidization in a genotype of qualitative resistance (*Php* gene), together with the presence of a high level of partial resistance, is among the objectives of the genetic improvement of Cuban tobacco. The SCAR Php marker can be an effective tool in the selection of the most promising genotypes, carefully interpreting the results when the breeding programs involve wild species belonging to the genus *Nicotiana*. Other molecular markers not included in this study and determined for other sources of resistance to *P. nicotianae*, such as the *Wz* region coming from *N. rustica* [43] or the *Phn15.1* region present in cultivar 'Beinhart 1000' [31], could also contribute to generate genotypes with a higher degree of resistance to this pathogen.

Multiplex marker

Tobacco Mosaic Virus (TMV)

From the Cuban cultivars, only 'Criollo 98', 'Corojo 99', 'Corojo 2006' and 'Corojo 2012' were identified as carriers of the *N* gene from *Nicotiana glutinosa* L., when producing amplification products (1.8 and 1.5 kb bands) similar to the resistant control (Figure 3, Table 2). This result is consistent with the resistance/susceptibility reported for the accessions evaluated [44].

It is well established the negative impact of genes linked to the introgressed region of *N. glutinosa* that confers resistance to TMV on yields and quality of Virginia and Burley tobacco, not so for the dark tobacco [45]. Lewis *et al*. [18] demonstrated that it is possible to obtain recombination within the introgressed region. Marker-assisted backcrossing may allow selection against the undesirable alleles of *N. glutinosa* and increases the probability of developing disease resistant cultivars of dark tobacco with higher yields and quality than the current ones carrying the *N* gene.

Nematodes Meloidogyne incognita (Kofoid and White) Chitwood

Only 'San Luis 22' was identified as a carrier of *Rk1* gene among all Cuban cultivars (Figure 3, Table 2), by amplifying only one band at 360 bp. The lack of the *Rk1* gene does not necessarily imply the susceptibility of the rest of the cultivars, although it is improbable, from the study of the parents, that they carry the *Rk2* gene, which is the other gene reported as conferring resistance in *N. tabacum* to *Meloidogyne* spp. [26].

The phenomenon of resistance breakdown in cultivars resistant to certain phytopathogenic fungi by nematodes has been widely documented [46-48]. In experiments with plants of double radical system, in which the nematodes were inoculated to one root system and the fungus to the other, the plant behaved as susceptible to the second. This suggested that metabolic and/or physiological changes induced by the nematodes could be responsible for resistance breakage and not the mechanical damage inflicted by it in the root of the host plant [49]. In this way, tobacco cultivars resistant to *P. nicotianae* could behave as susceptible in areas infested by root-knot nematodes such as *Meloidogyne* spp. Therefore, the introgression of genes *Rk1* and *Rk2* in Cuban cultivars, beyond conferring resistance to nematodes, would be a valuable support tool in the control of *P. nicotianae*.

Potato Virus Y (PVY)

The presence of the *va* gene in the 'Havana 142 Seed', 'Bergerac C', 'TB-2519', 'DH-3433-17' and 'Polalta'

15. Milla S, Levin J, Lewis R, Rufty R. RAPD and SCAR markers linked to an introgressed gene conditioning resistance to *Peronospora tabacina* DB Adam. in tobacco. Crop Sci. 2005;45(6):2346-54.

16. Cole D, Mapuranga N. Reaction of Indicator tobacco cultivars to races of *Pseudomonas syringae* pv. tabaciTox+. Beiträge zur Tabakforschung Int/Contrib Tobacco Res. 2001;19(7):353-9.

17. Trojak-Goluch A, Laskowska D, Kursa K. Morphological and chemical characteristics of doubled haploids of fluecured tobacco combining resistance to *Thielaviopsis basicola* and TSWV. Breed Sci. 2016;299:293-9.

18. Lewis R, Milla S, Levin J. Molecular and genetic characterization of *Nicotiana glutinosa* L. chromosome segments in tobacco mosaic virus-resistant tobacco accessions. Crop Sci. 2005;45(6):2355-62.

19. Wu X, Li D, Bao Y, Zaitlin D, Miller R, Yang S. Genetic dissection of disease resistance to the blue mold pathogen, *Peronospora tabacina*, in tobacco. Agronomy. 2015;5(4):555-68.

20. Li B-C, Bass W, Cornelius P. Resistance to tobacco black shank in *Nicotiana* species. Crop Sci. 2006;46(2):554-60.

21. Borrás-Hidalgo O, Thomma BP, Collazo C, Chacón O, Borroto CJ, Ayra C, *et al*. EIL2 transcription factor and glutathione synthetase are required for defense of tobacco against tobacco blue mold. Mol Plant-Microbe Interact. 2006;19(4):399-406.

22. García M, Andino V. Tecnología de bandejas flotantes en la producción de plántulas de tabaco en Cuba. Cuba Tabaco. 2002;3(1):30-3.

23. Julio E, Verrier J, De Borne F. Development of SCAR markers linked to three disease resistances based on AFLP within *Nicotiana tabacum* L. Theor Appl Genet. 2006;112(2):335-46.

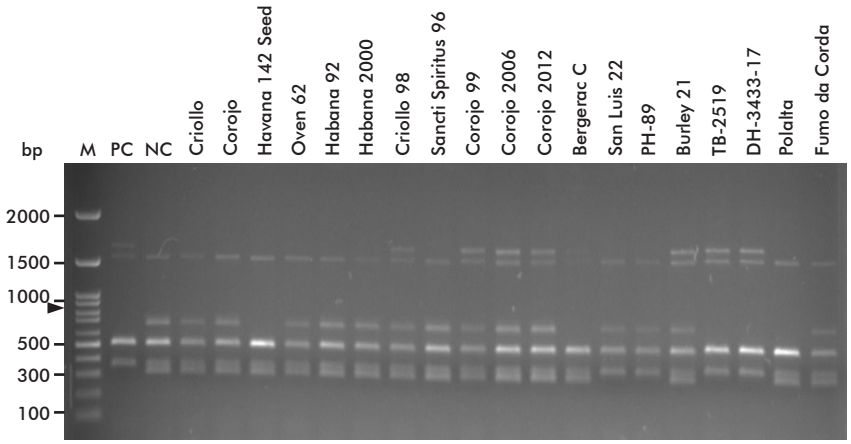

Figure 3. Agarose gel electrophoresis (1 %) showing amplification products generated by Multiplex marker. The number and expected length of the bands for the fragments linked to the resistant and susceptible genotypes of TMV were two fragments of 1800 and 1500 bp for the carrier and one fragment of 1500 bp for the non-carrier genotypes. Equivalently, for *M. incognita* race 1 y race 3, there was for the carrier one fragment of 360 bp, and two fragments of 360 and 320 bp for the non-carrier. Finally, for the Potato Virus Y (PVY), there were one fragment of 500 bp for the carrier and two fragments of 500 and 700 bp for the non-carrier. One amplification was visualized for each accession. M: 100 bp DNA ladder (Promega). PC: positive control ('Coker 371-Gold'). NC: negative control ('TB 2519'). bp: base pairs.

accessions was shown (Figure 3, Table 2). Although PVY is not a pathogen that causes severe losses in Cuba, it is remarkable the fact that none of Cuban cultivars was a carrier of this gene, in agreement with previous reports [44].

Such result was mainly expected by two causes: i) Resistance to PVY is not among the priorities of tobacco improvement in Cuba [1]; ii) several adverse effects of the va gene have been reported, despite its wide use. Resistant lines tend to have short and narrow leaves and less productivity [27], so this region could be discarded in the traditional improvement programs based on phenotypical selection.

Despite the effectiveness demonstrated by the va gene, no source has been able to provide complete resistance to all isolates of PVY [27]. An additional source of PVY resistance in tobacco has been found and transferred from *Nicotiana africana* Merxmüller and Buttler. The combination of va and the introgressed *N. africana* region (*Nafr*) seems to provide a good compromise in terms of resistance, yield and quality [50].

The analysis of the amplification products originated by the multiplex marker primers highlights the limitations of phenotypic selection. The *N*, *RK1* and *va* genes are not present in most of our cultivars, despite being contemplated in the improvement programs, *i.e.*, at least one of the parents was a carrier but its selection in the progeny was not effective.

Conclusions

Overall, this is the first study addressing the molecular characterization of Cuban tobacco germplasm in terms of resistance to the main diseases that affect its cultivation in Cuba. Promising genotypes carrying genes for resistance to the main diseases were identified, which will enhance their use in future breeding programs. Effective markers are now available for the genes and/or QTLs of resistance against the diseases targeted in this study, which is an essential step to routinely introducing MAS in Cuban tobacco improvement programs. Nevertheless, biological screening aimed to confirm disease resistance in advanced inbred lines will still be a critical step for the development of multiple disease resistant cultivars.

Acknowledgements

We gratefully thank to Mrs Tatiana Pérez and Mr Isidoro Martínez for their excellent technical assistance, and to Dr Yunior Miguel Morán for comments on the early manuscript version. This research was funded by the Cuban Ministry for Agriculture and British American Tobacco company.

Conflicts of interest statement

The authors declare that there are no conflicts of interest.

24. Drake K, Lewis RS. An introgressed *Nicotiana rustica* genomic region confers resistance to *Phytophthora nicotianae* in cultivated tobacco. Crop Sci. 2013;53(4):1366-74.

25. Lewis RS, Rose C. Agronomic performance of tobacco mosaic virus-resistant tobacco lines and hybrids possessing the resistance gene *N* introgressed on different chromosomes. Crop Sci. 2010;50(4):1339-47.

26. Pollok JR, Johnson CS, Eisenback J, Reed TD. Reproduction of *Meloidogyne incognita* Race 3 on flue-cured tobacco homozygous for Rk1 and/or Rk2 resistance genes. J Nematol. 2016;48(2):79.

27. Julio E, Cotucheau J, Decorps C, Volpatti R, Sentenac C, Candresse T, *et al*. A eukaryotic translation initiation factor 4E (eIF4E) is responsible for the "va" tobacco recessive resistance to potyviruses. Plant Mol Biol Rep. 2015;33(3):609-23.

28. Díaz M, Gil M, Mena EC. 'San Luis 22': Nueva variedad de tabaco Virginia resistente a las principales enfermedades y de alto potencial de rendimiento. Cuba Tabaco. 2008;9(2):22-6.

29. Knapp S, Chase MW, Clarkson JJ. Nomenclatural changes and a new sectional classification in *Nicotiana* (Solanaceae). Taxon. 2004;53(1):73-82.

30. Vontimitta V, Lewis RS. Mapping of quantitative trait loci affecting resistance to *Phytophthora nicotianae* in tobacco (*Nicotiana tabacum* L.) line Beinhart-1000. Mol Breed. 2012;29(1):89-98.

31. Ma JM, Heim C, Humphry M, Nifong J, Lewis RS. Genetic analysis of Phn7.1, a major QTL conferring partial resistance to *Phytophthora nicotianae* in *Nicotiana tabacum*. Mol Breed. 2019;39(1):11.

32. Xiao B, Drake K, Vontimitta V, Tong Z, Zhang X, Li M-y, *et al*. Location of genomic regions contributing to *Phytophthora nicotianae* resistance in tobacco cultivar Florida 301. Crop Sci. 2013;53(2):473-81.

33. Valleau W, Stokes G, Johnson E. Nine years' experience with the *Nicotiana longiflora* factor for resistance to *Phytophthora parasitica* var. *nicotianae* in the control of black shank. Tobacco. 1960;150(20).

34. Apple JL. Transfer of resistance to black shank (*Phytophtora parasitica* var. nicotianae from *Nicotiana plumbaginifolia* to *Nicotiana tabacum*. Phytopatology. 1962; 52(1):351-4.

35. Nelson R, Wiesner-Hanks T, Wisser R, Balint-Kurti P. Navigating complexity to breed disease-resistant crops. Nat Rev Genet. 2018;19(1):21.

36. Nifong J, Nicholson J, Shew H, Lewis R. Variability for resistance to *Phytophthora nicotianae* within a collection of *Nicotiana rustica* accessions. Plant Dis. 2011;95(11):1443-7.

37. Johnson E, Wolff M, Wernsman E, Atchley W, Shew H. Origin of the black shank resistance gene, *Ph*, in tobacco cultivar Coker 371-Gold. Plant Dis. 2002;86(10):1080-4.

38. Martínez-Pacheco J, Castro-Férnandez MdC, González-Toledo A, Toledo-Sampedro V. El gen *Php* no es una fuente de resistencia en el tabaco cubano. Cult Trop. 2019;40(1):e12.

39. Carlson SR, Wolff MAF, Shew H, Wernsman E. Inheritance of resistance to race 0 of *Phytophthora parasitica* var. *nicotianae* from the flue-cured tobacco cultivar Coker 371-Gold. Plant Dis. 1997;81(11):1269-74.

40. Bao Y, Ding N, Qin Q, Wu X, Martinez N, Miller R, *et al*. Genetic mapping of the Ph gene conferring disease resistance to black shank in tobacco. Mol Breed. 2019;39(9):122.

41. Dluge KL. Application of genomic approaches toward characterization of commercially important tobacco loci. North Carolina: North Carolina State University; 2017.

42. Silva YK, Muiño BL. Análisis de la virulencia en seis aislados de *Peronospora hyoscyami* f. Sp *tabacina*, agente causal del Moho azul del tabaco. Cuba Tabaco. 2012;13(2):3-11.

43. McCorkle KL, Drake-Stowe K, Lewis RS, Shew D. Characterization of *Phytophthora nicotianae* resistance conferred by the introgressed *Nicotiana rustica* region, Wz, in flue-cured tobacco. Plant Dis. 2018;102(2): 309-17.

44. Crespo JA, Dominguez M, Torrecilla G. Comportamiento de variedades de tabaco y especies del genero *Nicotiana* al Virus del grabado del tabaco, Virus del mosaico del tabaco y Virus Y de la papa. Cuba Tabaco. 2012;13(1):22-7.

45. Lewis R, Linger L, Wolff M, Wernsman E. The negative influence of N-mediated TMV resistance on yield in tobacco: linkage drag versus pleiotropy. Theor Appl Genet. 2007;115(2):169-78.

46. Colak-Ates A, Dincer D, Ata A. Determination of the interaction between *Fusarium* Crown-Root Rot Disease and Root-Knot Nematode on tomato genotypes. Fresenius Environ Bull. 2018;27:6785-91.

47. Kumar N, Bhatt J, Sharma RL. Interaction between *Meloidogyne incognita* with *Fusarium oxysporum* f. sp. *lycpersici* on tomato. Int J Curr Microbiol Appl Sci. 2017;6:1770-6.

48. Sumbul A, Mahmood I. Interactive effect of *Meloidogyne incognita* and *Macrophomina phaseolina* on the development of root–rot disease complex in relation to growth and physiological attributes of chickpea. Hellenic Plant Protect J. 2020;13(1):13-23.

49. Ros C, Lacasa CM, Martínez V, Bielza P, Lacasa A. Response of pepper rootstocks to co-infection of *Meloidogyne incognita* and *Phytophthora* spp. Eur J Horticult Sci. 2014;79(1):22-8.

50. Lewis R. Evaluation of *Nicotiana tabacum* genotypes possessing *Nicotiana africana*-derived genetic tolerance to Potato Virus Y. Crop Sci. 2007;47(5):1975-84.

Received in January, 2021.
Accepted in March, 2021.