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EVALUATION OF RESISTANCE TO FALSE OROBANCHE CAUSED BY *Nocardia* sp. ON *Nicotiana* spp.

Evaluación de la resistencia al falso Orobanche causado por *Nocardia* sp. en *Nicotiana* spp.

Yunior M. Morán Gómez[1]✉, Juan L. Pérez Rodríguez[2], Antonio Núñez Mansito[2], Rosario Domínguez Larrinaga[1], Gilberto Torrecilla Guerra[2], María del C. Córdoba Sellés[3] and Felipe L. Herrera Isla[4]

ABSTRACT. False Orobanche affects tobacco production in Cuba. Diseased plants development abundant shoot proliferation and tumour in the root, and they're showing dwarfism and rickets. Cuban Black tobacco cultivars are susceptible to *Nocardia* sp., causal agent of this disease. The incorporation of resistance genes into Cuban cultivars through genetic improvement will be an important element in the strategy for integrated management of this disease. However, there were not known sources of resistance to the disease and there isn't an available procedure for the assessment of potential resistance sources present in the tobacco germplasm of Cuba. The objective of this research is to develop a method of assessing the level of resistance to the causal agent of false Orobanche of tobacco germplasm accessions. Fourteen accessions of different species and types of tobacco were inoculated with T42 strain of *Nocardia* sp. From the showing of symptoms was developed an empiric visual scale for calculating the plant's damage degree. Accessions were located into four levels of reaction (resistant, moderately resistant, moderately susceptible and susceptible) according to plant's damage degree. Resistant genotypes were identified within the tobacco germplasm bank of Cuba. Also within susceptible evaluated cultivars, plants carrying resistance genes were found with which they could immediately begin a genetic improvement program by selection of pure lines.

Key words: bacteria, tumour, shoot proliferation, germplasm, resistance

RESUMEN. El falso Orobanche afecta a la producción tabacalera de Cuba. Las plantas afectadas desarrollan abundante proliferación de brotes y tumores en las raíces, muestran enanismo y raquitismo. Los cultivares de tabaco Negro cubano son susceptibles a *Nocardia* sp., agente causal de esta enfermedad. La incorporación de genes de resistencia a este agente fitopatógeno en los cultivares cubanos, mediante el mejoramiento genético tradicional, constituirá un elemento de peso en la estrategia de manejo integrado de esta enfermedad. Sin embargo, no se conocen fuentes de resistencia a la enfermedad dentro de *Nicotiana* spp., ni se dispone de un procedimiento para la búsqueda de estas fuentes en la amplia diversidad de accesiones presentes en el banco de germoplasma de tabaco de Cuba. El objetivo de esta investigación es desarrollar un procedimiento de evaluación del nivel de resistencia frente al agente causal del falso Orobanche de las accesiones del banco de germoplasma de tabaco de Cuba. Catorce accesiones de diferentes especies y tipos de tabaco fueron inoculadas con la cepa T42 de *Nocardia* sp. A partir de la manifestación de los síntomas se elaboró una escala visual empírica que permite calcular el grado de afectación de las plantas. En correspondencia con el grado de afectación se ubicaron las accesiones en cuatro niveles de reacción (resistente, moderadamente resistente, moderadamente susceptible y susceptible). Se identificaron accesiones resistentes a la enfermedad dentro del banco de germoplasma de tabaco de Cuba. También dentro de cultivares evaluados de susceptibles se encontraron plantas portadoras de genes de resistencia con las que se pudiera comenzar de inmediato un programa de mejoramiento genético por selección de líneas puras.

Palabras clave: bacteria, tumor, proliferación de brotes, germoplasma, resistencia

[1] Instituto de Investigaciones del Tabaco (IIT), carretera a Tumbadero, km 8 ½ San Antonio de los Baños. Artemisa. CP 32500.

[2] Estación Experimental del Tabaco de Cabaiguán, carretera a Santa Lucía, km 2, Cabaiguán, Sancti Spíritus. Cuba.

[3] Universidad Politécnica de Valencia (UPV), camino de Vera s/n, 46022 Valencia. España.

[4] Universidad Central "Martha Abreu" de Las Villas, carretera a Camajuaní, km 5 ½, Santa Clara. Villa Clara. Cuba.

✉ yunior.moran@gmail.com

INTRODUCTION

The disease known as False Broomrape is one of the factors affecting tobacco production in Cuba. In the early stages, affected tobacco plants develop small overgrowth in any position of the main root or in lateral secondary or tertiary roots[A].

With the development of symptoms, excrescence of whitish juicy irregular mass start differentiating small sprouts with aborted leaves without chlorophyll. In this stage, tumors can be seen in roots that can reach up to 8 cm of diameter. During this stage, that takes place underground, developing structures are similar to an *Orobanche ramosa* L. plant between the stages of nodules and emergence, in which the parasitic plant is seen as a fleshy bulb from which a stem emerges with one or two branches with some closed inflorescences; all these structures are light yellow[A].

Around 45 after transplanting tobacco seedling to the tocacco plantation, this disease induces the production of structures showing a clear difference with *O. ramosa*, hence this disorder is name "false broomrape". In this period, overgrowth over the soil surface exposed to sunlight, start taking a green color and the appearance of normal tobacco plant sprouts. These differentiated sprouts from the whitish masses can produce plant organs, like if it would be an independent tobacco plant and at this very moment, symptoms can be easily seen in the field[A].

The first report of the disease in Cuban tobacco (*Nicotiana tabacum* L.) plantations was in the second half of last century[A] and since then, its distribution has remarkably increased as well as the intensity of the damages (1).

In Cuba's central region, the black tobacco cultivar 'Pelo de Oro', showed economic losses that amounted to an average of $ 2 511,73 pesos per hectare characterized mainly by dwarfing, rickets and chlorosis of affected plants which negatively influence yields and crop quality[A].

It was recently shown that the causal agent of this disease is an actinomicete of the *Nocardia* genus (2). The identification of this causal agent opens new spaces to go into an integrated management of this disease. In this regard, the incorporation of resistance to commercial cultivars by breeding programs, will be a major element for its future management, like it has been for other crops affected by different pathogens (3, 4). The determination of resistance sources in the materials present in germplasm collections is greatly important since it will allow to incorporate resistance genes to cultivars of interest (5).

However, little progress has been made in the quest for materials of the *Nicotiana* genus showing resistance to False Broomrape. In the 90s of last century, it was determined that Cuban commercial black tobacco cultivars (*N. tabacum*) 'C-30', 'Cabaiguán-72', 'Habana-92', 'Burley-37' and 'Corojo', though affected by the disease, showed less damage than cultivar 'Pelo de Oro'[A]. This result indicates the possibility of finding sources of resistance to this disease.

At presente, there are not known sources of resistance to the disease within *Nicotiana* spp., and there is not either an available procedure for the quest of these resistance sources in the wide diversity of accessions at the Cuban Germplasm Tobacco Collection. In order to achieve this, it is necessary to have a scale to evaluate the resistance degree of the plants to the etiological agent of the disease.

Based on the above, this research has looked at developing an evaluation procedures of the resistance degree of accesions to the False Broomrape according to an empirical scale of symptoms observation of the disease under controlled conditions.

MATERIALS AND METHODS

Preparation of the assay

Germplasm used. Fourteen accessions of the *Nicotiana* genus present at the Germplasm Collection of the Cuban Tobacco Research Institute, located at the Tobacco Experiment Station of Cabaiguán, Sancti Spíritus province, were used. This place hosts one of the largest Germplasm Tobacco Collections of Latin America and the Caribbean, with over 800 accessions of *Nicotiana tabacum* L. and 40 wild species of the genus[B]. Seven accessions were of tobacco type Virginia (*N. tabacum* cv. 'Fogia D' Oro'; 'LAFC 53'; 'Virginia 110'; 'Virginia SL 32'; 'Novaga 768'; 'K 358'), two of the black type (*N. tabacum* cv 'Habana 92'; 'Corojo 2006'), one of the oriental type (*N. tabacum* cv 'Xanthi UR') and the other four were of wild species (*Nicotiana alata*; *Nicotiana nesophila*; *Nicotiana sylvestris*; *Nicotiana rustica* cv. '3001').

[A] Méndez, R. *Característica, distribución y daños del falso orobanche en el cultivo del tabaco* [Tesis de Maestría], Universidad Central de Las Villas, Las Villas, Cuba, 1998, 66 p.

[B] Torrecilla, G.G. y Cabrera, E.M. ''Colección del género *Nicotiana*, una experiencia al servicio de la ciencia y la sostenibilidad'', *Tobacco Irrigation*, vol. 1, no. 1, 2010, pp. 20-25.

These materials were selected for this study because they showed a different behavior to symptoms expression in tobacco growing areas with a natural presence of the pathogen. Some of them, as black tobacco and Virginia, showed a high quantity of tumors in the roots and an excessive development of aborted sprouts in roots and stem crown; while species did not show symptoms or its expression was very mild.

Preparation of the causal agent inoculum of the false broom rape. From a plate with soil D2 culture medium (6) sown by exhaustion 10 days before with the isolate *Nocardia* sp. T42, causal agent of False Broomrape (2), a pure colony was taken without agar and of 1 mm of diameter with a platinum handle. The colony was transferred to an Erlenmeyer flask of 500 ml of capacity with 200 ml of the D2 liquid medium. Following the same procedure, other two Erlenmeyer flasks were prepared under the same conditions. This preinoculum was incubated for seven days at 30 °C with constant agitation on an orbital screen at 150 rpm.

With this preinoculum, 100 Erlenmeyers flasks of 100 ml of capacity were inoculated, all of them contained 45 ml of the D2 liquid. Each Erlenmeyer flask was inoculated with 5 ml of the preinoculum. The inoculum was incubated for seven days at 30°C with constant agitation on an orbital screen at 150 rpm. The culture of the microorganism was done separately in aliquots of 45 ml to favor the aeration of the medium since this microorganism shows a high dependency from oxygen for development (2).

At the end of the incubation time, each of the cultures were filtered with slow-filtering quantitative paper (Whatman 390) using glass funnels. This procedure was done over the working table. In order to guarantee the homogeneity of the inoculum for all the microbial mass collected on the filter paper, it was vigorously resuspended on 1 000 ml of sterile saline solution (water solution of NaCl at 0,85 %) on a 5 000 ml beaker.

Through the procedure of counting viables on plates (7), made to the bacterial suspension, it was established that the cell concentration of *Nocardia* sp. isolate T42 was of 6,2 x 10^8 UFC mL^{-1}.

Inoculation procedure of the seedlings from each accesion of the bank. On a plastic tray of 50 cm x 40 cm x 10 cm, disinfected with sodium hypochlorite at 5 % for 30 min., nine litters of sterile saline solutions were poured and to this volume, one litter of inoculum was added, estimating a population concentration for the T42 isolate of around 6,2 x 10^7 UFC mL^{-1} in the new volume. The height of the poured liquid did not surpass 5 cm in the container used, so only seedling roots could be dipped into the inoculum.

Seedling accessions of 45 days were attained in seedbeds of floating trays with substrate (black peat- humic –Agricultural Substrates Ltda. Chile-, mixed with 10 % of Fertizol [Zeolite] –Empresa Geominera Oriente. Cuba-) sterilized in autoclave at 121 °C, 1 013 hPa, 45 min.

For each entry to the Collection, 20 seedlings were taken at random and tied up by their stem with a string to form a group. Each group was identified with the name of the entry and seedling roots were washed with abundant running water to remove all the substrate attached to them. The 14 groups of plants were dipped into the inoculum for 4 hours and at 1 hour intervals the content of the container was homogenized by smoothly shaking it manually for 5 min.

Transplanting inoculated plants. All inoculated seedlings were transplanted to black poly bags of 1 L filled with 500 g of Sialitic Brown soil without carbonate differentiation (8). It was retrieved from a tobacco plot historically affected by the Tobacco False Broomrape. Before filling the bags, all the soil was screened and exhaustively mixed, sterilized in autoclave at 121 °C at a pressure of 1 013 hPa for 45 min. The pH value of the soil after sterilization, determined as pH in KCl was of 6,05. This type of soil was used to guarantee the persistence of the agent after transplanting, because it has been observed that in affected tobacco growing areas, the disease shows up in some types of soils while not in others[C]. Each bag hosted two seedlings with a total of 10 bags per accession.

Other 10 seedlings of each accesion were also transplanted directly to bags with sterile soil taken from the same seedbed (two plants per bag). These non-inoculated seedlings were the control of the trial. All the plants were kept till evaluation under screening conditions at 30 ± 2 °C, and a maintenance irrigation was proviced every three days.

Evaluation of tobacco plants. The valuation was made 45 days after transplanting. For such purpose, plants were taken out from the bags and were washed with abundant running water to remove the soil from the roots. It was done very carefully not to detach any tumor or sprout. Measurements of the structure size (sprouts and tumors) were done with the aid of a slide gage.

[C] Morán, Y.M. *Caracterización del agente etiológico del «falso Orobanche» del tabaco (Nicotiana tabacum* L.*) en Cuba. Propuesta de un método para su detección* [Tesis de Doctorado], Universidad Central de Las Villas, Las Villas, Cuba, 2012, 100 p.

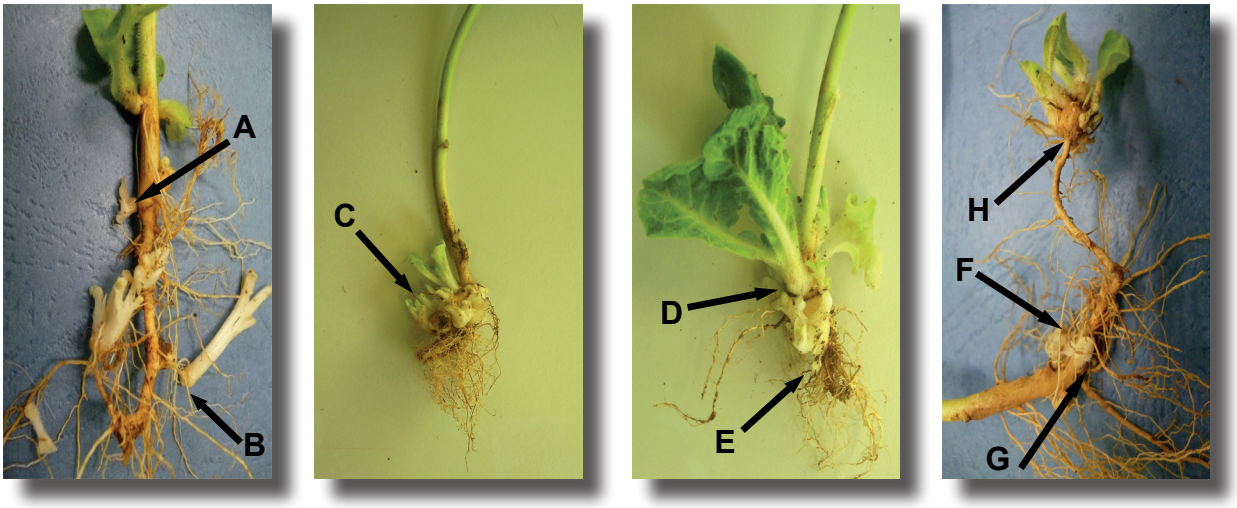
RESULTS AND DISCUSSION

The evaluated plants showed four types of expressing tumors and sprouts. These types of symptoms expression caused by the disease were defined by an empirical visual criterion that described the intensity of tumors development and sprouts proliferation in the roots and stem crown of the evaluated materials, taking into account the measurement of the size of these structures and their differentiation degree. The descriptive elements of each of the four types of expression are shown in Table I and Figure 1.

All accessions with symptoms showed the main root as the most affected organ. It was also observed that a plant could have tumors and sprouts of different types, but in general such tumors and sprouts of type 1 and 2 exceeded in number the tumors and sprouts of type 3 and 4.

Table I. Characteristics of each of the four types of tumors and sprouts seen in affected plants after 45 days of being transplanted to the seedbed and inoculated

Types of expression of tumors and sprouts	Structures size	Differentiation degree
Type 1	Ø≤4 mm	Simple white regrowth at the height of the stem crown; or globular compact mass; (tumor) of white color in the roots.
Type 2	4 mm< Ø≤12 mm	Branched regrowth of white-yellow color at the height of the stem crown or in roots; or globular compact mass of white-yellow color in the roots.
Type 3	12 mm< Ø≤24 mm	Branched regrowth green again at the height of the stem crown or in roots; or globular compact mass of white-yellow color in the roots with small proliferation of aborted white color sprouts.
Type 4	24 mm< Ø	Regrowth with complete developed foliar organs at the height of the stem crown or in roots; or globular compact mass of brown color with development of sprouts and foliar organs.



Arrows point at different structures. A, B, C and D: Sprouts with types 1, 2, 3 and 4 respectively. E, F, G y H: Tumors with types 1, 2, 3 and 4 respectively.

Different types of tumor expression and sprouts shown by tobacco plants inoculated with the causal agent of the False Broomrape 45 days after being transplanted from the seedbed

In order to prepare the evaluation scale, a measurement of the damage degree per accession was needed. This variable was named "Mean Damage Degree" (MDG) and was calculated from 4 types of tumors and sprout expression using the following formula:

$$GMA = \frac{\sum_{i=1}^{n} (T4i * 4 + T3i * 3 + T2i * 2 + T1i)}{n}$$

where:

- $T4_i$: total of tumors and sprouts type 4 of the plant i
- $T3_i$: total of tumors and sprouts type 3 of the plant i
- $T2_i$: total of tumors and sprouts type 2 of the plant i
- $T1_i$: total of tumors and sprouts type 1 of the plant i
- n: number of evaluated plants (only living ones)

Table II shows three examples of the cards for the MDG calculation that the three accessions used in the study showed.

Regarding dead plants found in the inoculated material, it is important to mention that in control plants there were some dead. In general, the symptoms found in these dead plants were described as wilt, blackening and root rot (symptoms that were never associated to the False Broomrape, but to soil fungi). Lab diagnostic confirmed the presence of *Phytophthora nicotianae* Brenda de Hann (causal agent of the tobacco black shank). It is possible that the infection cause might be due to the irrigation with non-sterile water with presence of fungus propagules, inoculum sources and mode of dispersion of this pathogen (9).

It is essential to have a minimum of 20 replicates of plants per accession to perform the study and for MDG calculation in each accession no less than 10 plants should be used.

Evaluation scale of the materials reaction to the causal agent of the False Broomrape. The designation of the resistance scale degree followed an emprirical criterion, but some elements that allowed to reasonably calculate the value for the "Medium Damage Degree" with the resistance responde of a certain material, was taken into account.

Due to the fact that Cuban commercial tobacco cultivars are a set of homocygotic genotypes (10), the possibility of finding within an entry evaluated as resistant, a small rate of individuals showing symptoms of the disease was considered. Thus, the rate of individuals with symptoms within the accession considered as resistant should not exceed 40 % of the total evaluated and the expression of

Table II. Data for the MDG calculation of the inputs of *N. sylvestris*, *N. tabaco* cv. 'Novaga 768' and *N. tabacum* cv. 'Habana 92'

Replicate	Material vegetal: *Nicotiana sylvestris* Type of expression				*N. tabacum* cv. 'Novaga 768' Type of expression				*N. tabacum* cv. 'Habana 92' Type of expression			
	1	2	3	4	1	2	3	4	1	2	3	4
1	1						1		X	X	X	X
2	X*	X	X	X					1	1	2	
3		1			1							
4	2				1	1	1			1	1	
5						3	1		1		2	1
6		1							X	X	X	X
7					X	X	X	X	X	X	X	X
8									6	2		
9		1						1		1	1	
10					1				X	X	X	X
11					1	2	1	1	1	4	1	
12	X	X	X	X			2		2			
13	X	X	X	X	1	3	1				2	1
14					X	X	X	X	X	X	X	X
15							2	2	X	X	X	X
16					1						2	1
17					2		2		2		1	2
18	X	X	X	X						1	2	
19									X	X	X	X
20					X	X	X	X	1	4		1
Total	3	3	0	0	8	9	11	4	14	14	14	6
	(3*1+3*2)/16				(8*1+9*2+11*3+4*4)/17				(14*1+14*2+14*3+6*4)/13			
	GMA: 0,56				GMA: 4,41				GMA: 8,31			

* X: Dead plant (non-evaluated)

sprouts and tumors in these individuals should not exceed type 2, nor the total of tumors and sprouts in each individual should surpass 2.

Based on the above, the "Medium Damage Degree" for an accesion be considered resistant should be lower or equal to the unit. The presence of some aberrant individual (extreme quantity of tumors or sprouts in advanced stages) within this group of resistant plants should not be considered for the calculation. Those accessions with MDG ≤ 1, not meeting the requirement that at least 60% of its individuals do nor show symptoms, will be allocated to the group of moderately resistant as those accessions with MDG values from 1 to 4.

Table III summarizes the four levels of the proposed empirical scale to evaluate the reaction of the resistance 45 days after genotypes from the tobacco germplasm collection were transplanted from the seedbed and inoculated with the causal agent of the False Broomrape based on the estimated medium damage degree.

Table III. Empirical scale of four categories

Type of reaction of the accession	Sigla	Values of MDG
Resistant	R	MDG≤ 1
Moderately resistant	MR	1<MDG ≤ 4
Moderately sensitive	MS	4< MDG<7
Sensitive	S	7≤ MDG

Empirical scales have been widely used to simply define the response degree of the materials present in germplasm collections of different crops to a particular pathogen. All of them, in their extremes, classify the reactions in resistant and sensitive. They mainly differ in the quantity of intermediate degrees used to adequately characterize the reaction of the material to face the pathogen, using, in some cases, up to nine degrees of reaction (5, 11, 12). This characterization is based on the observation of the symptoms shown by challenged plants; the evaluation can be done under field or greenhouse conditions (11, 13, 14, 15).

The four-degree scale developed in this study meets the characteristics mentioned above for empirical scales. This scale allows the characterization of the reaction present on tobacco germplasm to the causal agent of the False Broomrape and will turn into an important tool to determine the possible resistance sources present in Cuba's tobacco germplasm collection. This is the first procedure available to determine the resistance of the *Nicotiana* genus to the causal agent of the False Broomrape.

It is important to establish the inoculum pressure for this trial on a value close to 10^7 UFC mL^{-1} of the T42 isolate of *Nocardia* sp., since this variable is critical for the results be repeatable, reproducible and reliable. It has been estimated that the minimum concentration of propagules of this pathogen that should be present on the soil is to be above 1,2 x 10^3 UFC g^{-1}, for the tobacco plants transplanted to this soil express the disease[C].

The inoculum potential used in this study, for being superior (in more than 1000 times) to the minimum required concentration for transplanted tobacco to a contaminated soil express symptoms, is a guarantee that entries evaluated as resistant be really resistant (at least for the evaluation period) so their behavior would be the same when transplanted to a contaminated plantation.

Another essential element to take into account is the fact that the evaluation of materials be always done 45 days postinoculation with the causal agent to achieve the reproduction of results, so though the term resistance is being dealt with in this study, that condition is not really evaluated in all stages of the phenomenological development of the tobacco plant, hence the further behavior of the evaluated materials cannot be considered as "resistant". Plant's reaction to a pathogen is of immunity, that is, not even under the most favorable conditions it is attacked, or the resistance can vary from a very high level to be imperceptible or nil which provides a response of high sensitivity (16, 17).

Table IV shows the results of applying procedure's criteria proposed to the 14 accessions of the germplasm collection used in this study by evaluating the reaction of the materials 45 days after inoculation with the causal agent of the False Broomrape.

Out of the 14 materials evaluated, the specie *N. sylvestris* was resistant. The moderately resistant materials were: *N. nesophila, N. rustica* cv. '3001'*, N. tabacum* cv. 'Georgia 1469' and *N. alata*. Out of the cultivars of *N. tabacum*, 'Novaga 768', 'Corojo 2006', 'K 358', 'Virginia 110', 'Fogia D' Oro', 'Habana 92', 'Xanthi UR', 'Virginia SL 32' y 'LAFC 53', the first four reacted as moderately sensitive and the rest (five) were sensitive.

Table IV. Type of reaction of 14 accessions present in the Germplasm Collection to the causal agent of the False Broomrape 45 days postinoculation

Materials	Type of tobacco	Value	Reaction
Nicotiana sylvestris	specie	0,56	R
Nicotiana nesophila	specie	0,94	MR
Nicotiana rustica cv. '3001'	specie	2,60	MR
*N. tabacum*cv. 'Georgia 1469'	Virginia	2,69	MR
Nicotiana alata	specie	3,18	MR
N. tabacum cv. 'Novaga 768'	Virginia	4,41	MS
N. tabacum cv. 'Corojo 2006'	black	5,00	MS
N. tabacum cv. 'K 358'	Virginia	6,37	MS
N. tabacum cv. 'Virginia 110'	Virginia	6,86	MS
N. tabacum cv. 'Fogia D' Oro'	Virginia	7,24	S
N. tabacum cv. 'Habana 92'	black	8,31	S
*N. tabacum*cv. 'Xanthi UR'	oriental	8,70	S
N. tabacum cv. 'Virginia SL 32'	Virginia	9,43	S
N. tabacum cv. 'LAFC 53'	Virginia	13,89	S

R: resistant; MR: moderately resistant; MS: moderately sensitive; S: sensitive

Nicotiana nesophila, despite the GMA calculation had a value below 1, it was not included within resistant genotypes because it had a percentage of individuals with symptoms above 40 % (43,8 %; 7 symptomatic out of 16 evaluated).

The fact that *N. tabacum* cv. 'Habana 92' turns out as sensitive, is according to Méndez[A] and to what is observed in production (1).

In general, the existence of resistant genotypes within the species of the Germplasm Collection is a very important fact for breeders since they can have sources with resistant genes to this disease (5, 18, 19, 20, 21).

Though the presence of symptomless plants within an accession has been evaluated as sensitive, it not an element that directly indicates that material shows resistant genes to the disease (it could be due to difficulties attributable to other factors of the infection process beyond the plant), it should not be despised that such material, has indeed, those genes. As explained above, tobacco commercial cultivars are a mixture of homocygotic genotypes selected for certain stable characters (10) so it is possible to find in the set of individuals of this accession classified as sensitive, some plant with resistant genes to the disease to which no selection of this type for that character had been done before.

It could be the case of the third evaluated replicate of the cultivar 'Habana 92' (Table I), that proved to be symtompless throughout the experiment. After this study, this plant was inoculated three times on the root at different phenomenological stages and never showed symptoms of the disease. It maintained a normal development unlike other control plants of the same cultivars that did get the disease when inoculated with the causal agent. This type of plant is useful to immediately start a selection process of pure lines following the Johannsen's approach (22) and quickly incorporate resistance to this cultivar without looking for resistance sources through intraspecific crossings and much less throught interspecific crossings. The introduction of resistance genes from one specie to another by repeated crossing, is a long process in time which usually takes several hybrid generations before the cross be successful (20).

The introduction of this procedure into the agricultural practice will require validation by conducting field experiments in affected tobacco growing areas that allow to determine if the use of the ranges present in the scale under controlled conditions, fit to the behavior of accessions under field conditions and longer evaluation periods of the materials.

It has been stated that the accuracy and precision of the evaluation scales depend more on the evaluator's training than the type of interval selected; and that reproducibility is attained with the practice and discussion of results. Therefore, as this scale is tuned up with different assays and successive years, the necessary adjustments that will finally turn it into a tool to meet requested needs (23).

CONCLUSIONS

- The first procedure to determine the resistance response of Cuba's tobacco germplasm to the causal agent of False Broomrape, was developed, which will be established as an important tool for breeders.
- This procedure uses a four-level scale to evaluate accessions as to: resistant, moderately resistant, moderately sensitive and sensitive.
- For the results of this research be repeatable, reproducible and reliable, an inoculum pressure of approximately 10^7 UFC mL^{-1} of the T42 *Nocardia* sp. Isolate is required, and a minimum quantity of 20 replicates of plants per accession to perform the study and for GMA calculation in each accesion, no less than 10 plants should be used.

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