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COMPARATIVE ANALYSIS OF LEAF CHEMICAL INDICATORS AND DITERPENES FROM LEAF EXUDATES OF *Nicotiana tabacum* L.

Análisis comparativo de indicadores químicos de la hoja y diterpenos de exudados foliares de *Nicotiana tabacum* L.

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ABSTRACT. Plants are a great source for the isolation of natural products. The discovery of novel metabolites from plants is essential for the development of chemicals used in different industries. The objective of this research is to determine the chemical composition of leaf and ethanolic leaf exudates from 10 noncommercial accessions of *Nicotiana tabacum* L., two of them cuban, to propose their use for the production of bioactive natural products or their inclusion in breeding program to commercial purposes. The morphological and chemical indicators evaluated showed differences among accessions. The accession Nic 1016 showed the highest height and number of leaves, together with Nic 1061 and SNN while BHmN remained low compared with the rest of accessions. Accessions showed a chemical diversity of the crude extracts from leaf exudates and four of them (Nic 1017, Nic 1003, CE y BHmN) showed the presence of diterpene cis abienol, secondary metabolite of interest due to its potential application. The accession Nic 1017 showed the best indicators of leaf chemical composition with influence in quality and BHmN showed the indicators less adequate. These results allow a comparative morphological approach to determine the chemical characterization of the leaf composition and the obtained extracts using tobacco accessions not fully characterized before with possible biological activity.

Key words: isolation, chemical composition, indicator, genetic improvement, tobacco

RESUMEN. Las plantas son una fuente extraordinaria para el aislamiento de productos naturales. El descubrimiento de nuevos metabolitos es esencial para el desarrollo de químicos en diferentes industrias. El objetivo de esta investigación es determinar la composición química de la hoja y de los extractos etanólicos obtenidos a partir de exudados foliares de diez accesiones no comerciales de *Nicotiana tabacum* L., dos de ellas cubanas, para proponer su uso en la obtención de productos naturales bioactivos o en la inclusión de ellas en programas de mejoramiento genético con fines comerciales. Los indicadores morfológicos y químicos evaluados mostraron diferencias entre accesiones. La accesión Nic 1016 mostró la mayor altura y número de hojas, junto a las accesiones Nic 1061 y SNN mientras que la accesión cubana BHmN se mantuvo por debajo de todas las accesiones. Las accesiones evaluadas mostraron una diversidad química de los extractos crudos a partir de los exudados foliares y cuatro de ellas (Nic 1017, Nic 1003, CE y BHmN) mostraron la presencia del diterpeno cis abienol, metabolito secundario de interés por sus potenciales de aplicación. La Nic 1017 mostró los mejores indicadores de composición química de la hoja y la BHmN los menos adecuados, que influyen en la calidad. Estos resultados permiten realizar un acercamiento a una determinación morfológica comparativa entre accesiones y una caracterización química de la composición de la hoja y de los extractos obtenidos a partir de las accesiones de tabaco no antes caracterizadas, con posible actividad biológica.

Palabras clave: aislamiento, composición química, indicadores, mejoramiento genético, tabaco

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INTRODUCTION

Plants are an important source of natural diversity, because of the large number of compounds they synthesize; many of them are useful in agriculture, human medicine, food preservation and disease control (1, 2, 3, 4, 5, 6). Plant morphological aspects determined by genetics, among other factors, are closely related to the wide chemical diversity existing in the metabolite composition of different species (7). Such chemical diversity is a consequence of the evolutionary process that has led to select species with better defence against microbial attack or insect and animal predation (7). It is now known that these metabolites play an important role in plant defensive mechanism (8). Therefore, due to the negative impact caused by the overuse of synthetic pesticides on the profitable fauna, the man and, in general, on the environment (9, 10), plants are being recently reused as a source of safer pesticides (11, 12). These natural products are an alternative to substitute synthetic products for pest control (13).

Tobacco (*Nicotiana tabacum* L.) is a model plant from Solanaceae family, which includes other important crops, such as tomato and potato. Tobacco is a short-cycle environmentally-resistant crop that is easily cultivated. The curative and pesticide properties are known, due to its characteristics and compounds providing organoleptic features and high productivity, as well as the presence of leaf glandular trichomes involved in compound secretion, mainly diterpenes and sugar esters in leaf surface exudates (14). Such compounds isolated from leaf surface allow obtaining these products with a lower contamination degree of its inside constituents. All these characteristics make tobacco an attractive crop to study leaf chemical indicators, which are very important for product quality with commercial purposes, allelochemistry and its alternative uses to get natural products with different goals (15).

Despite all that is known about tobacco, the chemical and morphological characteristics of noncommercial accessions of *Nicotiana tabacum* L. are comparatively unknown. This work is aimed at determining leaf chemical composition and extracts derived from leaf exudates of *Nicotiana tabacum* L. accessions, so as to suggest their use to obtain bioactive natural products or to be included in breeding programs with commercial purposes.

MATERIALS AND METHODS

1. Morphological indicators determined in selected tobacco accessions

Ten tobacco accessions were selected for the experiment, which had previously shown different diterpene profiles in dichloromethane extracts (data not shown); eight out of them were provided by IPK-Gatersleben Germplasm Bank from Germany: Nic 1003 (“TI 66”), Nic 1006 (“TI 193”), Nic 1015 (“TI 1341”), Nic 1016 (“Incekara”), Nic 1017 (“Red Russian”), Nic 1019 (“TI 998”), Nic 1061 (“TI 1738”) and SNN (Samsun NN), whereas BHmN and CE (Special Corojo) accessions were given by the Genetic Engineering and Biotechnology Center from Havana, Cuba. Seeds were sown in nurseries and floating trays[A] filled with a mixture made up of soil-substrate-zeolite (55:40:5) (m:m:m) at the Tobacco Experimental Station of Cabaiguán, belonging to the Tobacco Research Institute, using a carbonated sialitic brown soil, which was disinfected by inoculating the antagonist pathogen *Trichoderma* sp. and filter cake with rice straw (3:2:1) (m:m:m) as substrate. Seven days after seeding, the fertilizer was poured to water, 3 kg of complete formula: nitrogen, phosphorus and potassium (NPK+Mg) 5-12-6-2,6; after 21 days, it was again poured to water, 100 g of the same fertilizer.

Plants were individualized after 45 days and transplanted to polyethylene bags of 6 kg capacity that were filled with soil-filter cake-zeolite (3:2:1) (m:m:m) respectively. Thus, 10 plants were transplanted per accession that grew up to 70 days.

Then, 14 days after being transplanted to bags, morphological indicators were evaluated every 14 days: leaf number and plant height, making a total of five evaluations up to 70 days.

2. Production, separation and identification of crude extracts rich in diterpenes obtained from fresh leaf exudates of tobacco accessions

2.1 Production of ethanolic crude extracts from tobacco leaf exudates

Crude extracts of leaf exudates were obtained in all leaves from three plants out of the 10 selected accessions after 70 days of being transplanted to bags by using 500 mL ethanol at 90 % as solvent and they were concentrated to a volume of 50 mL in the Metabolic Engineering Laboratory from the Bioplant Center. This extract was stored at 4 °C for its further analysis (16).

[A] Instituto de Investigaciones del Tabaco. *Instructivo Técnico para el cultivo del tabaco en Cuba*. Inst. Ministerio de la Agricultura, 2012, p. 37.

2.2 Separation and identification of crude extract diterpenes obtained from tobacco leaf exudates through thin layer chromatography (TLC)

To determine the compounds present in ethanolic extracts from leaf exudates, a thin layer chromatography was performed in silica gel plates on aluminum cards (DC-60 F 254, 20 x 10 cm, MERCK) at the Phytomedicine Institute from the University of Hohenheim, Stuttgart, Germany. Then, 20 µL of each extract prepared at a rate of 10 µg $µL^{-1}$ was applied and separated 1 cm between them. The following solvent system was used for chromatographic runs: Terbutyl Methyl Ether (TBME): Isooctane: Methanol (50:50:10; v:v:v). Plates were observed at 254 and 366 nm.

3. Chemical indicators determined in dry leaves of tobacco accessions

So, 30 leaves were randomly taken from different parts of tobacco plant in several plants per accession; they were strung by tobacco quills and placed inside the curing house to be dried and cured for 55 days. Later on, such samples were sent to the Tobacco Research Institute of Havana, in order to evaluate the following chemical indicators: K_2O (%), Na_2O (%), CaO, MgO, Cu (mg kg^{-1}), Zn (mg kg^{-1}), Mn (mg kg^{-1}), Fe (mg kg^{-1}), N (%), nicotine (%), Cl (%), ashes (%), pH and alkalinity. Analyses were performed according to the methods established in the Agrochemistry Laboratory from the Tobacco Research Institute[B].

Statistical analysis

Data were analyzed by means of the Statistical Package for Social Sciences (SPSS) (version 15 for Windows) (17) and parametric tests (single and bifactorial ANOVA) were also made. HSD Tukey tests were applied in the cases that ANOVA showed significant differences. It was previously demonstrated that each treatment data fulfilled the assumptions of normal distribution and variance homogeneity for p≤0.05, according to Kolmogorov-Smirnov and Levene tests respectively. Statistical treatment details are presented in all tables of Results and Discussion.

RESULTS AND DISCUSSION

1. Morphological indicators of selected tobacco accessions

All morphological indicators evaluated show differences between accessions at each evaluating time.

[B]Instituto de Investigaciones del Tabaco. *Manual de Procedimientos del Laboratorio de Agroquímica*. Inst. Ministerio de la Agricultura, Cuba, 2004.

Every plant increased its height and leaf number with time, according to plant growth (Tables I and II). Most of the accessions showed a similar height and leaf number 14 days after being transplanted to bags, except accession 1016 that showed the highest values.

Accession growth rate was different with time, as is the case of 1061, which increased its height and leaf number considerably 28 days after being transplanted to bags and became one of those with the greatest height and leaf number, together with 1016 and SNN at the end of the experiment, whereas BHmN height always remained below every accession, similar to Nic 1015.

Regarding growth dynamics, most accessions developed two to three leaves every 14 days (Table II). In the case of Nic 1061 and SNN, leaf growth and development was greater, since four to seven leaves were formed every 14 days, mainly within the first 42 days after being transplanted to bags (Table II).

Table I. Plant height under semicontrolled conditions out of 10 accessions evaluated 14 days after being transplanted to bags up to 70 days and its evaluation in time every 14 days

Accesions	Time after transplantation to bags (days)				
	14	28	42	56	70
	Height of plants (cm)				
.Nic 1016	29,9 *mnop*	41,6 *ghijk*	49,0 *efgh*	59 *abcd*	63,9 *ab*
Nic 1061	8,5 *wxy*	24,6 *pqrs*	50,3 *defg*	60,3 *abc*	65,5 *a*
SNN	18,4 *rstuv*	43,6 *ghij*	53,3 *cdef*	63,3 *ab*	68,3 *a*
CE	17,8 *stuvw*	29,3 *mnopq*	44,5 *fghi*	54,5 *bcde*	59,8 *abc*
Nic 1017	17,6 *stuvw*	31 *lmnop*	38,6 *ijklm*	48,6 *efgh*	53,6 *cdef*
Nic 1019	9,4 *vwxy*	19,1 *rstu*	33,1 *klmnop*	43,1 *ghij*	48,1 *efgh*
Nic 1006	10,8 *tuvwxy*	17,5 *stuvwx*	31,5 *lmnop*	40,3 *hijkl*	45,1 *efghi*
Nic 1003	8,1 *xy*	14,4 *tuvwxy*	26,5 *opqrs*	36,5 *ijklmn*	41,5 *ghijk*
Nic 1015	7,9 *y*	11,4 *tuvwxy*	19,9 *qrst*	29,9 *mnop*	34,9 *jklmno*
BHmN	6,13 *y*	10,4 *uvwxy*	17,8 *stuvw*	27,8 *nopqr*	32,8 *klmnop*

Means with different letters differ significantly (Bifactorial ANOVA, Tukey, p≤0.05, n= 8

Table II. Leaf number per plant growing under semicontrolled conditions out of 10 accessions evaluated 14 days after being transplanted to bags up to 70 days and its evaluation in time every 14 days

Accesions	Time after transplantation to bags (days)				
	14	28	42	56	70
	Number of leaves per plant				
Nic 1016	11	12	15	17	19
	ijklm	*hijkl*	*cdef*	*abc*	*a*
Nic 1061	5	9	14	16	18
	z	*pqrst*	*defg*	*bcde*	*ab*
SNN	5	12	14	16	18
	xyz	*ghijkl*	*defg*	*bcd*	*ab*
CE	6	8	9	11	13
	vwxy	*qrstu*	*opqrs*	*jklmn*	*fghij*
Nic 1017	5	8	10	12	14
	yz	*stuvw*	*mnopq*	*hijkl*	*efgh*
Nic 1019	5	8	9	11	13
	yz	*qrstuv*	*pqrst*	*klmno*	*fghijk*
Nic 1006	5	7	9	11	13
	yz	*tuvw*	*pqrs*	*klmno*	*fghijk*
Nic 1003	6	8	11	14	16
	wxy	*rstuvw*	*hijklm*	*defg*	*bcde*
Nic 1015	4	6	9	11	13
	z	*uvwxy*	*nopqr*	*ijklm*	*fghi*
BHmN	5	7	8	10	12
	yz	*uvwx*	*qrstu*	*lmnop*	*ghijkl*

Medias with different letters differ significantly for data transformed according to $x' = \sqrt{x}$ formula
(Bifactorial ANOVA, Tukey, $P \leq 0.05$, n= 8)

Leaf number per plant together with leaf area are important aspects in this crop, since leaves are the main plant organ, not only for photosynthesis, from which derives an efficient growth and development, but also because it is one of the few crops whose leaves reach the market.

Thus, 70 days after being transplanted to bags, accessions with greater height and leaf number were Nic 1016, SNN and Nic 1061 (Tables I and II). These two parameters generally tend to be closely related, because as plants grow higher, they have more leaves. So, the highest plants were observed in the above mentioned accessions, without differences with CE and showing values of about 60 to 68 cm, corresponding to the expected plant height for its age under house bag conditions. While mid plant height had values of about 45 and 55 cm. BHmN recorded the lowest height, without differences with Nic 1015 and Nic 1003 (Table I).

Regarding leaf number, three groups are defined at 70 days: a first group consisting of Nic 1016, SNN and Nic 1061, with the highest values between 18 and 19 leaves per plant; a second group having Nic 1003 with 16 leaves and a third one represented by the greatest accession number with values between 12 and 14 leaves per plant (Table II).

These results enable to get closer to a comparative morphological determination of a dynamic development among accessions, analyzing such morphological indicators for its partial characterization, but they do not determine the specific compound production in trichomes and leaf surface, nor the chemical composition inside leaves or their biological activity. Chemical composition is genetically determined according to specialized structures present in leaves involved in their metabolite synthesis and storage (14, 18, 19, 20). Certainly, once the accession shows the synthesis capacity of a given metabolite, a plant with a suitable height and, consequently, a great leaf number with a considerable surface could produce large amounts of interesting metabolites. Therefore, the knowledge of morphological aspects is important when establishing a production process.

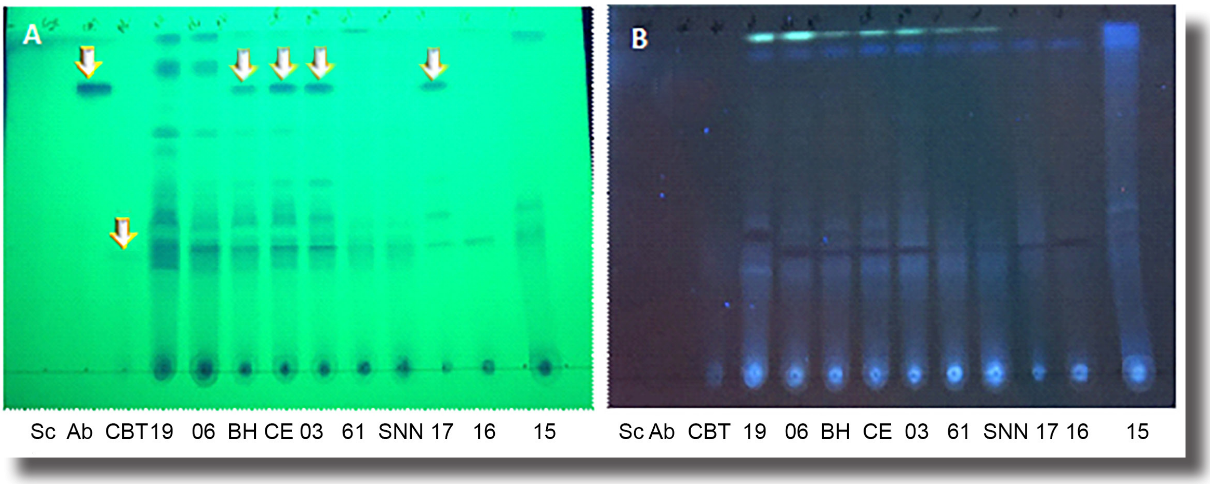
2. Separation and identification of compounds present in fresh leaf exudates

2.1 Production of ethanolic crude extracts from tobacco leaf exudates

Ethanolic crude extracts were obtained from tobacco leaf exudates of 10 accessions 70 days after being transplanted to bags, with differences in extract concentration and yield (16).

2.2 Separation and identification of ethanolic extracts by thin layer chromatography (TLC)

The thin layer chromatography of extracts revealed a chemical composition diversity given by each tobacco accession as well as the concentration of extracts and most compounds in all cases (Figure). When observing TLC at two wavelengths, a greater compound number was detected at 254 nm and a lower number at 366 nm, so that TLC observed at 254 nm enables to compare differences of several compounds. TLC showed that sclareol (Sc), a *Salvia sclarea* labdane diterpene used as pattern in this test, is not visible under ultraviolet light, so that, under these conditions, it is not possible to determine the presence of this diterpene in the ethanolic exudates obtained. The presence of cis-abienol, another labdane diterpene, was observed in Nic 1003, Nic 1017 and Cuban BHmN and CE.



A: CCF observed at 254 nm
B: CCF observed at 366 nm to separate ethanolic crude extracts obtained from leaf exudates of 10 tobacco accessions
19: Nic 1019, 06: Nic 1006, BH: BHmN, EC: Special Corojo, 03: Nic 1003, 61: Nic 1061, SNN: Samsun NN, 17: Nic 1017, 16: Nic 1016, 15: Nic 1015. Patterns: Sc: sclareol, Ab: cis-abienol, CBT: α-CBT diol

Thin layer chromatography (TLC)

There was no cis-abienol in other accessions evaluated. Maximum absorption for cis-abienol is 238 nm, so that it can be observed much better at 254 nm than at 366 nm; thus, under these conditions, a band can be seen in TLC at the corresponding height, according to run characteristics. Such ethanolic extracts prove the presence of this diterpene, except for SNN, which lack of it in these extracts, so the seeds obtained are not original SNN. The presence or absence of these compounds in trichomes and leaf surfaces is genetically determined; besides, it is an indicator that characterizes each accession and allows together with other descriptors to verify plant material authenticity. By determining the presence of cis-abienol in non-commercial tobacco accessions, it is possible to find its natural sources, which is very important for the new prospects of metabolic engineering concerning the multiple applications of this compound. Its properties are taken into account, as a precursor of important tobacco flavor and aroma compounds during leaf curing process (20). In addition, such application to perfume industry for its qualities, based on the chemical structure and enzymes involved in its synthesis (21), often provide a better cis-abienol starting material than the sclareol already used for these purposes (22). Its relationship with tobacco plant defense against pathogens is highlighted (8), as well as its inhibitory activity against wilt bacterial disease caused by *Ralstonia solanacearumen* in tomato, tobacco and Arabidopsis, among others (23).

For the case of α-CBT diol pattern, a light band was observed in TLC at 254 nm. Maximum absorption for cembrene diterpenes (α, β-CBTdiols and α, β-CBT oles) is 210 nm, so that the expected band is light at 254 nm, even when applying a high amount. Consequently, it is difficult to determine the presence of this compound in leaf exudate extracts under these running and developing conditions, besides the presence of other bands around the expected height.

Fewer compounds, under the running conditions used, are retained at the application point, whereas others escape with the run front; however, this system enables to separate a large amount of interesting compounds from crude extracts, identify cis-abienol and show the chemical diversity of extracts, which presupposes a wide diversity of their potential applications. Separation and identification by HPLC would allow proving the presence of these interesting diterpenes and other compounds.

Similar results in the chemical composition of leaf exudates have been found in a large number of *Nicotiana* accessions pertaining to USDA (United States Department of Agriculture) germplasm and to collections from Bergerac Tobacco Institute of France, which showed a considerable variation among them as for the presence and content of different types of diterpenes (20). It is believed that this diversity results from an intraspecific variation; it has been observed that Burley varieties only produce cembranoid diterpenes, while eastern varieties produce labdanoid diterpenes, generally cis-abienol and cembranoids (14, 20).

3. Chemical indicators in dry leaves of selected tobacco accessions

Leaf chemical composition is determinant on tobacco quality as a smoking product, but also on plant growth and development; therefore, on its characterization. Leaf analysis is also a useful tool to manage crop nutrition in general, which leads to a specific sample processing depending on the crop, so that the analysis may be effective (24). Table III shows chemical characterization results of all accessions evaluated. The variability of every chemical indicator studied could be valuable for tobacco germplasm banks, as a basis for future breeding programs to obtain new varieties with commercial purposes.

The accessions evaluated in most indicators presented values with significant differences between them, proving that such accessions are contrasting for leaf exudate diterpenes and also for these indicators (Table III).

One of the properties appreciated by smokers is ash percentage, quality and leaf combustibility. This is determined by several factors; thus, various chemical indicators influence on it. The main ash constituents are K, Ca and Cl, other elements like Mg, Na, etc. also influence. Ash percentage in the evaluated accessions was from 24 to 29 %. Nic 1003 presented the highest percentage with an average value of 29,33 %, meanwhile Nic 1015 had the lowest value with an average value of 24,07 %. In addition, ash quality and combustibility are also important. The essential ash constituent is K and the second one is Ca, which are antagonistic, so that when K increases combustibility, Ca decreases it. A suitable balance of both influences a better leaf structure and elasticity given by K and a whiter ash given by Ca. Results indicate that there were no deficiencies of both elements in the accessions evaluated when values of K_2O were above 2 % and CaO above 1 %. Nic 1017 was highlighted with the highest value of K_2O (4,86 %), whereas Nic 1006 had the lowest value (3,42 %). It is stated that values above the optimum may even serve to compensate excessive harmful elements, such as N and Cl, although it is not recommendable that these values surpass 6 %, since leaves become fragile and inappropriate (25). None of the accessions evaluated exceeds this value. The highest CaO value is shown by accession SNN with 8,86 % and the lowest one by BHmN with 3,15 % (Table III).

Nutrients play an important role in tobacco plant, only a balanced nutrition can lead to obtain a great harvest of high quality leaves. Tobacco absorbs a relatively large amount of nutrients that varies depending on the type of tobacco grown (26).

Table III. Chemical indicators of tobacco leaves that influence its quality with commercial purposes for each evaluated tobacco accession

Chemical indicators	Accessions									
	SNN	BHmN	1061	1015	1016	1006	1019	1003	CE	1017
K_2O (%)	4,17 d	3,84 e	4,20 d	3,67 f	3,51 g	3,42 h	4,48 b	4,17 d	4,30 c	4,86 a
Na_2O (%)	0,02 b	0,00 c	0,01 b	0,01 b	0,01 bc	0,01 bc	0,00 c	0,01 bc	0,01 bc	0,15 a
CaO (%)	8,86 a	3,15 h	6,49 e	7,56 d	5,24 f	7,59 d	3,16 h	7,67 c	8,29 b	5,18 g
MgO (%)	1,00 d	0,60 g	1,02 d	1,17 b	0,70 f	0,91 e	0,53 h	1,32 a	1,14 c	1,02 d
Cu (mg kg^{-1})	18 b	11 e	14 cd	20 a	16 bc	20 a	12 de	22 a	22 a	15 bc
Zn (mg kg^{-1})	89 a	33 f	47 e	51 d	45 e	53 d	32 f	66 c	70 b	53 d
Mn (mg kg^{-1})	15 d	6 e	16 cd	18 bcd	17 bcd	19 b	8 e	15 d	18 bc	78 a
Fe (mg kg^{-1})	75 d	57 e	90 a	84 b	57 e	73 d	75 d	81 c	86 b	40 f
Nitrogen (%)	5,17 b	5,23 a	4,39 e	5,13 b	4,84 c	4,59 d	4,55 d	4,28 g	4,34 f	3,78 h
Nicotine (%)	2,15 b	0,87 h	1,60 d	1,26 e	1,08 g	0,88 h	1,21 f	1,97 c	1,24 ef	3,78 a
Cl^- (%)	1,55 e	1,19 h	1,56 e	1,30 g	1,60 d	1,45 f	1,81 b	2,28 a	2,32 a	1,68 c
Ashes (%)	24,24 h	24,11 i	25,24 g	24,07 j	27,42 d	25,43 f	28,41 b	29,33 a	28,30 c	25,87 e
pH	5,8 a	5,38 cd	5,49 b	5,45 bc	5,43 bc	5,29 d	5,32 d	5,35 cd	5,36 cd	5,52 b
Alcalinity	1,21 b	1,14 c	1,08 d	0,85 f	0,73 g	1,08 d	1,23 b	1,09 d	1,01 e	1,26 a
K^+/Cl^-	2,68 c	3,21 a	2,69 c	2,82 b	2,19 e	2,37 d	2,48 d	1,83 f	1,85 f	2,89 b

Medias with different letters represent significant differences between accessions (Anova of a factor and Tukey in a statistical analysis between accessions for each indicator, $P \leq 0.05$, n= 3)

Evaluated accessions show Cl^- values from 1.19 % (BHmN) to 2.3 % (CE and Nic 1003). A suitable amount of Cl^- is approximately 0,5 %, which gives a better leaf texture and management. Cl^- values above 1,1 % may cause leaf combustibility troubles, since they do not burn above 3 %; therefore, the values of accessions evaluated are not adequate, although none of them exceeds 3 %.

It is known that a high K^+/Cl^- relationship usually means a high combustibility, a very appreciated property by smokers. The highest values are shown by BHmN, whereas the smallest ones by CE and Nic 1003, mainly due to Cl^- values, which demonstrates that BHmN is the accession that could present less combustibility problems, although this relationship does not determine this property by itself.

MgO values of BHmN, Nic1016, Nic 1006 and Nic 1019 were below 1 %. MgO values between 0.5 and 2 % are considered normal and determine leaf mass quality and elasticity (26), as well as a compact and white ash, which was only observed in the other six accessions out of 10 evaluated. Nic 1003 presented the highest value (1.32 %).

The highest Cu values between 20 and 22 mg kg^{-1} are presented by Nic 1015, Nic 1006, Nic 1003 and CE, whereas the lowest ones by BHmN together with Nic 1019, without significant differences between them (11 and 12 mg kg^{-1} respectively). The highest Zn values were recorded by SNN with 89 mg kg^{-1} and the lowest ones by BHmN and Nic 1019 with 33 and 32 mg kg^{-1}, respectively, without significant differences between them. The highest Mn values were shown by Nic 1017 with 78 mg kg^{-1} and the lowest ones by BHmN and Nic 1019 with 6 and 8 mg kg^{-1}, respectively.

These three elements have been linked to certain plant physiological processes, mainly enzymatic as cofactors, but its direct influence on tobacco quality has not been yet clearly established. It is noteworthy that in the three cases, BHmN and Nic 1019 present the smallest quantities. On the other hand, Fe values were from 90 mg kg^{-1} with Nic 1061 to 40 mg kg^{-1} with Nic 1017. Fe is indispensable for chlorophyll formation and involved in brown leaf color after fermentation; besides, it is an enzymatic component related to respiration and protein synthesis regulation.

BHmN also presents a greater N percentage of 5,23 %, while NIC 1017 shows a lower value of 3,78 %. The normal N percentage depends on the type of tobacco, but in general, values between 2 and 5 % are concerned and deficiency symptoms begin to appear when values are below 1,5 %. Excessive leaf N leads to excessive protein content, which is harmful to combustibility and indicates the lack of maturity in young leaves, besides causing a bitter taste to smokers. Moreover, this element is also linked to chlorophyll and nicotine formation (26). Thus, a chemical composition analysis was performed to tobacco leaves under controlled conditions, taking into account different leaf levels and its relationship to quality indicators (24). In general, their nutritional status showed higher values of N, Ca, Mg and K in the evaluated accessions than those reported for the entire leaf that represents all its parts at the central level of *Criollo-98* cv. plant, with a nice vegetative growth and nutritional appearance (24), while Na, Fe, Mn, Cu and Zn values were much lower in all accessions evaluated.

Another important characteristic determining tobacco strength is nicotine percentage; when this alkaloid is higher than 3 %, it may be the main cause of a very strong tobacco, which fits with maturity and contrary to N and protein content (26). The highest nicotine values are found in Nic 1017 with 3,78 %, the only one that is higher than 3 %, while the lowest ones are presented by BHmN and Nic 1006 with 0,87 and 0,88 %, respectively.

Then, pH values were between 5,8 by SNN and about 5,3 by BHmN, Nic 1019, CE, Nic 1003 and Nic 1006 that showed no significant differences between them; the second highest pH values were recorded by Nic 1017 and Nic 1061, with approximately 5,5 and without significant differences with the remaining accessions (Nic 1015 and Nic 1016) with similar values. Green tissue has an acid pH between 4,8 and 5,6; during drying process, acid destruction causes a pH increase, so that when tissue is dried, tobacco leaf pH is between 5,0 and 6,8, depending on the type of tobacco (Virginia, Eastern or Black). A greater pH is adequate, as it increases ammonia volatilization and irritating substances disappear. Alkalinity values were between 1,26 by Nic 1017 and 0,73 by Nic 1016.

Some researchers also conducted a study on other chemical indicators of tobacco leaves, in addition to those already mentioned (24), and provided more detailed information about the role of chemical constituents upon leaf quality (26).

It should be noted that none of these accessions is commercial, except CE, which is a discontinued commercial variety, since they had not been cultivated under their best conditions to develop adequate foliage with the required quality and it is expected that most of all these quality indicators evaluated are not suitable, but leaf chemical characterization is interesting, to search for accessions with traits to be considered in a breeding program, so as to obtain new tobacco varieties that also combine with diterpenes and sugar esters present on leaf surface, which are also involved to provide leaf organoleptic properties and tobacco plant resistance to diseases, such as cis-abienol in the brown leg caused by *Phytophthora nicotianae* (8).

These results prove that, in general, Nic 1017 showed the best leaf chemical composition indicators influencing the quality and BHmN presented the least adequate ones. Consequently, Nic 1017, due to its dry leaf chemical composition as well as the presence of cis-abienol in its exudates, could be taken into account in breeding programs to obtain new varieties with commercial purposes.

Regarding ethanolic extracts of leaf exudates, all accessions, due to their chemical diversity, are interesting in the search of new compounds with specific biological activities, highlighting Nic 1006 and Nic 1003 for their high yield, and Nic 1015 for its specific antibacterial activity against *Xanthomonas campestris* and *Pectobacterium carotovorum* (16).

CONCLUSIONS

♦ Nic 1016 reached the greatest height and leaf number, along with Nic 1061 and SNN, 42 days after being transplanted to bags. BHmN remained below the others in plant height and leaf number.

♦ Ethanolic crude extracts of Nic 1017, Nic 1003, BHmN and CE showed the presence of cis-abienol in its leaf exudates and all accessions showed a chemical diversity in their crude extracts.

♦ Nic 1017 showed the best leaf chemical composition indicators influencing quality whereas BHmN the least adequate.

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