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Department of Plant Breeding and Biotechnology, Institute of Soil Science and Plant Cultivation – State Research Institute, Czartoryskich 8, 24-100 Puławy, Poland
* e-mail: anngol@iung.pulawy.pl

ANNA TROJAK-GOLUCH, MARTA KOZIARA-CIUPA

Improvement of *Nicotiana tabacum* L. for low conversion of nicotine to nornicotine and its effect on morphological traits and chemical composition

Doskonalenie *Nicotiana tabacum* L. w kierunku obniżenia konwersji nikotyny do nornikotyny i jego wpływ na cechy morfologiczne i skład chemiczny

Abstract. Nornicotine is a secondary metabolite formed in tobacco leaves by the oxidative N-demethylation (conversion) of nicotine. Its high level is undesirable because this alkaloid is a precursor of N-nitrosonornicotine, which has been shown to have carcinogenic properties. The aim of the study was to assess the nicotine and nornicotine content in four successive generations of ten tobacco cultivars/breeding lines. The possibility of reducing potentially harmful compounds in the cultivars/breeding lines was also determined. The study was conducted as field experiments between the years 2014 and 2018. The alkaloid content in the leaves was determined by the gas chromatography/ mass spectrometry (GC/MS) method. The systematic assessment of the alkaloid profile of tobacco and eliminating converter plants in four successive generations, particularly within breeding lines characterized by a wide conversion range, made it possible to reduce the nornicotine content and, thus, the potentially carcinogenic compounds in the leaves. Three lines, ZD2, TNX1, and WGLB with a stable conversion rate of ≤3% and low content of nornicotine were obtained. Furthermore, the morphological traits of the isogenic lines ZD2, TNX1 and WGLB, which exhibit markedly different conversion capacity were evaluated. The greenhouse experiment showed that there were significant differences in some morphological traits. The non-converting lines TNX1 and ZD2 produced longer and wider 9th and 15th leaves than the converting analogues. A relationship has been identified between the traits that determine the phenotype of tobacco cultivars/lines and their ability to convert nicotine to nornicotine.

Keywords: tobacco, alkaloid, non-converter genotypes, isogenic lines, morphological traits

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INTRODUCTION

The cultivation of tobacco, *Nicotiana tabacum* L., is an important component of crop production in several regions of the world, providing farmers with a satisfactory income even under difficult habitat conditions. However, tobacco consumption remains a major public health challenge [Burns et al. 2008]. The World Health Organization [2015] has been working diligently for years to reduce tobacco consumption. In 2015, the organization published, recommendations to decrease the nicotine concentration in tobacco filler to 0.04%. Therefore, ensuring the high quality of raw tobacco and, above all, its compliance with increasingly stringent addictive content requirements has become a priority task for tobacco growers and breeders.

The quality of raw tobacco is determined by the content of major alkaloids [Shen and Shao 2006]. These compounds have physiological effects on humans and animals and affect the taste of tobacco products [Mishra et al. 2015]. The alkaloid content of tobacco leaves can vary widely due to genetic variation in cultivars, nitrogen fertilization, mechanical damage, air temperature, and sunlight [Tso 1990]. The content of secondary metabolites is also dependent on various factors such as plant density, topping practices, sucker control, the method of leaf curing, and the technological treatment of the raw materials [Zou et al. 2021]. Air-cured Burley tobacco generally contains higher levels of alkaloids than flue-cured Virginia tobacco. The predominant alkaloid in low-converter tobacco, accounting for 90 to 95% of the total alkaloid pool, is nicotine [Wang and Bennetzen 2015]. It is a heterocyclic compound formed by the combination of a six-membered pyridine ring and a five-membered pyrrole ring. It is produced primarily in the roots and is transported to the leaves and flowers as the plants grow. Its presence in raw material and tobacco products within recognized limits is beneficial and expected, as it promotes the release of dopamine in the brain. Nicotine *per se* is not recognized as a carcinogen. Nevertheless, it should be noted that it is primarily responsible for cigarette addiction, which, in turn, promotes cardiovascular disease [Benowitz 2010]. The second most abundant alkaloid in tobacco that strongly affects human health is nornicotine. It is formed by nicotine conversion i.e. oxidative N-demethylation of nicotine into nornicotine mainly during the ripening and curing of tobacco leaves [Siminszky et al. 2005, Chakrabarti et al. 2008]. The content of nornicotine typically ranges from 2% to 5% of the total alkaloids in low-converter tobacco. However, some seed lots in which the conversion of nicotine to nornicotine occurs to a high degree, and the proportion of nornicotine accounts for more than half of the total alkaloid content. They mainly occur in Burley tobacco cultivars, whose leaves are air-cured. The high content of nornicotine is undesirable as it is a precursor of N'-nitrosonornicotine (NNN), one of the major tobacco-specific nitrosamines (TSNAs) [Cai et al. 2012]. NNN is formed during tobacco curing, storage and smoking. Nitrosamines have been found to have carcinogenic properties and alter the lipid profile of the blood [Fant et al. 1999, Andra and Marris 2011].

The conversion of nicotine to nornicotine is catalyzed by nicotine N-demethylases, enzymes encoded by a group of cytochrome P450 genes belonging to the *CYP82E* subfamily [Shoji et al. 2009]. There are at least five genes belonging to *CYP82E* in tobacco. These are: *CYP82E4*, *CYP82E5* and *CYP82E10*, which encode functional nicotine N-demethylases [Siminszky et al. 2005, Chakrabarti et al. 2007, Lewis et al. 2010], as well as *CYP83E2* and *CYP82E3* genes encoding inactive enzymes [Chakrabarti et al. 2007]. Lewis et al. [2010] suggest that these genes spontaneously undergo genetic modifications, resulting in the emergence of individuals that convert nicotine to nornicotine. Hence, in

successive generations of many cultivars and breeding lines considered stable for many functional traits, individual variation in nornicotine content is recorded. This phenomenon is particularly frequent in Burley, where a change in phenotype from low nornicotine to high nornicotine affects up to 20% of the population [Gavilano et al. 2006].

There are several methods to reduce the amount of potentially dangerous compounds in tobacco. One of these is the chemical extraction of nicotine from dry leaves, which reduces the content of this alkaloid to a very low level but also contributes to the reduction of aromatic components in the raw material. Some agronomic practices are also used, such as harvesting only part of the leaves from the plant or abandoning topping and sucker control, which reduces the accumulation of nicotine in the leaves. It is also recommended to reduce the N fertilization. However, besides reducing the content of major alkaloids, these practices result in a marked decrease in yield [Henry et al. 2019]. Mutation breeding is an equally helpful tool in controlling N-nitrosonornicotine in tobacco. The public widely accepts the resulting genetic variation. The technique of mutagenesis of the nicotine N-demethylase gene (*NtabCYP82E4*) of the strongly converting Burley cultivar 'BB16NN' was used by Julio et al. [2008]. The resulting mutants were crossed with high-quality tobacco cultivars, and breeding lines were obtained. These lines were distinguished by a stable, non-converting phenotype conditioned by the inactivation of *NtabCYP82E4* alleles. In contrast, Lewis et al. [2010] induced knockout mutations in the nicotine demethylase genes (*CYP82E4*, *CYP82E5v2*, and *CYP82E10*) of the breeding line DH98-325-6. The resulting single-point mutants were then crossed to combine three mutations in one genome. Genotypes were obtained in which the conversion of nicotine to nornicotine was very low, averaging 0.55%. The oxidative N-demethylation of nicotine to nornicotine can be reduced using the RNA interference technique. This involves inserting and activating small interfering double-stranded RNAi fragments [Chintapakorn and Hamill 2003, Gavilano et al. 2006, Kajikawa et al. 2011]. The CRISPR-Cas genome editing technique provides significant opportunities for modifying the alkaloid profile [Schachtsiek and Stehle 2019]. However, modern molecular biology techniques such as RNAi and CRISPR-Cas and the cultivars obtained through these techniques are not yet widely applicable in agriculture due to legal regulations and low public acceptance.

The reduction of N-nitrosonornicotine in tobacco can also be achieved by selecting appropriate parental forms for crossing, followed by selecting individuals with low nornicotine content in the progeny. Using rouging is a fairly simple and cost-effective strategy, but it requires systematic control of the alkaloid profile of parental forms and progeny. Any individuals that exhibit more than 3% conversion of nicotine to nornicotine should be excluded from seed production. Jung et al. [2005] conducted studies to eliminate converting genotypes from tobacco materials. The initial material consisted of Burley tobacco breeding lines KB 9118, which exhibited a varying proportion of converters in the population, ranging from 25% to 56%. The technique of self-pollination of non-converters was used and the progeny plants were tested to select non-converters in the next generation. In the first year after selection, non-converting lines accounted for 75% (12 out of 16 tested). However, the authors suggest that eliminating converters from subsequent populations should be carried out annually before seed harvesting to ensure the production of pure non-converter lines.

The study aimed to evaluate the content of major alkaloids and the conversion rate of nicotine to nornicotine in the leaves of four successive generations obtained through a selection and self-pollination of non-converting and highly converting genotypes in the

tobacco cultivars and breeding lines. Additionally, the possibility of reducing potentially carcinogenic compounds in tobacco cultivars and lines was determined. Moreover, the study also includes a comparison of isogenic lines differing extremely in their ability to convert nicotine in order to assess the correlation between the conversion capacity and tobacco morphological traits.

MATERIALS AND METHODS

Plant material

The initial experimental material included four Polish cultivars and breeding lines of Virginia tobacco: 1) 'WAC120' a black root-rot resistant cultivar, 2) 'WAC121' a newly developed cultivar with recognized high-quality of leaves, 3) 'Sybilla' a cultivar described as nicotine-free, and 4) WGLB breeding line with *N. glauca-type* resistance to black root rot [Trojak-Goluch and Berbeć 2011]. The study also examined Burley tobacco, including the popular American cultivar 'TN90' with good disease resistance, as well as five high-yielding breeding lines ZD2, TNX1, TNX2, TNX3, and TNX4, which have good agronomic characteristics and were derived from a cross between 'TN90' and Polish Burley cultivars. All tobacco cultivars and lines were sourced from the collection maintained in the Institute of Soil Science and Plant Cultivation in Puławy, being the part of the National Centre for Plant Genetic Resources: Polish Genebank, Radzików, Poland.

Field procedure

Field experiments were conducted at the Experimental Station (51°27'N 22°03'E), of the Institute of Soil Science – State Research Institute, Puławy, Poland, from 2014 to 2018. The aim was to select individuals that do not convert and those that convert strongly in subsequent generations. Seedlings were prepared in 160 multicellular trays measuring 60 × 40 cm under greenhouse conditions. Tobacco transplantation was carried out in May. Mineral fertilization (NPK) was completed before planting. A 50 kg^{-1} of N, 125 kg^{-1} of K_2O, 100 kg^{-1} of P_2O_5 and 112 kg^{-1} of SO_3 per 1 ha dose of minerals was applied. The plot area was 17.82 m^2 and included 44 plants spaced at 0.9 m × 0.45 m. The plot was formed by four rows, each with 11 plants. Tobacco management practices were appropriate for the type of tobacco used, with the exception of removing the main inflorescence and suckers. Mature leaves were harvested in five rounds from 30 randomly selected plants per plot. The leaves were air-cured independently of the type of tobacco to ensure conditions favorable for oxidative N-demethylation of nicotine. All leaves from individual plants were analyzed using a gas chromatography. The content of nicotine and nornicotine, as well as the rate of conversion of nicotine to nornicotine, were determined for each of the 30 plants. The flowering plants' inflorescences were protected with paper bags to enable self-pollination for seed setting. However, only plants with the most favorable nicotine to nornicotine ratio, i.e. with the highest nicotine content and the lowest nornicotine content, were used for seed collection within each plot. The seeds were then sown, and the resulting plants were evaluated for their alkaloid profile. The same procedure was followed in subsequent years of the study.

Sample preparation for GC/MS analysis

To evaluate the alkaloid profile, plant material consisting of all leaves collected from individual plants was used [Jack et al. 2003]. Dried and ground leaves weighing 1 ±0.01 g were incubated at room temperature for 15 min in 7 ml of 2 M NaOH. Subsequently, the leaves were extracted in 25 ml of tetr-butyl methyl ether (HPLC purity) with an internal standard (quinoline 0.4 mg ml^{-1}). The extraction process was carried out for 2 h and at 130 rpm. The extracted solution was separated into an organic layer, and 1 ml was transferred into vials. The concentration of the alkaloids was determined using gas chromatography-mass spectrometry.

Analysis of the alkaloid content in tobacco leaves

For the study of alkaloids, an Agilent Technologies GC System 7890A gas chromatograph coupled with an MS 5975C mass detector and an Agilent Technologies 19091S-433UI model HP-5MS polar chromatographic column (30 m × 0.250 mm, stationary phase film thickness 0.25 μm) was used. We select chromatographic separation conditions to achieve optimal separation of the analyzed substances. The temperature program was as follows: the initial temperature was set at 115 °C for 10 min, followed by an increase of 5 °C per min until it reached 200 °C, where it was held for 2 min. The temperature was then increased to 280 °C at a rate of 50 °C per min and held for 10 min. Separation was carried out using helium at a flow rate of 1 ml per min. Samples were injected through an injector heated to 220 °C onto the chromatography column at a rate of 1 μl of sample (split, 20 : 1).

The concentration of nicotine and nornicotine were determined by comparing the mass spectrum of the recorded chromatographic peaks and the retention time of the tested compounds with the standards used, namely nicotine (Sigma Aldrich CAS 54-11-5) and nornicotine (Toronto Research Chemicals CAS 5746-86-1). The internal standard method was used for quantitative analysis with quinoline (Sigma-Aldrich CAS 91-22-5) as the constant concentration at 0.4 mg ml^{-1}. Alkaloids were prepared as separate stock solutions in extraction solvent with nicotine at 10 mg ml^{-1} and nornicotine at 10 mg ml^{-1}. Calibration solutions were then created by combining the appropriate amounts of each stock solution in a volumetric flask and diluting to the desired volume, resulting in six different concentrations. The calibration curve solutions covered a concentration range of 0.6–0.02 mg ml^{-1} for nicotine and 0.08–0.002 mg ml^{-1} for nornicotine. The linear coefficient of determination for nicotine was R^2=0.999, while for nornicotine, it was R^2 = 0.998. The total content of nicotine and nornicotine in tobacco leaves was determined using the equation:

$$AC = \% \text{ chromatogram} / \text{d.w.} \times 10$$

where: AC – alkaloid content (mg g^{-1} d.w.); d.w. – the dry weight of the sample (g); % chromatogram – is a mass of nicotine or nornicotine for each test portion aliquot calculated using the coefficient of the linear regression.

Analysis of the alkaloid content in tobacco leaves

Data on the nicotine and nornicotine content in leaf dry weight were used to calculate the conversion rate. The following equation [Jack et al. 2003] was used for the calculation:

$$\text{conversion rate (\%)} = \text{nornicotine content} / (\text{nicotine content} + \text{nornicotine content}) \times 100$$

nornicotine content in dry weight (%); nicotine content in dry weight (%).

The percentage of plants with a conversion rate in the range from 0 to 100 was determined. Then, based on the results, the tobacco cultivars and breeding lines were assigned different levels of conversion (Tab. 1).

Table 1. Range of conversion of nicotine to nornicotine and the corresponding conversion levels in cultivars and breeding lines of tobacco

Range of conversion (%)	Level of conversion
≤3	no conversion*
3–10	low
10–30	medium
30–60	strong
60–90	very strong
>90	total

* The term „no conversion" has been established according to the recommendations of tobacco companies while the other levels are an in-house study

Evaluation of the relationship between nicotine conversion capacity and morphological traits of tobacco

In 2018, a pot experiment was conducted in the greenhouse under partially controlled conditions. The experiment included three breeding lines, ZD2, TNX1, and WGLB, which were selected through the roguing process. For each of them, a converting population C (range of conversion >60%) and a non-converting population N (range of conversion ≤3%) were also selected. Six treatments were selected, each represented by 10 plants in 3 replicates. Tobacco seedlings were prepared in the greenhouse at a temperature of 17–25 °C. Then, after 40 days, the plantlets were transferred to 33 cm diameter pots filled with peat substrate (Solvika) at 7 kg each. The soil reaction ranged from 5.5 to 6.0 pH_{KCl}. Each plant received an equal amount of water throughout the experiment. Additionally, the plants were fertilized once with an 8% Yara Tera^{TM} Kristalon Special, NPK 18-18-18 (Yara International ASA) at a rate of 0.5 L per plant pot. Thermal conditions were natural, with a mean daily temperature ranging from 18.9–22.9 °C between May and August. Biometric measurements were taken of all plants, including height (from the base of the plant to the tip of the inflorescence), number of leaves per plant, length, and width of the 7th, 9th, and 15th leaves, and the number of days from planting into pots to the appearance of the first flower. Once the plants reached the generative stage, statistical calculations were made on the basis of the measurements of 10 plants per replication.

Statistical analysis

The plant biometric data and nicotine and nornicotine contents in the leaves were statisticaly analyzed using Statistica 13.3 StatSoft software. One-way analysis of variance

(ANOVA) was used to test the significance of differences. Tukey's test was used to compare morphological traits and chemical parameters of leaves of the tested cultivars/breeding lines at $P \leq 0.05$. The rate of conversion of nicotine to nornicotine was subjected to an arcsine transformation. The statistical analysis was performed using non-parametric Kruskal-Wallis test.

RESULTS

Analysis of the nicotine and nornicotine content in the tobacco leaves

In 2014, 10 tobacco cultivars and breeding lines belonging to Virginia and Burley tobacco were evaluated for the major alkaloids and the conversion rate of nicotine to nornicotine. The qualitative GC/MS analysis showed that nicotine was the predominant alkaloid in most tested accessions. However, the studied accessions showed significant variation in terms of the content of this alkaloid (Fig. 1).

The nicotine content was particularly high in the cultivars 'WAC121' and 'WAC120' (1.11 and 1.85% d.w., respectively) of Virginia tobacco, as well as in the cultivars/breeding lines 'TN90', TNX2, and ZD2 (1.29, 1.23, 1.00% d.w., respectively) of Burley tobacco 'Sybilla', WGLB (Virginia), and TNX3, TNX4 (Burley) had significantly lower nicotine levels than the others, with nornicotine being the leading alkaloid (Fig. 2). The highest content of nornicotine (0.79%) was found in the TNX4 breeding line.

The study found that the nicotine content of tobacco leaves increased in subsequent years due to the selection and elimination of converting individuals. In most cases, the differences were statistically significant, with the highest alkaloid content recorded after the first year of selection. However, it is important to note that the weather conditions during the 2015 growing season, particularly the high air temperature and low rainfall in August, favored the production and accumulation of alkaloids in tobacco. This resulted in considerable differences in nicotine content between 2015 and 2014 as well as between 2015 and other years. The exception was the cultivar 'TN90', for which the nicotine content in 2017 was significantly lower than that found in the initial plants (Fig. 1).

Analysis of the conversion capacity in the tobacco leaves

The range of conversion and the rate of conversion for individual tobacco cultivars/ breeding lines between 2014 and 2018 are shown in Tables 2 and 3. The analysis in the initial populations (2014) revealed individual variability in terms of oxidative N-demethylation of nicotine to nornicotine. High individual variation was observed, particularly in ZD2, TNX1, TNX3 (Burley) – Table 2, and WGLB (Virginia) breeding lines (Tab. 3). In these accessions, there were individuals with strong conversion rates, as well as those with low conversion rates and some who did not convert at all. These lines provide valuable material to breed and select in subsequent generations of stable, non-converting tobacco genotypes. The remaining accessions showed much less individual variation in this regard. TNX2 (Tab. 2) and 'WAC120' (Tab. 3) were homogeneous populations with very low conversion rates. In contrast, TNX3 and TNX4 (Tab. 2) were strong converters, while 'Sybilla' (Tab. 3) exhibited the highest conversion range and rate values in 2014. This suggests that selecting non-converting individuals exhibiting the desired alkaloid profile was challenging.

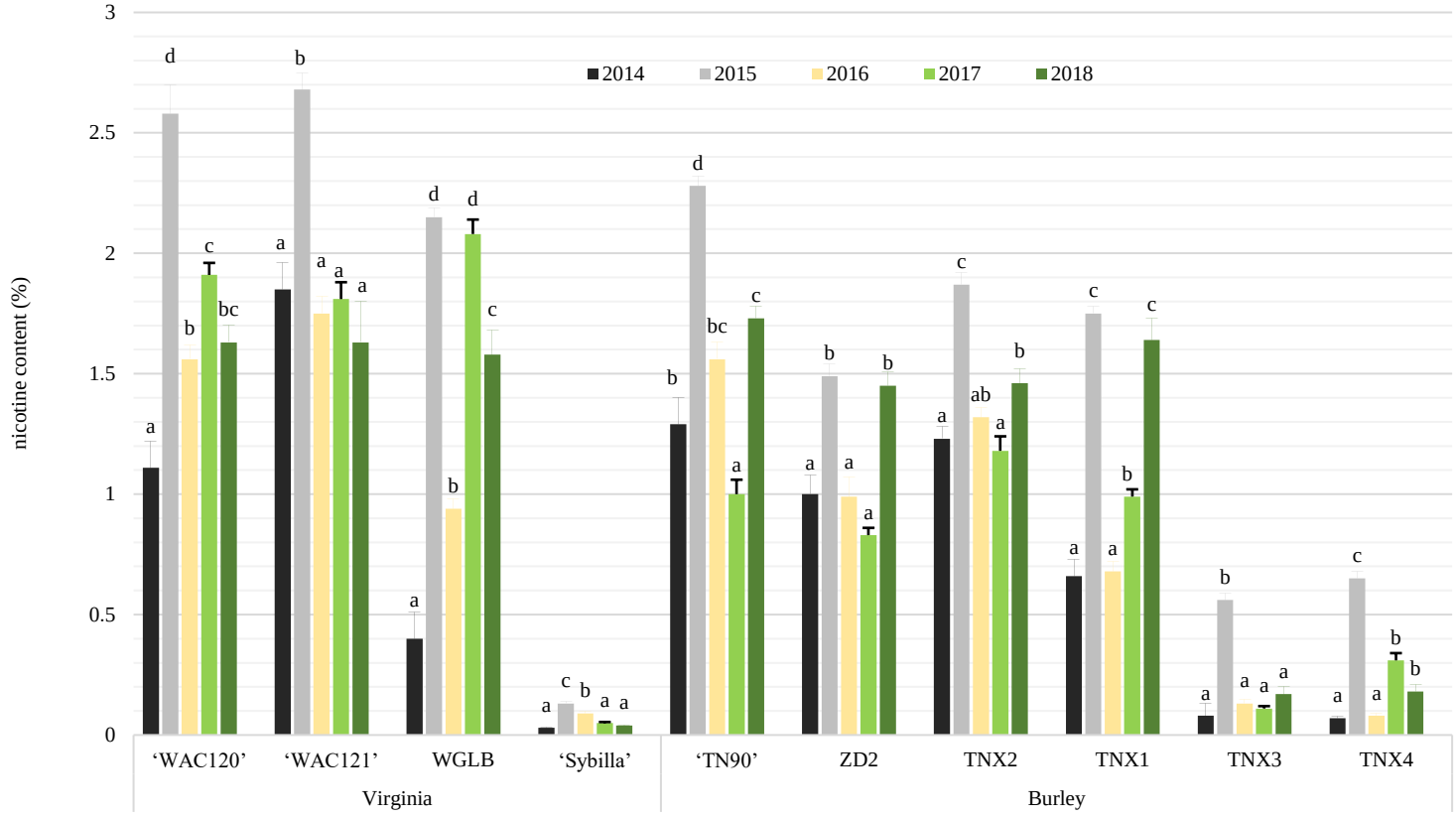

Fig. 1. Nicotine content (mean ±SE) in cultivars and breeding lines of tobacco in the years 2014–2018; different letters denote significant differences among the years (2014–2018) in the same cultivar/breeding line, according to Tukey's test at $p \leq 0.05$

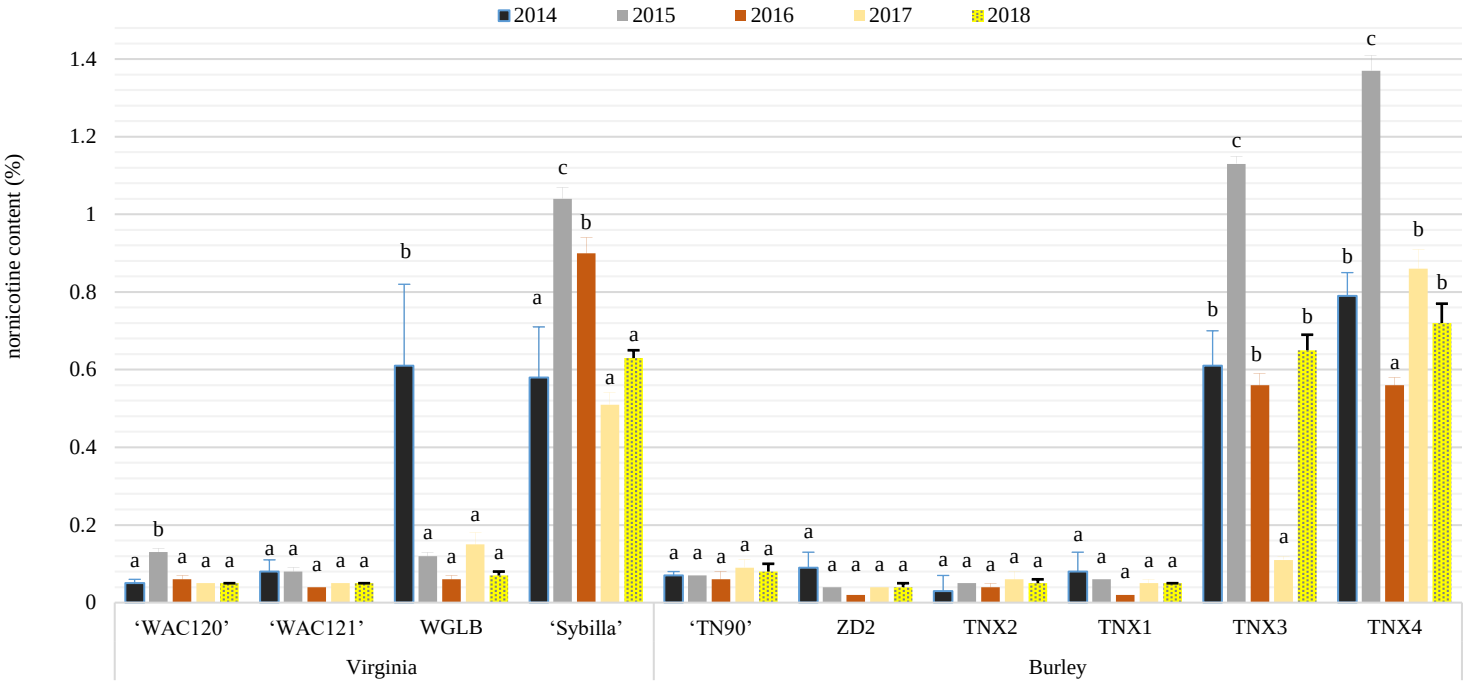

Fig. 2. Nornicotine content (mean ±SE) in cultivars and breeding lines of tobacco in the years 2014–2018; different letters denote significant differences among the years (2014–2018) in the same cultivar/breeding line, according to Tukey's test at $p \leq 0.05$

Table 2. Conversion rate and conversion range in cultivars and breeding lines of Burley tobacco in the years 2014–2018

Con-version	Year	Cultivar/Breding line – Burley						
		'TN90'	ZD2	TNX2	TNX1	TNX3	TNX4	$\bar{x}$
Rate (%)	2014	5.3^{ab*}	9.9^{b}	2.3^{a}	11.0^{b}	87.0^{b}	92.2^{d}	34.6
	2015	2.8^{a}	2.8^{a}	2.4^{a}	3.4^{ab}	67.2^{a}	68.1^{a}	24.4
	2016	4.0^{a}	1.5^{a}	2.4^{a}	2.6^{a}	82.0^{b}	87.5^{c}	30.1
	2017	9.1^{b}	4.4^{ab}	4.2^{a}	4.8^{ab}	85.1^{b}	74.1^{b}	30.3
	2018	4.8^{a}	2.9^{a}	3.3^{a}	3.2^{a}	80.5^{b}	75.1^{b}	28.3
Range (%)	2014	2.3–21.6	1.4–55.7	1.5–6.2	2.1–85.7	2.2–96.9	83.5–94.6	1.5–96.9
	2015	2.3–4.1	2.2–5.6	2.1–3.4	3.0–3.8	58.3–76.1	60.9–75.7	2.1–76.1
	2016	1.6–22.7	1.1–1.8	1.8–15.9	2.2–3.2	66.9–90.0	82.2–90.9	1.1–90.9
	2017	2.9–49.3	3.0–10.8	2.8–16.3	3.1–14.1	78.7–92.4	63.3–81.4	2.8–92.4
	2018	2.7–23.8	2.4–3.5	3.0–3.8	2.8–3.6	66.2–91.6	66.4–83.2	2.4–91.6

$\bar{x}$ – average;
* mean values not sharing a letter within the column are significantly different between the years (2014–2018), according to the Kruskal-Wallis test at $p \leq 0.05$

Table 3. Conversion rate and conversion range in cultivars and breeding lines of Virginia tobacco in the years 2014–2018

Conversion	Year	Cultivar/Breding line – Virginia				
		'WAC120'	'WAC121'	WGLB	'Sybilla'	$\bar{x}$
Rate (%)	2014	4.2^{bc*}	4.3^{a}	60.7^{b}	93.8^{bc}	40.7
	2015	5.0^{c}	3.0^{a}	5.2^{a}	88.8^{a}	25.5
	2016	3.7^{abc}	2.4^{a}	4.4^{a}	91.2^{ab}	25.4
	2017	2.5^{a}	2.7^{a}	6.5^{a}	91.1^{ab}	25.7
	2018	3.3^{ab}	3.5^{a}	4.5^{a}	94.2^{c}	26.4
Range (%)	2014	2.6–8.8	1.5–22.5	2.7–97.2	89.9–97.0	1.5–97.2
	2015	4.4–6.1	1.8–5.1	3.4–10.8	84.0–95.4	1.8–95.4
	2016	2.2–7.1	2.2–2.6	2.7–11.5	87.2–93.4	2.2–93.4
	2017	2.2–2.7	2.3–3.0	2.5–15.8	88.6–92.8	2.2–92.8
	2018	2.7–4.8	2.7–8.2	2.6–7.6	91.2–96.2	2.6–96.2

$\bar{x}$ – average;
* mean values not sharing a letter within the column are significantly different between the years (2014–2018), according to the Kruskal-Wallis test at $p \leq 0.05$

Based on the rate of conversion at each accession, the initial population was divided into three main groups (Fig. 3). The first group consisted of the cultivars/lines 'WAC121', 'TN90', and TNX2, within which mainly non-converting individuals were identified. The

frequency of genotypes showing low to medium conversion (range of conversion 3–30%) was no more than 27%. Most individuals in this group had a rate of conversion only slightly above the threshold value of 3%, including cultivar 'WAC120'. The second group consisted of ZD2, TNX1, and WGLB breeding lines. The individuals in this group belonged to different conversion levels, ranging from non-conversion (≤3%) to those with low and medium conversion, as well as total conversion (>90%). The WGLB line obtained by interspecific hybridization of *N. tabacum* × *N. glauca*, was of particular interest. This line was represented by individuals belonging to all conversion levels. The data showed that total converters accounted for 46.7% of the total, with very strong converters at 10%, strong converters at 10%, medium converters at 23.3%, low converters at 6.7%, and non-converters at 3.3%.

The third group consisted of strong converters, comprising the breeding lines TNX3, TNX4 and the cultivar 'Sybilla', in which most individuals converted very strongly or completely (conversion range 60–100%) – Figure 3.

Selection efficiency of non-converters in the group I

The analysis of the frequency of plants showing different rates of conversion in the initial populations (in 2014) and self-pollinated offspring populations in 2018 revealed a substantial increase in the proportion of non-converters (Figs 3 and 4). 'WAC120' showed a 33.3% increase in the proportion of genotypes that do not convert nicotine to nornicotine after 4 years of selection. However, the differences in mean conversion rate observed for this cultivar in 2014 and 2018 were not statistically significant (Tab. 3).

In the subsequent cultivar 'WAC121', individuals exhibiting medium conversion were eliminated after 4 years of selection. However, the study found an increased frequency of individuals with low conversion (3–10%) compared to that recorded in 2014 (Figs 3 and 4). Nevertheless, a detailed assessment of the conversion ability scores in this population revealed that the majority of individuals classified in this range were converters with conversion level only slightly above the 3% threshold. The selection for reduced conversion ability in the 'TN90' cultivar and the TNX2 breeding line was slightly less effective. In 2018, the number of individuals with medium conversion decreased slightly compared to the initial populations. However, there was an increase in the frequency of individuals with low conversion.

Selection efficiency of non-converters in the group II

The selection of stable, non-converting populations gave the best results in group II. For the WGLB line (Virginia), which initially had plants belonging to all conversion ranges, significant changes were observed after the fourth year of selection in the frequency of non-converters as well as individuals showing low and medium conversion. In 2018, 20% of the population were non-converters, while the remaining 80% were low converters (Fig. 4). The differences in the rate of conversion observed in 2014 and 2018 were statistically significant (Tab. 3). Additionally, in the successive generations obtained from self-pollination of the ZD2 (Burley), there was an increase in the number of non-converting plants, with their proportion being very high at 80% (Fig. 4). Based on the analysis of the alkaloid profile, satisfactory results were obtained from the plant selection process and self-pollination of non-converters of the TNX1 breeding line. The conversion rate in the 2018 population was significantly lower than that of the 2014 population (Tab. 2). Furthermore, 60% of the total population were low-converting individuals, while 40% were non-converters. There were no plants that showed a higher rate of conversion (Fig. 4).

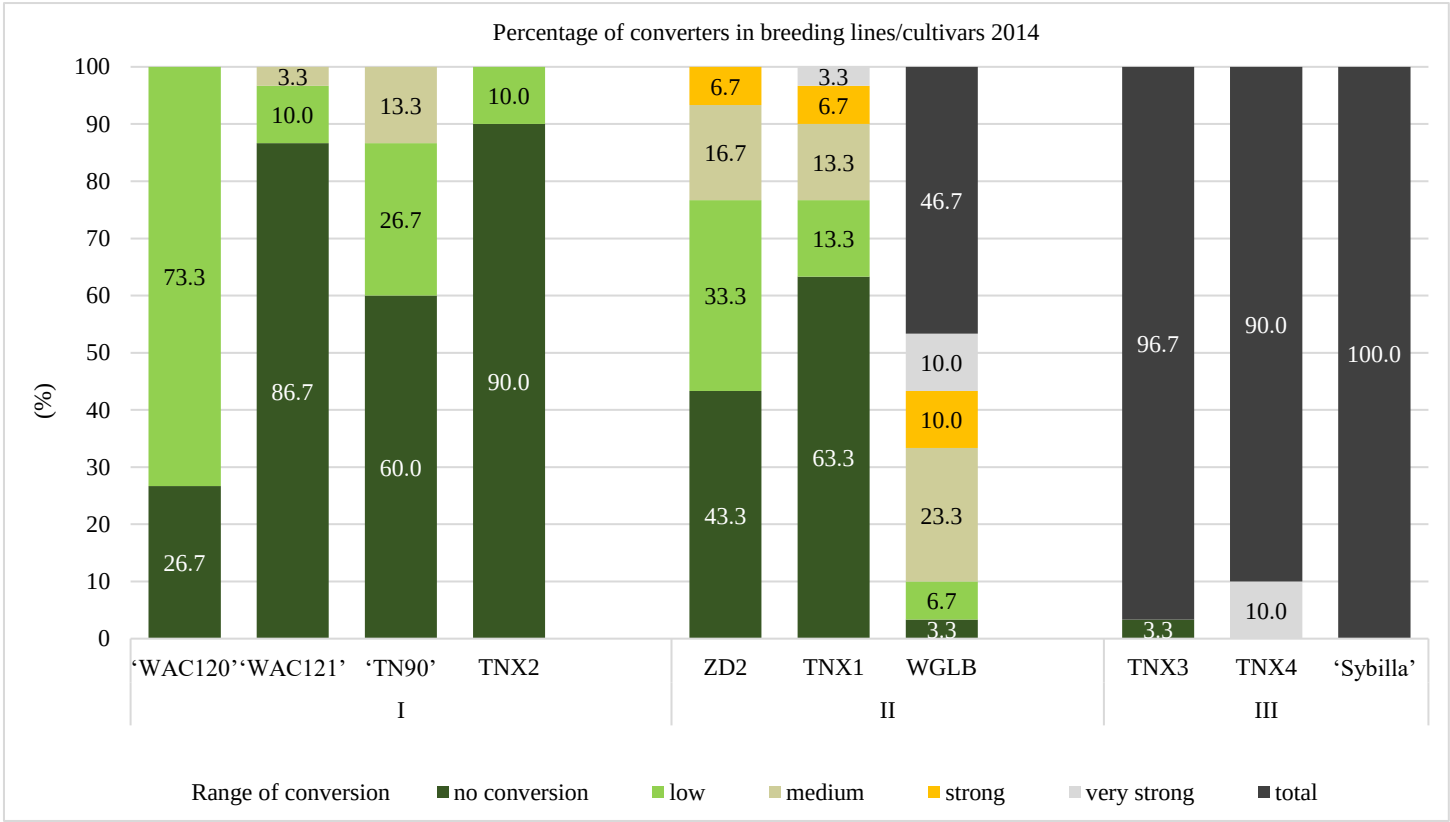

Fig. 3. The percentage of genotypes showing different conversion rates in cultivars and breeding lines of tobacco in 2014

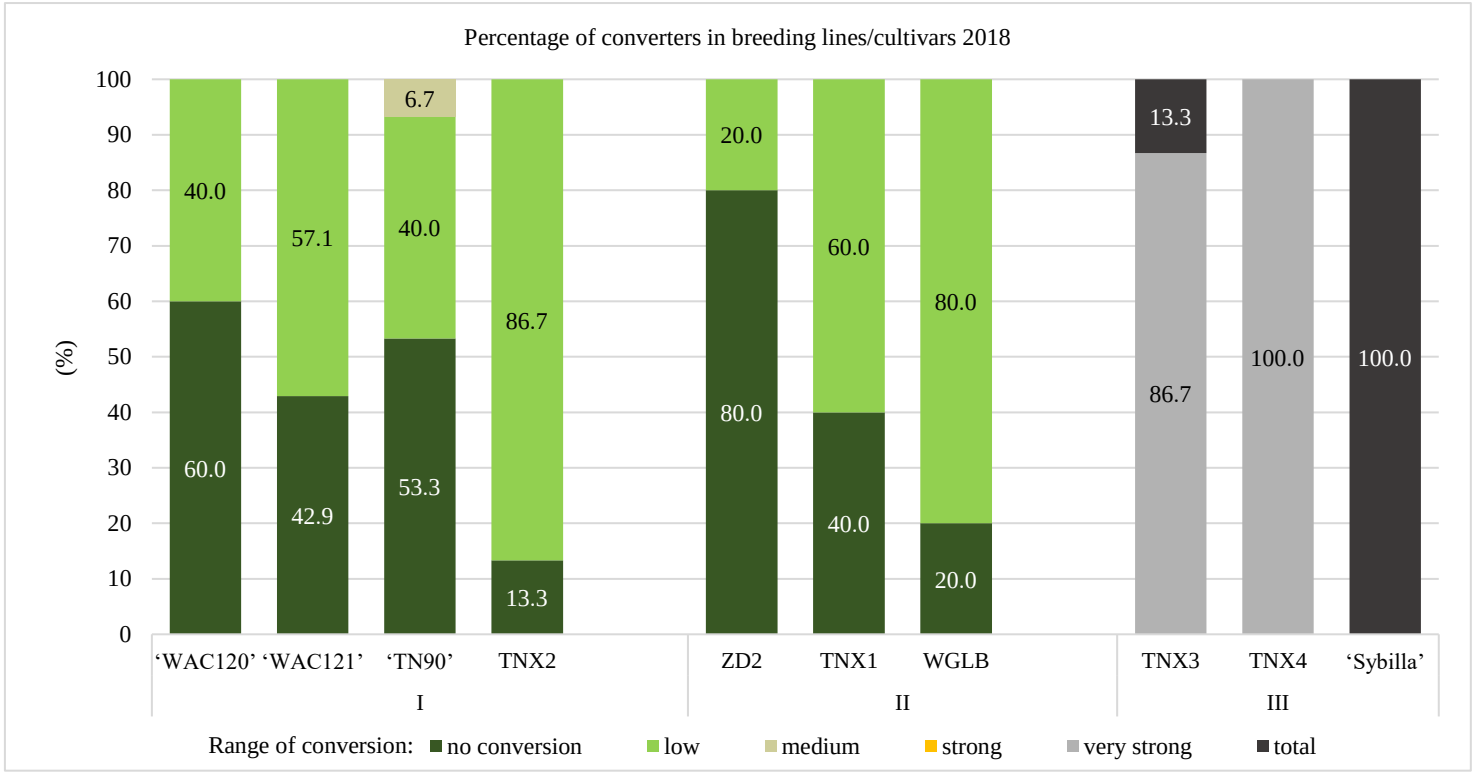

Fig. 4. The percentage of genotypes showing different conversion rates in cultivars and breeding lines of tobacco in 2018

Selection efficiency of non-converters in group III

The initial population of the 'Sybilla' had a very low content of nicotine and a high content of nornicotine (Figs 1 and 2). Most of the plants converted nicotine to nornicotine to a very strong or total level. Attempts to select non-converting plants were unsatisfactory. In 2018, the frequency of plants with total conversion was 100% (Fig. 4) and the average conversion rate was 94.2% (Tab. 3). Burley tobacco breeding lines TNX3 and TNX4 exhibited a similar characteristic. No non-converting or low-converting genotypes were found in 2018 (Fig. 4). However, due to lack of suitable breeding material, lines with better chemical parameters could not be obtained in subsequent years of selection. In 2018, 86.7% of the population of the TNX3 breeding line were very strong converters and 13.3% were total converters. In contrast, all plants in the TNX4 line, showed a very strong conversion level.

Evaluation of the relationship between conversion rate and agronomic traits of tobacco breeding lines

A greenhouse experiment was conducted in 2018 to determine the relationship between conversion capacity and morphological traits in tobacco. The study compared the most important phenotype traits, which have a strong influence on yield quantity and quality, of isogenic tobacco breeding lines with different conversion ability. Plant measurements revealed that the progeny of non-converters differed significantly from the progeny of converters in five of the nine studied traits (Tab. 4). It was higher than the progeny obtained from the self-pollination of converters. However, statistically significant differences were observed only for the ZD2 breeding line, and not for the others. The comparison of leaf size showed that the non-converting lines tended to produce wider and longer leaves than the converters. However, the differences were only statistically significant for the length and width of the 9th and 15th leaf of the TNX1 and ZD2 lines.

Table 4. Plant height, number of leaves per plant, dimensions of leaves, and days to flower of the tobacco isogenic lines TNX1, ZD2, WGLB that differ in their conversion capacity

Trait	TNX1		ZD2		WGLB	
	N	C	N	C	N	C
Plant height (cm)	192.1 ±1.3^a	198.5 ±4.4^a	226.3 ±2.4^b	209.9 ±2.2^a	208.5 ±1.1^a	206.9 ±1.6^a
AW 7 (cm)	31.8 ±0.5^a	31.1 ±0.6^a	35.5 ±0.5^a	36.7 ±0.4^a	36.2 ±0.4^a	35.7 ±0.8^a
AL 7 (cm)	50.1 ±1.5^a	52.2 ±0.6^a	57.0 ±0.7^b	55.3 ±0.6^a	52.3 ±0.4^a	51.5 ±0.6^a
AW 9 (cm)	28.7 ±0.6^b	26.0 ±0.7^a	31.8 ±0.6^a	31.4 ±1.2^a	34.7 ±0.7^a	32.7 ±0.9^a
AL 9 (cm)	53.4 ±0.7^b	50.9 ±0.8^a	53.9 ±0.8^a	52.9 ±0.5^a	49.6 ±0.9^a	48.1 ±1.9^a
AW 15 (cm)	20.6 ±0.4^b	15.8 ±0.4^a	23.8 ±0.4^b	21.5 ±0.6^a	21.5 ±0.5^a	21.0 ±0.7^a
AL 15 (cm)	42.9 ±0.9^b	38.1 ±0.7^a	42.5 ±0.5^a	40.7 ±0.8^a	34.91.3^a	38.3 ±1.2^a
Flowering*	58.9 ±0.4^a	59.6 ±0.7^a	68.2 ±1.1^a	65.3 ±2.2^a	60.7 ±0.5^a	60.6 ±0.3^a
Number of leaves	22.0 ±0.3^a	22.8 ±0.3^a	23.5 ±0.3^a	23.9 ±0.3^a	19.0 ±0.7^a	20.4 ±0.3^a

N – non-converter line, C – converter line, AW7 – average width of the 7th leaf on the stalk, AL7 – average length of the 7th leaf on the stalk, * – number of days from planting into pots to beginning of flowering
Data represent mean ± (SD) of three replicates. Different letters in the row denote significant differences between isogenic breeding lines N and C, according to Tukey's test at $p \leq 0.05$

No similar patterns were found in the other breeding lines. No significant differences were found between the isogenic lines in 7th leaf size, number of leaves and flowering date. Virginia tobacco isogenic lines WGLB N and WGLB C were found to be the most homogeneous in terms of the morphological traits. The progeny of converters were on average 1.6 cm lower than the progeny of non-converter. It also produced slightly smaller leaves but the differences obtained were not statistically significant. The WGLB N and WGLB C lines reached the flowering stage at a similar time indicating similar rates of plant development and reaching maturity.

DISCUSSION

In the present study, the GC-MS technique was used to determine the alkaloid profile in the initial populations of 10 Burley and Virginia tobacco cultivars/breeding lines. On average, Burley tobacco accessions had lower nicotine content than Virginia tobacco. Most literature reports indicate that Burley cultivars generally have higher nicotine levels than Virginia cultivars [Burton et al. 1992, Siminszky et al. 2005]. This difference is likely due to genetic factors, higher nitrogen fertilization and air curing. The observed differences in the main alkaloid content between Burley and Virginia cultivars/breeding lines in our study are likely due to their origin. Burley breeding lines such as ZD2, TNX3 and TNX4 were obtained through breeding programs that did not consider the strict requirements of regulatory agencies, such as the World Health Organization [2015] and tobacco companies. They were produced by crossing 'TN90' – a moderately stable converter according to Jack et al. [2004], with local Polish Burley cultivars, unstable converters according to Berbeć [2014]. The main alkaloid found in these plants was nornicotine, with nicotine accounting for only a small proportion. In contrast, populations of Virginia cultivars and breeding lines were far less diverse in terms of major alkaloid content. Most of the accessions were characterized by a relatively high content of nicotine and a low content of nornicotine. This was achieved through a breeding process that involved identifying and removing the cherry-red individuals with high levels of nornicotine.

The results of the gas chromatography analysis revealed varying abilities of cultivars/breeding lines to convert nicotine to nornicotine. Based on this, the initial plant material was classified into three groups of converters. Four cultivars/lines with predominantly non-converting individuals were classified into group I. This group included Virginia cultivars 'WAC120' and 'WAC121', as well as Burley 'TN90' and TNX2. A comparison of the range of conversion, as well as the rate of conversion of individual plants in the initial populations and in the progeny obtained after the first year of selection, did not reveal the presence of strong or very strong converters. However, the study revealed a high proportion of non-converters in this group, which corresponded with the result previously obtained by Burk and Jeffrey [1958] in the progeny of the stable and non-converting Burley 11A as well as with the proportion of non-converters found in stable, low-converting selections of Burley tobacco cultivars 'TN90' and 'KY9' [Lewis et al. 2008]. Nevertheless, in the following years of the study, group I exhibited a reduction in the percentage of non-converters and the appearance of individuals with low (range 3–10%) and medium (range 10–30%) conversion. It is important to note that this trend was only observed in Burley accessions, as Virginia cultivars 'WAC120' and 'WAC121' consisted mostly of non-converting plants. The results are consistent with the data presented by

Gavilano et al. [2006]. They evaluated the conversion capacity of commercial tobacco cultivars and showed that up to 20% of converters may arise in a single generation from parents that are non-converters, in the case of Burley cultivars. The study and results of Jung et al. [2005] clearly demonstrate that Burley cultivars exhibit genetic instability at the nicotine conversion locus and revert to the converter phenotype. In contrast, Virginia cultivars show much greater stability in terms of conversion. Therefore, roguing using self-pollination of low-converting single-plant selections of Virginia is more effective in creating low-converter, stable selections.

The second group consisted of three breeding lines, ZD2, TNX1, and WGLB, which exhibited considerable individual variation in terms of the rate of conversion. The WGLB line showed the greatest variability in this respect. The high content of nornicotine and the significant proportion of high-converters in WGLB in 2014 were surprising results. This breeding line was produced by interspecific hybridization of *N. tabacum* 'Wiślica' and *N. glauca* [Trojak-Goluch and Berbeć 2011]. None of the parental species used for the production of WGLB showed any tendency toward conversion. The analysis of the leaf alkaloid profile of *N. tabacum* 'Wiślica', places it in the group of low converters [Berbeć 2014]. The evaluation of the *Nicotiana glauca* species indicates low nornicotine content, with anabasine being the predominant alkaloid (86.7%) [Sisson and Severson 1990]. The probable cause of the high levels of nornicotine in the WGLB line can be explained by analyzing the alkaloid biosynthesis pathway in tobacco and its wild relatives of *Nicotiana* [Lewis et al. 2015]. It was found that 3,6-dihydronicotinic acid, one of the quinolinic acid conversion products used for the production of anabasine, can also be used for the production of nicotine. The synthesis of anabasine depends on the activity of the berberine bridge enzyme-like (BBL) genes and the availability of the precursor. In the case of the initial WGLB lines having *N. glauca* in their ancestry [Trojak-Goluch and Berbeć 2011], nicotine production takes place instead of anabasine production, most likely due to a high supply of 3.6-dihydronicotinic acid and modification of the alkaloid biosynthetic pathway, which is then converted to nornicotine. It is important to note that, the selection of WGLB non-converters and, their self-pollination in the following years of the study resulted in the near-complete elimination of individuals metabolizing nicotine to nornicotine. This led to the development of a stable breeding line in terms of its alkaloid profile. Jack et al. [2005] conducted a study on the inheritance of conversion factors and the obtaining of stable non-converting populations. The analysis involved segregation of conversion factors in the F_1 and F_2 generations obtained from crosses between converters and non-converters. The majority of the F_1 population was homogeneous and strongly converting, while two of them were heterogeneous in terms of conversion, containing strongly, moderately, and weakly converting individuals. Segregation of conversion factors in a 3:1 ratio, in the F_2 generation, showed that the process of N-demethylation of nicotine is controlled by a single dominant allele and a corresponding recessive non-converting allele. The authors state that non-conversion is characterized by recessive homozygotes. In this study, almost all selected WGLB plants were classified as non-converters, indicating that the selected population is homozygous and stable.

The ZD2 and TNX1 (Burley) lines used in this study exhibited significant individual variation in their ability to convert nicotine to nornicotine. The applied technique of selection and self-pollination of non-converters in heterogeneous populations resulted in a stable generation with very low conversion capacity. The results confirm that this technique can be used to generate Burley tobacco that is almost free of nornicotine and has a slightly

reduced risk to human health. Previous studies have investigated the stability of conversion in Burley cultivars. Wernsman et al. [2000] and Jung et al. [2005] reported that selection and self-pollination of Burley non-converters resulted in reduced conversion rates in subsequent generations. The authors point out, however, the possibility of a small number of converters arising spontaneously due to the genetic instability of recessive „converter" alleles in the *Nicotiana tomentosiformis* genome (T) of tobacco. Gavilano et al. [2006] reported that up to 20% of the Burley tobacco population exhibits the metabolic pathway leading to nicotine N-demethylation in non-converter progeny.

During the experiment, a third group was identified, consisting exclusively of individuals with a high conversion rate. This group consisted of the Virginia tobacco cultivar 'Sybilla' and the two breeding lines TNX3 and TNX4 (Burley). Data analysis on the proportion of converters among these accessions revealed that 'Sybilla' was the strongest converter. In this cultivar, there were almost exclusively individuals manifesting complete conversion, indicating potential problems in minimizing the raw material's carcinogenic properties. Additionally, it has been shown that inconsistent monitoring of alkaloid levels in the plant materials used for breeding programs results in the selection of converting individuals, ultimately leading to a decrease in the quality of the raw material. Unfortunately, attempts to self-pollinate selected individuals and select non-converting plants did not yield the expected results.

A detailed analysis of the morphological traits of isogenic breeding lines that differed in conversion ability showed statistically significant differences between converting and non-converting lines. The converting ZD2 C line was significantly lower than its non-converting counterpart, ZD2 N. Additionally, the 15th leaf of the converting line was shorter and narrower. The most common response for the converting TNX1 C line was a reduction in leaf size compared to the BPTN N. There is a lack of literature reporting comprehensive characteristics of plants with different conversion capabilities. Jung et al. [2005] identified converting plants among KB9118 Burley breeding lines using a thin-layer chromatographic method. Based on the agronomic characteristics of plants, it can be concluded that converters KB9118-7, KB9118-21, and KB9118-23 had smaller and narrower leaves compared to non-converters KB9118-2 and KB9118-4. In addition, it can be concluded that some converting lines were significantly shorter than low converters. No significant differences were found in the days to flower. In contrast, Chaplin and Burk [1984] studied the agronomic and chemical characteristics of BC_5F_7 generations obtained from the crossing of three flue-cured cultivars 'Cocker139', 'NC95', 'SC58' with a low alkaloid line. They also evaluated DH lines obtained from the BC_5F_1 generation. Their field experiments showed that total alkaloid levels were not associated with days to flower, plant height flower, plant height, and number of leaves per plant. However, they observed that in populations derived from the cultivars 'NC95' and 'SC58', some lines, especially the non-converting ones (with nornicotine levels ranging from 0.02–0.08%), had statistically lower yields than the recurrent parent. Generally, lines with low nornicotine had the lowest grade index. They also produced less reducing sugars than the parental forms, but the differences were not statistically significant in this case. The authors concluded that lines with low total alkaloid levels are likely to have lower grade indexes and may be less acceptable to smokers than those with high levels. The relationship between total alkaloid content and agronomic and chemical traits of isogenic lines of flue-cured tobacco was the subject of a study by Chaplin and Weeks [1976]. The study showed that alkaloid level had little association with plant height, number of leaves per

plant, or days to flower. Additionally, the authors found that the low alkaloid lines (approximately 0.20%) had lower yield, grade index, total N, and reducing sugars content than the „normal" flue-cured cultivars. Explaining the association between low alkaloid content and reduced yield is challenging. Chaplin and Weeks [1976] suggest that tobacco leaves from the low alkaloid lines are probably thinner than leaves from the „normal" alkaloid lines. The results of our study suggest that one of the causes of reduced yield in converters may also be a reduction in leaf size. However, considerable variation in cultivar has been observed for this trait.

CONCLUSION

Taking into account the rate of conversion of nicotine to nornicotine in both Burley and Virginia tobacco cultivars/breeding lines and the harmfulness of N-nitrosonornicotine to humans, there is a strong need to systematically control the alkaloid profile of tobacco cultivars and breeding lines. By conducting a controlled elimination of converting individuals, especially within breeding lines characterized by a wide conversion range, it was possible to reduce the potentially carcinogenic compounds in tobacco leaves. As a result of the studies, three breeding lines, ZD2, TNX1 (Burley tobacco), and WGLB (Virginia tobacco), exhibiting a stable rate of nicotine conversion of <3% in successive years, were obtained. There is a relationship between the traits determining the phenotype of tobacco cultivars/lines and the content of particular alkaloids. The length and width of the 9th and 15th leaves of the non-converters were longer and wider than those of the non-converting analogues of the TNX1 and ZD2 lines. By conducting selection based on the removing from seed populations of individuals converting nicotine to nornicotine, an improvement in the technological utility of raw tobacco can be expected.

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