EXPERIMENTAL PAPER

Determination of nicotine levels in the leaves of some *Nicotiana tabacum* varieties cultivated in Syria

GHALEB TAYOUB*, HUDA SULAIMAN, MALIK ALORFI

Department of Molecular Biology and Biotechnology
AECS, Damascus
PO Box 6091, Syria*corresponding author: phone: +963 11 2132580. fax: + 963 11 6112289,
e-mail: ascientific@aec.org.sy

Summary

Introduction: Tobacco is the most widely grown non-food crop in the world. Nicotine is the most abundant volatile alkaloid in tobacco leaves. **Objectives:** This work aimed at measuring nicotine levels in the leaves of seven different varieties of *Nicotiana tabacum*, namely: Virginia, Burlip, Katrina, Shk al-bent, Zegrin, Basma and Burley, cultivated in Syria. **Methods:** Nicotine was extracted according to approved method and its concentration was determined by LC/MS/MS in comparison with a standard material dilution series. The percentage of nicotine concentration was calculated manually. Statistical analysis was used to assess the significance of differences among variables and to perform multiple comparisons. **Results:** The amount of nicotine in dry weight of tobacco leaves represented 6.7% in Virginia variety, 4.9% in Burlip, 4.84% in Katrina, 4.67% in Shk al-bent, 4% in Zegrin, 3.3% in Basma and <3% in Burley. Significant differences in nicotine concentration were found among the different varieties as determined by LSD test at a level of 0.05. **Conclusion:** This study shows the importance of tobacco varieties grown in Syria, particularly Virginia, Burlip, Katrina, as a cheap and wealthy source for nicotine to be used in some industries.

Key words: nicotine, *Nicotiana tabacum*, alkaloid, leaves, LC/MS/MS

INTRODUCTION

Tobacco is a valuable cash crop, it is the most widely grown non-food crop in the world. The leaves are the most valuable part of this plant. It is a member of

Solanaceae family, which includes many crop species like tomatoes, potatoes and peppers [1].

Within the genus of *Nicotiana*, there are more than sixty species of tobacco; among those, only *Nicotiana rustica* and *Nicotiana tabacum* are used by humans. *N. tabacum* is the principal species for commercial production of tobacco [2]. Smoking is a leading cause of mortality related to drug use worldwide. Besides nicotine, tobacco contains about 4000 substances including particles and gases, 1000 of them are produced during smoking. These include many known carcinogens in addition to carbon monoxide which impair the oxygen carrying capacity of blood [3]. Nicotine level is a key index for the evaluation of the quality of tobacco, and is closely correlated with many factors, such as tobacco species or type, soil type, nitrogen nutrition, topping height and time, degree of maturity or ripening of tobacco, curing and yellowing period of tobacco [4–8].

Nicotine, (S)3(1-methyl-2-pyrrolidinyl) pyridine is the most abundant volatile alkaloid in tobacco leaves comprising about 95% of the total alkaloid content. The primary commercial source of nicotine is its extraction from *N. tabacum* or *N. rustica*, and – in lower quantities – from tomato, potato, eggplant, and green pepper. It is also found in the leaves of the coca plant [9, 10]. The nicotine content of *N. tabacum* varieties grown commercially generally ranges from 0.3 to 3% of dry weight of the leaves, although 5% and even 7% have been reported [11].

Nicotine is a very toxic substance, and serious or fatal poisoning may occur as a result of its ingestion, inhalation or skin absorption in small amounts. The lethal dosage of nicotine is 50 mg/kg for rats and 3 mg/kg for mice. For adult humans, 40–60 mg can be a lethal dosage. This makes it an extremely deadly poison according to the Encyclopedia of Occupational Health and Safety [12] as well as to Keith and Chinn [13]. Nicotine acts on nicotinic cholinergic receptors, affecting most of body organs, and is a highly addictive drug. Nicotine normally makes up about 5% of a tobacco plant by weight. Each cigarette contains 8 to 20 mg of nicotine (depending on the brand), but only 1 mg is actually absorbed by the human body [14].

Many HPLC methods have been described for the determination of nicotine levels in pharmaceutical formulations [15]. HPLC equipped with tandem mass spectrometry methods were used for measuring nicotine levels in biological samples [16,17], while GC/MS methods were used for the determination of nicotine levels in tobacco leaves [18].

Tobacco is cultivated in Syria as an economic crop. The estimated cultured area is 15 817 hectare with an annual production of about 11 117 tons of tobacco [19]. However, no data is available about nicotine levels in tobacco varieties cultivated in Syria. This study is to report nicotine concentrations in the dried leaves of seven of the most popular varieties locally cultivated.

MATERIAL AND METHODS

Sampling

Leaf samples of seven different varieties of tobacco (*Nicotiana tabacum*) cultivated in coastal region of Syria (Lattakia region), namely: Virginia, Burlip, Katrina, Shk al-bent, Zegrin, Basma and Burley, were collected in the harvest season of 2013. The varieties were documented by the General Organization of Tobacco (GOT). Whole leaves were dried and ground to fine powder, (to 60 mesh size), and stored at -20°C for analysis.

Sample preparation

The samples were extracted according to Vlase *et al.* [14] with some modifications. Briefly, about 50 mg dried tobacco leaves were accurately weighted and introduced into a 15 ml centrifuge tube. 2 ml of H₂SO₄ (0.1 M solution), were added and the mixture was kept in ultrasonic bath for 10 s. Then, the centrifuge tube was incubated in a water bath at 70°C for 10 min. 50 µl of extract were diluted in 1 ml with mobile phase and centrifuged for 6 min at 5000 rpm. Then, 50 µl of supernatant were diluted in 1 ml mobile phase. 3 µl from the final solution were injected in the LC-MS/MS system.

Chemicals, standards, solution analysis

A 98% pure nicotine standard material was purchased from Sigma-Aldrich. Acetonitrile Lichrosolv, methanol Lichrosolv, formic acid 98% and sulfuric acid (0.1 M) were purchased from Merck. A Stock solution of nicotine (10 mg/ml) and intermediate solution 1 µg/ml were prepared in methanol. Four work solutions levels (100, 250, 500 and 1000 ng/ml) were obtained by diluting appropriate volumes of intermediate solution with methanol.

Apparatus and chromatographic conditions

The LC-MS/MS system from Agilent technologies (1260 series LC and 6410 series Triple Quad LC/MS) was used in samples analysis. Analytical column was Eclipse Plus C₁₈, 3.5 µm, 4.6x100 mm obtained from Agilent. The mobile phase was a mixture 80:20 (v/v) of methanol:water acidified with 2% formic acid. The flow rate was 0.3 ml/min and the injection volume of 3 µl. The mass analyzer operated with an ESI source, ion mode: positive, V cap: 4000 V, nebulizer: 30 psi, drying flow gas:

8 l/min, drying gas temperature: 250°C. The precursor to product ion transitions of 163>132, Fragmentor :120, Collision energy (CE):15V.

STATISTICAL ANALYSIS

Data were expressed as mean ± standard deviation (SD). The percentage of nicotine concentration was calculated manually. One way analysis of variance (ANOVA) was used to assess the significance of differences among variables. SPSS software (v. 17) was used to perform multiple comparison test by applying the least significant difference LSD at *p* values less than 0.05. Microsoft Excel was also used to generate statistical histograms.

Ethical approval: The conducted research is not related to either human or animal use.

RESULTS AND DISCUSSION

LC-MS/MS results of nicotine concentrations in the leaf extracts of the seven tobacco varieties are shown in table 1. Typical HPLC chromatograms of chemical standards and Basma tobacco leaves are shown in Figure1. Retention times of A and B were 3.1 min. In the range of 100–1000 µg/ml, good correlation of linearity has been achieved (n=3; R²=0.9999).

Table 1.

Nicotine content in tobacco varieties studied [mg/g]

Nicotine concentration	Varieties
67.27 ± 1.21 (6.7%)	Virginia
49.7 ± 1.23 (4.9%)	Burlip
48.37 ± 0.7 (4.84%)	Katrina
46.7 ± 1.15 (4.67%)	Shk al-bent
40.23 ± 1.6 (4%)	Zegrin
33.27 ± 1.83 (3.3%)	Basma
28.64 ± 1.22 (2.86%)	Burley

Values are the means ±SD (n=3).

Figure 1.

Typical HPLC chromatograms of chemical standards and sample. A: chemical standards for nicotine; B: Chromatogram of nicotine extracted from Basma tobacco leaves ($t_R = 3.1$ min)

Using this analytical method, nicotine content, expressed as mg nicotine/g dry weight of tobacco, was measured in 21 samples, three replicates for each variety. The mean nicotine concentration of three replicates and the percentage

of nicotine content for each variety is presented in table 1. Results showed that amount of nicotine in dry weight of tobacco leaves represented 6.7% in Virginia variety, 4.9% in Burlip, 4.84% in Katrina, 4.67% in Shk al-bent, 4% in Zegrin, 3.3% in Basma and <3% in Burley. Total nicotine concentration was quite variable among the different varieties analyzed as indicated by LSD test ($p < 0.05$).

The comparison of our results with those reported by other authors they were different. For example: the mean concentration of nicotine in whole leaves of Virginia variety was significantly higher than the mean concentration recorded in the same variety cultivated in Italy (3.01%), China (2.998%), India (2.979%), Brazil (3.122%), Zimbabwe (3.172%), and Ethiopia (3.26%) according to the reports issued in the years 1995, 1998, 1999, 2001, 2006 and 2007 [20]. This increase might be due to the differences in the localization of leaves on the plant stem. It seems that the apical leaves contain higher concentrations of nicotine as demonstrated by Tasew [20] who measured a 5.03% level of nicotine in those leaves.

In another study by Hossain and Salehuddin [18] it was found that the nicotine concentration in leaves of some Bangladesh tobacco varieties ranged between 0.9–3.6%, and the highest concentration (3.6%) was in Halsha, Kushtia variety. In a study by Tasew [20], nicotine concentration represented 0.65% in Burly variety and 1.11% in Gaya variety. Differences found are most likely due to the kind of variety, soil type, nitrogen nutrition, topping height and time, degree of maturity or ripening of tobacco, curing and yellowing period of tobacco. These prospects were confirmed by other researchers [4–8].

This study shows the importance of tobacco varieties grown in Syria, particularly Virginia, Burlip, Katrina, which are cheap and wealthy source for nicotine to be used in some industries.

ACKNOWLEDGEMENTS

The authors would like to express their thanks and gratitude to Professor Ibrahim Othman, General Director of Atomic Energy Commission of Syria and Professor Nizar Mir Ali for support and encouragement.

Conflict of interest: Authors declare no conflict of interest.

REFERENCES

1. Wendie H, Mickel WK. Handbook of cultural practice for flue-cured tobacco, Unpublished, 1973; 1-3.
2. Benowitz NL. Pharmacology of nicotine: addiction and therapeutics. *Ann Rev Pharmacol Toxicol* 1996; 36:597-613.
3. Shekhar S, Hem RP, Atul A. Alcohol and Drug Abuse, New age International P. Ltd. 2003:69-73.
4. Tso TC, Jeffrey RN. Studies on tobacco alkaloids. II. The formation of nicotine and nor-nicotine in tobacco supplied with N15. *Plant Physiol* 1956:86-93.

5. Tso TC. Physiology and biochemistry of tobacco plants. Plant Science Research Division U.S. Department of Agriculture, Dowden, Hutchinson and Ross, Inc. Beltsville Maryland USA, 1972.
6. Hawks SNJ. Principle of Flue-cured tobacco production, 2nd ed., John Wiley and Sons, 1978:32-35.
7. De Roton C, Tan Cogne J, Bazin R. Agro-Phyto Groups, Montreux, AP3. Effect of maturity on the leaf characteristics of flue-cured varieties K 326 and ITB 31612. CORESTA Meet, SEITA, Institut du Tabac, Bergerac, France 1997.
8. Tsotsolis NC, Kanellopoulos GP. Nitrogen fertilization trials for flue-cured tobacco. CORESTA Meet. Agro-Phyto Groups, Montreux, AP33. Tobacco Institute of Greece, Drama, Greece 1977.
9. Andersson C, Wennström P, Gry J. Nicotine alkaloids in solanaceous food plants. Tema Nord 2003; 531:1-37.
10. Hukkanen J, Jacob P, Benowitz NL. Metabolism and disposition kinetics of nicotine, Pharmacol Rev 2005; 57(1):79-115.
11. Blakely T, Bates M. Nicotine and Tar in cigarette tobacco: a literature review to inform policy development, A report for the Ministry of Health of New Zealand, Institute of Environmental Science and Research Limited (ESR), 1998.
12. Encyclopedia of Occupational Health and Safety: International labor office, Geneva, 3rd ed. 1983; 2:1440.
13. Keith AT, Chinn BA. Nicotine. Drugs and the brain, California State University, USA, 1996.
14. Vlase L, Filip L, Mîndruțau I, Leucuța SE. Determination of nicotine from tobacco by LC–MS–MS. Studia Universitatis Babes-Bolyai Physica 2005; 4:19-24.
15. Tambwekar KR, Kakariya RB, Garg S. A validated high performance liquid chromatographic method for analysis of nicotine in pure form and from formulations, J Pharm Biomed Ana 2003; 32:441-450.
16. Tuomi T, Johnsson T, Reijula K. Analysis of nicotine, 3-hydroxycotinine, cotinine, and caffeine in urine of passive smokers by HPLC-tandem mass spectrometry. Clin Chem 1999; 45:2164-2172.
17. Byrd GD, Davis RA, Ogden MW. A rapid LC-MS-MS method for the determination of nicotine and cotinine in serum and saliva samples from smokers: validation and comparison with a radioimmunoassay method. J Chromatogr Sci 2005; 43:133-140.
18. Hossain AM, Salehuddin SM. Analytical determination of nicotine in tobacco leaves by gas chromatography–mass spectrometry. Arabian J. Chem 2013; 6:275-278.
19. Ministry of Agriculture. Annual Agricultural statistical Abstract (<http://moaar.gov.sy/main/archives/12850>) 2013.
20. Tassew Z. Levels of nicotine in Ethiopian tobacco [dissertation] School of Graduate Studies. Addis Ababa University 2007:48-50.

OZNACZENIE ZAWARTOŚCI NIKOTYNY W LIŚCIACH NIEKTÓRYCH ODMIAN *NICOTIANA TABACUM* UPRAWIANYCH W SYRII

GHALEB TAYOUB*, HUDA SULAIMAN, MALIK ALORFI

Department of Molecular Biology and Biotechnology
AECS, Damascus
PO Box 6091, Syria

*autor, do którego należy kierować korespondencję: tel.: +963 11 2132580,
faks: + 963 11 6112289, e-mail: ascientific@aec.org.sy

Streszczenie

Wstęp: Tytoń jest najczęściej uprawianą niejadalną rośliną na świecie. Głównym lotnym alkaloidem występującym w liściach tytoniu jest nikotyna. **Cel:** Celem pracy było oznaczenie zawartości nikotyny w siedmiu różnych odmianach *Nicotiana tabacum* uprawianych w Syrii: Virginia, Burlip, Katrina, Shk al-bent, Zegrin, Basma i Burley. **Metody:** Ekstrakcję nikotyny prowadzono według obowiązujących metod, a jej stężenie określono za pomocą LC/MS/MS w odniesieniu do wzorca. Procentowe stężenie nikotyny zostało obliczone ręcznie. Analizy statystycznej użyto do określenia istotności różnic zmiennych i dla porównań wielokrotnych. **Wyniki:** Zawartość nikotyny w suchej masie liści tytoniu wynosiła 6.7% w odmianie Virginia, 4.9% w Burlip, 4.84% w Katrina, 4.67% w Shk al-bent, 4% w Zegrin, 3.3% w Basma i <3% w Burley. Istotne różnice w stężeniu nikotyny u różnych odmian zostały określone za pomocą testu LSD na poziomie 0,05. **Wnioski:** Otrzymane wyniki wskazują, że odmiany tytoniu uprawiane w Syrii, zwłaszcza Virginia, Burlip i Katrina mogą być tanim i bogatym źródłem nikotyny stosowanej w różnych gałęziach przemysłu.

Słowa kluczowe: nikotyna, *Nicotiana tabacum*, alkaloid, liście, LC/MS/MS