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Original article

Reduced N supply limits the nitrate content of flue-cured tobacco

Fabio CASTELLI¹, Enrico CEOTTO^{2*}, Renato CONTILLO³

¹ CRA-CAT, Unità di ricerca per le colture alternative al tabacco, via Canton 14, 37051 Bovolone (VR), Italy

² CRA-CIN, Centro di ricerca per le colture industriali, via di Corticella 133, 40128 Bologna, Italy

³ CRA-CAT, Unità di ricerca per le colture alternative al tabacco, via Vitiello 108, 84018 Scafati (SA), Italy

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Abstract – Among tobacco components, nitrate is one of the most harmful for human health because during tobacco burning nitrate reacts with alkaloids to form tobacco-specific nitrosamines. Nitrosamines are carcinogenic compounds identified in tobacco products and smoke. Despite a general consensus on the relevance of nitrate on human health, nitrate is currently not considered in the routine analysis of flue-cured tobacco, unlike total alkaloids, reducing sugars, total nitrogen, pesticides and chemicals for sucker control residues. In implementing strategies for tobacco cultivation, it is critical to minimize the nitrate content of the harvested product. On the other hand, the agronomic practices devised to reduce nitrate content should not adversely impact either the yield or commercial characteristics of the product. We carried out a field experiment over three years at one site of the Veneto plane, Northern Italy. We studied the influence of nitrogen supply on productivity and relevant chemical characteristics of flue-cured tobacco. Treatments consisted of four rates of nitrogen supply and one unfertilized control. The rates of nitrogen applied were 0, 15, 30, 45 and 60 kg N ha⁻¹ in 1997, and 0, 20, 40, 60 and 80 kg N ha⁻¹ in both 1998 and 1999. Our results show that annual fertilization rates within 40 kg N ha⁻¹ fulfilled the crops' requirements for productivity, with no adverse effects on product quality. Higher rates of nitrogen supply resulted in undesired increment in nitrate concentration and reduced sugar content in the cured product, without any gain in productivity. Moreover, we found no correlation between total nitrogen and nitrate concentrations of tobacco cured leaves. Since our data provide evidence that nitrogen content is not a reliable estimator of the harmful nitrate content, we suggest that nitrate should be taken under control in the routine analysis for assessing flue-cured tobacco quality.

product quality / harmful compounds / productivity / nitrate / cancer / nitrosamine / tobacco

1. INTRODUCTION

Nitrogen is often the most important growth-limiting factor for all crop plants. Thus, nitrogen fertilizers are commonly applied to increase crop yield and quality. Most of the nitrogen absorbed by the plants is incorporated into protein, while a fraction remains in the form of non-protein nitrogen, mainly amino acids and nitrate (Blumenthal et al., 2001).

When input of nitrogen exceeds the plant's needs, nitrate tends to accumulate in the leaves (Cardenas-Navarro et al., 1999). High nitrate accumulation in plants, notably in fresh vegetables, is a general concern for its human health implications (Anjana and Iqbal, 2006). Unlike food and feed crops, tobacco typically produces leaves used for smoking, and this implies specific concern for human health. In both plants and animals, nitrate can be reduced to nitrite, by which nitrosation reactions produce carcinogenic N-nitroso compounds (Wolfe and Patz, 2002). The so-called tobacco-specific N-nitrosamines are recognized to have special significance

among the class of carcinogenic compounds identified in tobacco and in the smoke of tobacco (Hoffmann and Hoffmann, 1997). Green and freshly harvested tobacco leaves usually contain nitrate but are virtually free of tobacco-specific N-nitrosamines. It is during the post-harvest processing, and also during tobacco combustion, that nitrate is reduced to nitrite which then reacts with tobacco alkaloids to form tobacco-specific N-nitrosamines (Nestor et al., 2003). High nitrate content increases the generation of nitrogen oxides (NO_x) that, in turn, enhance the formation of tobacco-specific N-nitrosamines. In fact, the concentration of nitrogen oxides in tobacco smoke is predominantly given by the nitrate content, although a secondary fraction is formed from amino acids and proteins during smoking (Hoffmann and Hoffmann, 1997; Norman et al., 1983). Investigations carried out on a vast collection of cigarette brands, ranging from low to very high contents of both nitrate and tobacco-specific N-nitrosamines, concluded that nitrate concentration is linearly related to tobacco-specific N-nitrosamine content (Fischer et al., 1989; Hoffmann and Hecht, 1985; Hoffmann et al., 1997). Thus, a

*Corresponding author: enrico.ceotto@entecra.it

possible avenue for reducing nitrosamine levels in cigarette smoke is to reduce nitrate content in tobacco leaves (Hoffmann and Hecht, 1985).

However, an additional aspect merits consideration. Tobacco is a non-essential good, and its primary scope is to meet consumer satisfaction. The quality assessment for flue-cured tobacco in objective terms is currently based on the concentrations of leaf total alkaloids, reducing sugars, total nitrogen and their balance (Akehurst, 1981; Flower, 1999; Hawks and Collins, 1983). Therefore, any fertilization strategy intended to control nitrate levels should take into account possible detrimental effects on the relevant components.

The objectives of the present study were: (i) to evaluate the effect of nitrogen fertilization on yield, nitrate content and relevant chemical traits of flue-cured tobacco; and (ii) to assess the relationship between total nitrogen and nitrate content in tobacco leaves.

2. MATERIALS AND METHODS

2.1. Site characterization and agronomic details

A field experiment was conducted from 1997 to 1999 at the Experimental Farm of CRA, located in Bovolone (Verona), Northeast Italy (Lat. 45° 16' N, Long. 11° 07' E). The soil of the site was classified (Costantini et al., 2002) as coarse-loamy, mixed, mesic, superactive Fluventic Haplustept (Soil Survey Staff, 1999). The location is representative of a vast area of flue-cured tobacco cultivation in Northeast Italy. Five levels of nitrogen fertilization were applied each year to flue-cured tobacco (cultivar K 326). In the year 1997 the treatments were named N0, N15, N30, N45 and N60, in kg N ha⁻¹. After evaluation of the 1997 results, in both 1998 and 1999 the nitrogen rates were slightly increased, so that treatments were named N0, N20, N40, N60 and N80, in kg N ha⁻¹. The nitrogen fertilizer was applied as calcium nitrate, in one localized application, two weeks after the transplanting of the crop. No phosphorus fertilizer was applied due to the abundance in the soil. The potassium was applied before transplanting at the uniform rate of 250 kg ha⁻¹ as potassium sulfate. For each year, a preceding crop of barley was harvested in July, and the soil remained fallow until tobacco transplantation. The crop was transplanted in mid-May; precisely, 15 May 1997, 13 May 1998 and 14 May 1999, by using a transplanting machine. A weed chemical control (Pendimethalin+Metobromuron+Metalachlor) was applied in May before transplanting. Topping was performed in the second half of July at the full flowering stage, followed by three applications of chemicals for sucker control (two treatments with fatty alcohol, one with maleic hydrazide). Chemical treatments to control aphids and blue mold (Imidacloprid or Acephate, and Metalaxyl) were applied before transplanting and in the field. Sprinkler irrigation, with volumes of 20 mm, was applied during the growing season. This modest amount was imposed by the low infiltration capability of the soil. Total irrigation amounts were 220 mm in 1997, 250 mm in 1998 and 158 mm in 1999.

Following the usual practice of the region, leaves were harvested, when ripe, in four primings, starting from the end of July until the end of October. The experimental design was a randomized block with two replications for a total of 10 plots. The dimension of an individual plot was 200 m², the distance between the rows was 1 m and between plants within rows 0.45 m, corresponding to 444 plants per plot (22 222 plants per ha). The experiment was specifically designed for collecting frequent destructive plant samples throughout the growing season. Therefore, priority was given to big plots at the expense of number of replications.

2.2. Crop measurements

Crop measurements were performed on two sampling scales: (i) a larger area, corresponding to one plant row in the central area of each plot, including 50 individuals, equivalent to 22.5 m², was collected, on the mentioned four crop harvest dates per year, aiming to measure cumulated yield and nitrate, total nitrogen, reducing sugars and total alkaloids concentration in cured leaves; (ii) a smaller sample of two plants per plot, corresponding to 0.9 m², was collected every week, starting in early June, with the scope of measuring the time courses of nitrate and total nitrogen content of leaves. In each individual plot a two-row buffer zone was excluded from sampling.

On the higher sampling scale, for each harvest the fresh leaves from the selected crop row were gathered and flue-cured on curing cells based on the system DELTA-T, in which humidity, temperature and air flux are constantly regulated in order to enhance the quality of the tobacco. Samples of the cured product were oven-dried at 65 °C for 48 h to determine dry weight, and subsequently, sub-samples were collected for laboratory analysis: nitrate, total nitrogen, total alkaloids, and reducing sugars contents. On the lower sampling scale, the leaves of the two collected plants were detached and oven-dried at 65 °C for 48 h. Subsequently, sub-samples were selected for laboratory analysis to determine nitrate and total nitrogen contents over time. It is important to state that the small-sampling area was treated with the same agronomic management as the rest of the plot, including the four subsequent harvests.

2.3. Chemical analysis

Nitrate, total nitrogen, total alkaloids and reducing sugars contents were determined by colorimetric methods with a continuous flow automatic chemical analyzer (Technicon AutoAnalyzer, Technicon Industrial System, Tarrytown, NY 10591). Total nitrogen was determined as ammonia, after Kjeldhal digestion of tobacco powder, by the Berthelot reaction; ammonia reacts with sodium salicylate in the presence of hypochlorite to form a blue indophenol. Nitrate, free ammonia, total alkaloids and total reducing sugars were extracted in a 5% acetic acid solution. Nitrate was analyzed after reduction to nitrite by hydrazine sulfate and Cu²⁺ followed by formation of a red azo compound via diazonium salt. Free ammonia was determined using the same method for total nitrogen,

without mineralization. Total alkaloids were determined after a reaction with on-line-generated cyanogen chloride and sulfanilic acid to form a yellow compound (Lidzey and Savage, 1986, modified by Contillo). Reducing sugars were analyzed through the formation of a yellow complex after a reaction with p-hydroxybenzoic acid hydrazide (Tonini et al., 1981).

2.4. Statistical analysis

Data of the year 1997 were analyzed separately, because of the specific treatments applied in the first year. Combined analyses were performed for the years 1998 and 1999, since common N rates were applied and no interactions between treatments and years arose. A combined analysis of variance and appropriate F tests were performed using the restricted maximum likelihood (REML) estimation method in the Proc Mixed procedure of SAS (Littell et al., 1996). The auto-correlation of subsequent harvests, applied to the same plants sampled in the large area with the intent of measuring dry matter yield, nitrate, total nitrogen, reducing sugars and total alkaloids concentrations of cured leaves, was taken into account using the REPEATED statement. The REPEATED statement was also used to consider the time and space auto-correlation of the smaller samples of two plants collected weekly with the intent of measuring the time course of total nitrogen and nitrate content of leaves. Denominator degrees of freedom were calculated using the Kenward-Roger approximation method to determine appropriate degrees of freedom. Mean separation comparison was performed by computing least-squares means through the LSMEANS SAS statement. Effects were considered significant at $P < 0.05$.

With the intent of evaluating whether, and how, the total nitrogen is correlated to nitrate content the linear regression was calculated, using the PROC REG procedure of the SAS system (SAS, 1989). As a first step we calculated the linear regression over a large data set, including all the weekly sampling of the leaves, managed in individual plots during the growing seasons of the three years of the experiment ($n = 1058$).

Aiming to evaluate characteristics of the commercial product, the linear regression between nitrogen and nitrate content was also evaluated for the harvested and flue-cured leaves ($n = 119$). It is important to reiterate here that each experimental plot was harvested four times per year, and the commercial product was flue-cured.

3. RESULTS AND DISCUSSION

3.1. Climatic conditions

An overview of the prevailing climatic conditions throughout the growing seasons of the years 1997, 1998 and 1999 is reported in Figures 1 and 2, where daily average air temperature and cumulated precipitation are shown, respectively. The most important climatic difference among years was the amount and distribution of rainfall, which might have determined differentiated conditions of soil water content, nitrogen mineralization and nitrate leaching. Overall, the three

Figure 1. Time course of daily average air temperature for the three years of the experiment.

Figure 2. Daily values of cumulative precipitation for the three years of the experiment.

subsequent years of the experiment were representative of well-articulated growing seasons. In particular, the climatic conditions during spring 1997 were favorable for a massive aphid attack, which occurred in the whole region during the early phase of tobacco growth. Despite the chemical treatment against aphids normally applied, such an unusual aphid attack resulted in a widespread diffusion of tobacco viruses, affecting the crop for thousands of hectares in the region (European Commission, 2000; Forcato, 1997).

3.2. Productivity and nitrate concentration

The annual dry matter yield, nitrate, total alkaloids, reducing sugars and total N concentrations in cured leaves for individual treatments and the 3 years of the field experiment are

Table I. Analysis of variance and mean values of yield, nitrate concentration, total alkaloids, reducing sugars, and total nitrogen in tobacco cured leaves. Separate analyses were carried out on 1997 data, due to the different amount of N supplied to the crops with respect to 1998 and 1999.

Year	Effects	N rates	Yield DM (Mg ha ⁻¹)	NO ₃ -N (g 100 g ⁻¹)	Total alkaloids (g 100 g ⁻¹)	Reducing sugars (g 100 g ⁻¹)	Total N (g 100 g ⁻¹)
Analysis of variance							
1997	N rate		NS	NS	NS	NS	NS
1998–99	N rate		NS	***	NS	**	NS
	Year		*	***	NS	***	NS
	Year x N rate		NS	NS	NS	NS	NS
Comparisons among N rates							
1997		N0	1.95 ab	0.4 a	3.0 a	6.8 a	4.2 a
		N15	2.46 a	0.8 ab	3.4 a	7.0 a	4.6 a
		N30	2.17 ab	0.7 ab	3.4 a	6.2 a	4.7 a
		N45	1.31 b	0.8 ab	3.0 a	4.9 a	4.4 a
		N60	2.00 ab	1.1 b	3.3 a	6.8 a	4.8 a
		Means	1.98	0.8	3.2	6.3	4.5
1998–99		N0	3.75 a	0.3 a	2.4 a	10.9 a	2.3 a
		N20	3.95 a	0.4 a	2.4 a	10.3 ab	2.3 a
		N40	4.08 a	0.7 b	2.6 a	8.5 bc	2.6 a
		N60	3.98 a	1.0 bc	2.5 a	7.5 cd	2.7 a
		N80	3.85 a	1.2 c	2.6 a	6.6 d	3.0 a
		Means	3.92	0.7	2.5	8.8	2.6
1998		Means	4.10 a	0.3 a	2.8 a	4.8 a	2.9 a
1999		Means	3.74 b	1.1 b	2.2 a	12.7 b	2.2 a

NS, *, ** and *** refers to not significant at the 0.05, and significant at the 0.05, 0.01 and 0.001 probability levels, respectively. Mean comparisons: means followed by the same letter are not significantly different at the 0.05 probability level. DM: Dry Matter.

reported in Table I. These data represent the cumulative, integrated values of four subsequent harvests performed throughout each individual growing season. Overall, both productivity and nitrate concentration were strongly influenced by the growing seasons. The year 1998 was characterized by the highest productivity, +107% compared with 1997 and +10% compared with 1999, respectively. The highest productivity of the year 1998 was associated with the lowest nitrate concentration. Compared with 1998, the average nitrate content was +166% in 1997 and +266% in 1999.

Under our experimental conditions, the rates of nitrogen supplied had no significant effect on crop yield. Conversely, the effect of fertilization treatments in increasing nitrate content was quite evident. The rate N60 significantly increased nitrate content compared with N0 in 1997, while in both 1998 and 1999 the rate N40 significantly increased nitrate content compared with N0.

3.3. Alkaloids content

No significant effect on total alkaloids was found in the present experiment. However, the year 1997 was characterized by the highest average value of total alkaloids, +14% compared with 1998 and +45% compared with 1999, respectively. The highest content of alkaloids observed in 1997 is in good

agreement with Latorre et al. (1984), who indicated that nicotine content in tobacco is increased by virus infections. However, the concentrations of alkaloids observed are within the normal range, from 1.5 to 4.5 g 100 g⁻¹, reported for flue-cured tobacco by Fisher (1999).

Our data indicate that alkaloids content is not significantly influenced by nitrogen fertilization. Our findings are in accordance with Chamberlain and Chortyk (1992) who observed, in an experiment with nitrogen rate applications ranging from 0 to 300 kg N ha⁻¹, little influence of nitrogen fertilization on alkaloids content. In contrast, Elliot and Court (1978), who applied fertilization rates ranging from 0 to 60 kg N ha⁻¹, observed an increment in total alkaloids leaf content of +36% for the higher rate compared with the control.

From the standpoint of tobacco smokers the alkaloids play an extremely important role in tobacco quality because they provide a physiological stimulus that makes the use of tobacco products pleasurable to the smoker (Bush, 2000). Normally, nicotine accounts for 93–95% of total alkaloids (Tso, 1990).

3.4. Reducing sugars content

Both years and nitrogen rates determined significant effects on reducing sugars concentration.

The highest average concentration of reducing sugars was observed in 1999, +165% compared with 1998 and +102%

compared with 1999. In the years 1998 and 1999 nitrogen rates from N40 to N80 determined an undesired reduction of reducing sugars concentration. Hence, the effects of raising nitrate content were coupled with a reduction of reducing sugars in 1998 and 1999. We reiterate that the content of reducing sugars is considered an important quality trait for flue-cured tobacco, because of its influence on the characteristic sweet aroma of tobacco smoke. In fact, in blends with other tobacco types, the flue-cured component is used to enhance this characteristic (Peedin, 1999).

3.5. Total nitrogen content.

According to Akehurst (1981) and Hawks and Collins (1983), the flavor and pleasing qualities of the taste of smoke are directly correlated with the content of nitrogen. The higher the total nitrogen, the stronger and more pungent the taste will be. Conversely, if the content of nitrogenous constituents is too small the smoker regards the smoke as being flat and lacking pleasing qualities and flavor.

No significant effect on total nitrogen content was found in this experiment. However, higher values of total nitrogen were observed in the year 1997, +55% compared with 1998 and +104% compared with 1999. The rate of nitrogen fertilization had little if any effect on total nitrogen concentrations. As previously stated, the detrimental effects were to increase the nitrate concentration while decreasing reducing sugars content.

3.6. Time course of nitrate concentration in tobacco leaves during the growing season

Additional understanding of tobacco response to nitrogen fertilization can be gained from the time course of nitrate content of tobacco leaves during the growing season. These data were collected weekly on the smaller sampling area of each experimental plot for the three years of the experiment and provided an extensive data set. In Table II the analysis of variance, the mean values for each individual treatment, the intercept and the slope of the regression lines calculated between time, expressed in weeks after transplant, and the nitrate concentration of individual treatments are reported. These data confirm that nitrogen supply had a pronounced effect on nitrate content: in 1997 the treatments N15 and N30 were already different from N0, and the treatments N45 and N60 were different from both N15 and N30; in 1998–1999 the treatments N40, N60 and N80 were different from N0, and N60 and N80 were also different from N20. Overall, statistical differences among individual treatments are more evident in this data set, with respect to the results shown in Table 1, and this was likely due to the higher number of observations.

The slopes of the regression lines are negative for all treatments, and this indicates that nitrate content always decreased over time (Fig. 3). However, the interaction N rate x week was not significant, and this implies that individual regression has similar slopes. Hence, differences among treatments are attributable to differences in intercept, i.e. nitrate content at the outset of the growing season induced by rate of fertilization.

Table II. Analysis of variance and mean values of nitrate concentration in tobacco dried leaves collected during the growing seasons. Separate analyses were carried out on 1997 data, due to the different amount of N supplied to the crops with respect to 1998 and 1999.

Year	Effects	N rates	NO ₃ -N (g 100 g ⁻¹)		
Analysis of variance					
1997	N rate		***		
	N rate x week		NS		
1998–99	N rate		***		
	N rate x week		NS		
	Year		NS		
	Year x N rate		NS		
Comparisons among N rates					
			mean	intercept	slope
1997		N0	0.8 a	1.10 ***	–0.04 *
		N15	1.1 b	1.44 ***	–0.04 **
		N30	1.0 b	1.46 ***	–0.05 **
		N45	1.3 c	1.90 ***	–0.07 ***
		N60	1.3 c	1.91 ***	–0.07 ***
		Mean	1.1		
1998–99		N0	0.4 a	0.77 ***	–0.04 NS
		N20	0.7 ab	1.12 ***	–0.05 **
		N40	0.9 bc	1.26 ***	–0.04 *
		N60	1.1 c	1.42 ***	–0.04 *
		N80	1.2 c	1.47 ***	–0.03 *
1998		Mean	0.5 a		
1999		Mean	1.2 a		

NS, *, ** and *** refers to not significant at the 0.05, and significant at the 0.01 and 0.001 probability levels, respectively. Means followed by a common letter are not significantly different at the 0.05 probability level.

This is consistent with the general knowledge that solar irradiance reduces nitrate concentration (Cardenas-Navarro et al., 1999). Our data indicate that nitrogen fertilization, applied two weeks after transplant, sharply increased the nitrate concentration of the leaves at the outset of the growing season. Because the nitrate content decreased in the subsequent part of the growing season for all treatments, the initial effect was never cancelled, and at the end of the crop cycle the differences in nitrate concentration induced by fertilization still remained.

3.7. Nitrate and total nitrogen content relationship

In Figure 4 the linear regression between total nitrogen and nitrate content in the dried leaves is shown. These data refer to samples collected weekly during the growing season. Overall, the linear regression explains only 30% of the observed variability. In fact, high nitrate content is often associated with low total nitrogen content and vice versa. Hence, total nitrogen can be considered as a poor predictor of the nitrate content of tobacco leaves throughout the growing season. However, one might notice that leaves of flue-cured tobacco are harvested, normally on 4 subsequent dates, during the final part of the growing season, when nitrate content normally declines.

Figure 3. Lines of best fit for the relationship between time and nitrate content in the leaves for individual fertilization treatments applied in the year 1997 and in the subsequent years 1998 and 1999.

Moreover, the product used for smoking is flue-cured, and this treatment could further influence the nitrate and nitrogen content. Consequently, it is appropriate to consider the relationship between total nitrogen and nitrate concentration in the harvested and cured product. In Figure 5 the linear regression between total nitrogen and nitrate content in the harvested and flue-cured product is shown. We found no significant relationship between total nitrogen and nitrate concentration.

4. CONCLUSION

Under our environmental conditions fertilization rates within 40 kg N ha^{-1} fulfilled the crop's need for productivity with no adverse impact on product quality. This fertilization level should not be exceeded because higher rates of nitrogen supply resulted in undesired increment in nitrate concentration and reduced sugar content in the cured product, without any gain in productivity. The level of N supply that should not be exceeded might be different under diverse climatic and soil fertility conditions. Therefore, it is crucial to take the content of

Figure 4. Relationship between leaf nitrate and total nitrogen content in tobacco leaves. Data refers to dried leaves sampled throughout the growing season. $\text{N-NO}_3 = -0.27^{***} + 0.33^{***} \text{ Total N}$; $R^2 = 0.30$, $n = 1055$.

Figure 5. Relationship between leaf nitrate and total nitrogen content in tobacco leaves. Data refers to the harvested and flue-cured product. $\text{N-NO}_3 = 0.68^{***} + 0.014^{\text{NS}} \text{ Total N}$; $R^2 = 0.001$, $n = 119$.

harmful nitrate under control. Our data provide evidence that total nitrogen is not correlated with the harmful nitrate content in the flue-cured product. The routine analysis of commercial flue-cured tobacco currently determines total alkaloids, reducing sugars, total nitrogen, pesticides and chemicals for sucker control residues, but not nitrate content. Because total nitrogen is not a reliable estimator of nitrate, we suggest that nitrate content should be included in the analysis for assessing tobacco quality.

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