Using a Chlorophyll Meter to Evaluate the Nitrogen Leaf Content in Flue-Cured Tobacco (*Nicotiana tabacum* L.)

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Abstract

In flue-cured tobacco N fertilizer is commonly applied during pre-planting, and very often applied again later as a growth-starter. It is generally held that the efficiency of N-fertilizer use can be improved by evaluating the leaf N-status after transplanting and until flowering stage. N use efficiency in this context does not refer merely to the yield but also to the quality, in the meanwhile minimizing the negative effects on the environment. To investigate these aspects, we evaluated the capacity of a Minolta model SPAD-502 chlorophyll meter to estimate the N-status in flue-cured tobacco. The aims was to verify if a relationship exists between SPAD readings and leaf N content, and if a single leaf, in a well defined stalk position, could represent the nitrogen content of the whole plant. During the years 1995 and 1996, a pot experiment was conducted using two flue-cured tobacco varieties. SPAD values, total chlorophyll, total N contents and leaf area were measured throughout the growing season, on each odd leaf stalk position. SPAD values were well-correlated with both total chlorophyll and total N leaf concentration, and the regression coefficients were higher when relationships were calculated on a leaf-area basis. For both relationships, SPAD-total chlorophyll and SPAD-total N, the best fittings were obtained with quadratic equations.
One leaf stalk position alone is able to monitor the N-status of the whole plant during the first six weeks after transplanting, without distinction of year and variety effects. The SPAD measurement of one leaf per plant, throughout the vegetative growing season, is therefore a valid tool to test the N-status of the crop in a period when a required N supply is still effective.

Key-words: chlorophyll, N-status diagnosis, SPAD, Specific leaf nitrogen.

Introduction

The cultivation of flue-cured tobacco (*Nicotiana tabacum* L.) requires careful regulation of nitrogen availability with respect to both the quantities and the timing. The most acceptable results from a tobacco crop are to be expected from nitrogen fertilization practices that ensure a high availability of soil nitrogen during the early plant growth stage. Soil nitrogen depletion soon after topping is desirable to ensure that leaves can ripen correctly, to avoid a corresponding decrease in tobacco quality and an increase in production costs (McCants and Woltz, 1967; Goenaga et al., 1989; Flower, 1999). It should be added that excessive nitrogen application may contribute to groundwater pollution.

Recent studies on flue-cured (Marchetti et al., 2001) and Burley tobacco (Contillo et al., 2001) in Italian tobacco growing areas have shown that the mineral N content of cultivated soil, far from having a spatially uniform and temporally steady level, shows patchy distribution and fluctuates rapidly, with localized bursts of very high values lasting for a few days. This episodic behaviour, combined with sudden heavy rainfalls in late spring or early summer, can increase the risks of nitrate leaching. The risk of environmental pollution is high in the areas most suited to flue-cured production, as they

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are characterized by light sandy soil and frequent rain or a plentiful supply of irrigation water (Akehurst, 1981).

Strategies for improving N use efficiency should not be aimed at attaining the highest yield per unit of fertilizer applied, but rather at identifying the lowest fertilization rate required to achieve a satisfactory yield in a given environment. To minimize costs and the potential quantity of N that may escape the root environment, N-fertilizer should be applied only after it has been determined whether it will be beneficial (Turner and Jund, 1991), possibly using a plant need-based application of N, to improve yields and N use efficiency (Singh et al., 2002). The application of N-fertilizer on an "As Needed" basis, rather than using a "Lump Sum" approach, has advantages which are both environmentally friendly and economic (Schepers et al., 1992). For example, N fertilization can be improved through side-dressing, avoiding application before transplanting when demand is low.

In flue-cured tobacco, current N fertilization rates are not based on the level of nitrogen in the soil, but on other properties of the soil, such as depth, clay content, amount of percolating water, and finally also according to personal experience (Smith and Wood, 2005). To fine-tune plant-need based N application, a quick, in-field method of determining the N-status of the crop was envisaged, to detect the need for added N applications till flowering/topping time, i.e. during the period when applications are most beneficial. Since a large portion of the N in a leaf cell resides in the chloroplast (Makino and Osmond, 1991) it should be possible to use total leaf chlorophyll content to estimate crop N-status.

The green colour of a leaf canopy perceived by the human eye under natural light depends on the relative quantities of light transmitted through the leaves and reflected by the leaf epidermis, the position of the sun, cloudiness of the sky, and many other unpredictable factors (Turner and Jund, 1991). Visual observations are therefore a relative and non-quantitative method to determine the need for more N. The Munsell Color Chart for Plant Tissues, proposed by Kelley et al. (1990) to determine the pigment concentrations (chlorophyll *a*, chlorophyll *b*, etc.) in senescing leaves of burley tobacco through a visual colour rating, was not successful when a more accurate estimation was required. Currently, the most widely-used instrument for non-destructive total leaf chlorophyll content determination is the chlorophyll meter SPAD-502 (Soil-Plant Analysis Development, Minolta Camera Co., Osaka, Japan). The ability to predict total chlorophyll content on a leaf-area basis from a chlorophyll meter reading has been demonstrated for several crops and also used to estimate N-status in corn and cotton, both fertilised with different nitrogen rates. In corn experiments, one chlorophyll reading per leaf has been made, at a defined growing stage (six leaf stage, before silking, after silking, from the end of August to mid-September), on one to four leaves per plant (some bottom leaves, ear leaf, uppermost fully expanded leaf), and on five to sixty plants per plot (Piekielek and Fox, 1992; Piekielek et al., 1995; Schepers et al. 1992). On cotton, Feibo et al. (1998) made measures of leaf chlorophyll content at seven to eight successive growth stages, one reading on the fourth full expanded leaf from the apex, on twenty plants per plot. In this study, the authors found that the relationship between leaf N concentration and SPAD was stronger when N was expressed on a leaf-area rather than on a dry-weight basis. The ability to monitor leaf N-status and to predict the need for N-fertilizer top dressing through the relation between chlorophyll meter readings and N concentration on a dry-weight basis has also been demonstrated for rice (Hussain et al., 2000; Singh et al., 2002). A study to predict N-status and sucker growth in flue-cured tobacco claimed that the SPAD can be considered a valuable tool for non-destructive N determinations in tobacco leaves (Yelverton, 1995). More recently, Kowalczyk-Jusko and Koscik (2000) demonstrated that a SPAD chlorophyll meter could be used, with relatively high precision, in fixing the N doses used in growing flue-cured tobacco. In these experiments no attempt has been made to specify the reason for the leaf, or leaves, chosen for chlorophyll measures, other than possibly an *a priori* selection of an easy to identify leaf (some bottom leaves, ear leaf, uppermost fully expanded leaf, fourth leaf from the top), in a reproducible physiological status (six leaf stage, before silking, after silking). Furthermore, to related leaf SPAD measurements with N-status of the crop, chlorophyll data were normalized relative to an adequately N-fertilized area of the field.

Therefore, in the above-mentioned studies the feasibility of using the SPAD to determine leaf N concentration was confounded by: (i) leaf age; (ii) its metabolic state (sink or source); (iii) the intrinsic discrepancy between *in vivo* measures of optical absorbance of chlorophyll in foliar tissue and actual pigment concentration determined *in vitro* after extraction from the foliar tissue in a suitable solvent. We used a new approach, based on an accurate description of the relationships between SPAD measurements and chlorophyll or nitrogen leaf contents along all the leaf stalk positions, i.e., varying leaf development stages, in plants growing in optimal conditions, during the vegetative growing season. The objectives of our study were: (i) to verify the relationships between SPAD chlorophyll meter readings and total chlorophyll content determined by solvent extraction, and total N content; (ii) to test the validity of the above relationships in a range of different conditions, such as variety, crop phenological status, leaf position on the stalk, i.e. leaf physiological status; (iii) to verify if the SPAD reading on a single leaf, in a well defined stalk position, could represent the nitrogen content of the whole plant, allowing the use of a chlorophyll meter as an in-field diagnostic tool in estimating N-status of the crop.

Materials and methods

Plants of tobacco were grown in 1995 and 1996 at CRA, Consiglio per la Ricerca e la sperimentazione in agricoltura, branch of Bovolone, near Verona, Italy. Two varieties, K 326 and NC 27 NF (No Flowering), were used in the study. The variety K 326 has a day-neutral flowering habit and NC 27 NF requires a short photoperiod for flowering (Jones and Reed, 1991). The two varieties also differ in the onset of maturity-senescence processes, presenting a wider range of chlorophyll and possibly N content, mainly in proximity to the topping and harvesting time. For each variety 20 pot-grown plants were grown under a plastic shield, to avoid any uncontrolled rainwater supply in excess of the pot water holding capacity and possible leaching of nutrients from the pot. The 40 plastic pots, each measuring 0.014 m^3, were spaced at a distance of 1 m between rows and at 0.5 m within the row, thus forming a density of 20,000 plants per hectare. Pots were filled using a substrate prepared by mixing field topsoil with 10-20% oligotrophic peat with P and K fertilizer as detailed below. The peat was added to increase the water holding capacity of the substrate, to avoid water stress due to the finite volume of the pots. The main traits of the substrate were: sand 46%, silt 33%, clay 11%, organic C 4%, N 1‰, P_2O_5 56 mg kg^{-1}, K_2O 180 mg kg^{-1}, pH 7, water retention capacity 21%.

Plants were transplanted into the pots on 17 May in 1995 and 16 May in 1996. In addition to the nitrogen content of the substrate, N fertilizer in the form of $CaNO_3$ was side dressed 20 days after transplanting, as usual in tobacco flue-cured crop, at a rate of 20 kg ha^{-1}. Irrigation was applied as needed to avoid water stress; a volume corresponding to 10 mm of water was added each time the soil surface was dry. Pests and diseases were effectively controlled by Acephate and Metalaxyl + Mancozeb treatment (1 in 1995, 2 in 1996). The sampling procedures during the two years of experiment were: starting at 41 days after transplanting in 1995 and 26 days in 1996, two plants for each variety were randomly selected and odd leaves (minimum 150 mm length) were measured and sampled every 15 days in 1995 and every week in 1996. Samplings were suspended at the appropriate time for topping, when almost all of the N had been absorbed (Peedin, 1999). For K 326 topping (at 20-22 harvestable leaves) occurred at flowering time, and for NC 26 NF when plants had reached the same number of harvestable leaves, according to the usual cropping practices in this area. In 1995 there were 3 sampling dates, at 41, 57 and 72 days after transplanting (DAT); in 1996 the samples were collected at 5 DAT, at 26, 33, 40, 47, 50 days. It was not possible to perform the SPAD measurements on the last sample of 1995 (72 DAT).

Two measurement methods of the total chlorophyll leaf content were compared: *in vivo* using the SPAD, and *in vitro* using spectrophotometrical analysis. SPAD determines the relative quantity of total chlorophyll present in leaf tissue by measuring the transmittance of the leaf in the red region at a wavelength of about 650 nm, and in the infrared region at about 940 nm (Minolta, 1989). Given the very small area of the SPAD sensor compared to the large sur-

face of a tobacco leaf, ten chlorophyll readings were taken around each blade (20 mm from the edge) of the leaves in the odd positions before they were removed from the stalk, and averaged to represent the mean SPAD value of each leaf. The large number of leaves consequently provided a wide range of SPAD measurements. After the SPAD readings were recorded, each measured leaf was detached from the stalk and subjected to further investigations. Three disks of leaf lamina, close to the position of SPAD readings, each with a diameter of 8 mm and a total surface area of 151 mm^2, were taken for analytical determination of total chlorophyll content. To determine the specific leaf weight (SLW) and calculate the specific leaf N content (SLN), 3 leaf lamina disks with a diameter of 33 mm and total surface area of 2566 mm^2, were collected from the leaves, close to the position of the samples used for chlorophyll extraction. The dry weights of disks were measured after oven-drying at 70 °C for 48 h.

For total chlorophyll content determination, the 8 mm leaf disks were placed in vials containing 5 mL of N,N-dimethylformamide (DMF) for pigment extraction. Chlorophyll concentration was determined via spectrophotometer reading at 647 nm and 664 nm, in accordance with the method proposed by Moran and Porath (1980) and ascertained on tobacco by De Simone and Lombardi (1982). Subsequent conversion into total chlorophyll content was performed using Moran's formula (Moran, 1992) and expressed as mg dm^{-2}. After samplings and midrib removal, the remaining lamina of the detached leaves were stored at -20 °C and then freeze-dried and ground. Total N was determined on the obtained powder using an automatic chemical analyzer Technicon AutoAnalyzer II. The analysis of total ammonium N, produced after Kjeldahl digestion of the leaf tissue, was based on a colorimetric method in which an emerald-green complex is formed by the reaction of ammonia, sodium salycilate, sodium nitroprusside and sodium hypoclorite in a buffered alkaline medium at a pH of 12.8-13.0. The ammonia-salycilate complex was read at 660 nm. The total N content calculated according to unit weight was transformed into leaf area estimate, using the SLW values.

Leaf area of sampled leaves was calculated by fitted regression from the measurements of length and width. The large size and wrinkled surface of tobacco leaves would have caused some difficulties in using a leaf area meter. Thus, according to Suggs et al. (1960) and Farah (1974), the area of individual leaves was calculated with the relation LA = L x W x C, where LA is the leaf area, L is the leaf length, W is the maximum leaf width and C is a coefficient for assessing leaf area of flue-cured tobacco. The C value (0.59) used for calculations of leaf area has been previously determined at the same site for a similar tobacco variety (unpublished data).

SPAD values correlation with leaf total chlorophyll and total N contents, both referred to leaf surface and to leaf dry weight unit, was performed using the CORR procedure (SAS Institute, 1989). To investigate the variety and year effects over SPAD-total chlorophyll and SPAD-total N leaf contents relationships, a mixed model was accomplished using SAS PROC MIXED (Littell et al., 1996). In this model, year effect was considered as random and variety as fixed effect. In addition, existing correlation among the leaves belonging to the same plant was accounted for using the REPEATED statement to state covariance parameters. Moreover, for both SPAD-total chlorophyll and SPAD-total N leaf contents relationships, orthogonal comparisons among different leaf positions on the stalk were performed with the CONTRAST statement with the aim to ascertain statistical differences between each single odd leaf and the whole plant mean value. Finally, linear and quadratic regression analyses were calculated using the REG procedure (SAS Institute, 1989) to define the relationships between SPAD value as dependent variable and total chlorophyll or total N content as independent variables.

Results and discussion

SPAD-total chlorophyll and SPAD-total N relationships

SPAD values were correlated (Pearson's correlation coefficient) to a large extent with total chlorophyll content and the correlation coefficient was higher when total chlorophyll content was referred to leaf surface unit (r = 0.96, n = 298) than in reference to leaf dry weight unit (r =

0.84, n = 280). Two explanations for this are possible. First, given that SPAD readings are intrinsically on a leaf surface basis, it should be expected that SPAD measurements correlate better with total chlorophyll content on leaf surface unit than on leaf dry weight unit. Secondly, total chlorophyll expressed as mg g^{-1} leaf dry weight was actually a derived variable, obtained by combining the measured total chlorophyll as mg dm^{-2} with the measured SLW, each with its own experimental error. The lower correlation exhibited by SPAD-total chlorophyll as mg g^{-1} should thus be due to the higher variability of the last variable (the variation coefficient for total chlorophyll was 54% when expressed as mg dm^{-2}, and 59% when expressed as mg g^{-1}). The two reasons should apply together. SPAD values showed a high correlation with total N leaf content as well, although at a lower level than with total chlorophyll. As for the SPAD-total chlorophyll relationship, the SPAD-N relationship was also better when total N content was expressed in terms of SLN, i.e. on leaf surface units (r = 0.79, n = 278) rather than in terms of leaf dry weight unit (r = 0.51, n = 296).

The SPAD readings in response to both total chlorophyll and total N contents per leaf surface unit were well expressed by polynomial regressions (linear and quadratic components of regression model significant at the 0.001 probability test), with notably higher R^2 coefficients for total chlorophyll (R^2 = 0.93, n = 298) (Fig. 1) than for total N content (R^2 = 0.77, n = 278) (Fig. 2). These achievements demonstrated the expected good relationship between two methods to measure the same quantity – leaf total chlorophyll measured *in vivo* (SPAD) and *in vitro* (via solvent extraction), compared to the degree of association that exist between two different quantities, total chlorophyll and total N.

Considering the SPAD vs. total chlorophyll relationship, a curvilinear trend is evident at higher values of both variables, corresponding to the leaves in higher stalk position (Fig. 1). A curvilinear component between *in vivo* and *in vitro* measurements of photosynthetic pigments is expected, due to the so called "sieve effect", which is greater in younger leaves (higher leaf stalk positions). It was already recognized that a given quantity of pigment shows lower absorbance value when the pigment distribution is uneven (the sieve effect), as occurs in the intact leaf where chlorophyll molecules are organized in green chloroplasts immersed in a colourless matrix, in comparison with a homogeneous solution of chlorophyll (Fukshansky, 1981; Vogelmann, 1994). The sieve effect is greater in younger leaves of tobacco because they show an higher chlorophyll density in the chloroplast, rather than an higher number of chloroplasts per cell, with a reduction in pigment distribution homogeneity, as hypothesized by Castelli et al. (1996). On the contrary, cells of older leaves have a less organized structure, and discrepancies due to the sieve effect are less important.

Analytical total chlorophyll content, on a leaf surface basis, showed a significant and positive correlation with SLN (r = 0.85, n = 332). The good results of the polynomial regression fitting (linear and quadratic regression model

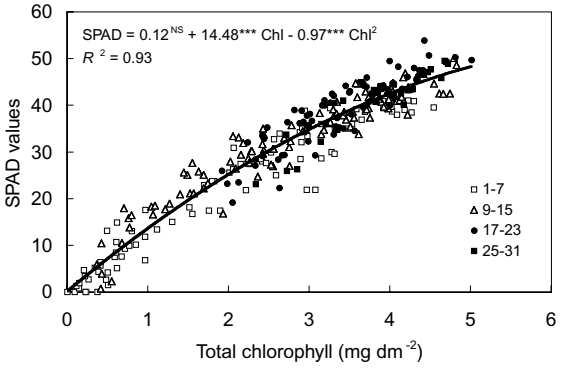

Figure 1. Relationship between SPAD values and total chlorophyll concentration in leaves. Leaf stalk positions, from bottom to top, are highlighted using different symbols.

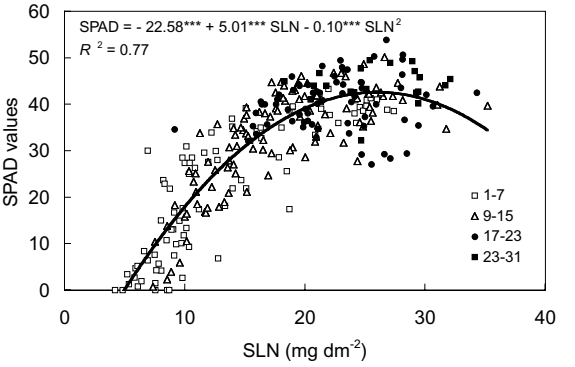

Figure 2. Relationship between SPAD values and SLN (specific leaf N). Leaf stalk positions, from bottom to top, are highlighted using different symbols.

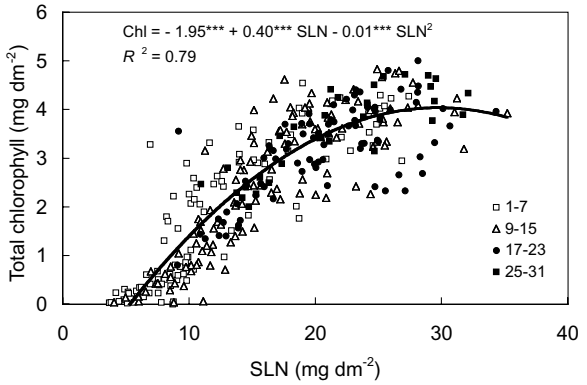

Figure 3. Relationship between total chlorophyll concentration in leaves and SLN (specific leaf N). Leaf stalk positions, from bottom to top, are highlighted using different symbols.

components significant at the 0.001 probability test) of total chlorophyll on total N (Fig. 3), both expressed in mg dm^{-2} (R^2 = 0.79, n = 332), confirmed that the total chlorophyll content reflects the total N content (Makino and Osmond, 1991) as does SPAD-SLN.

The curvilinear trend observed in the SPAD vs. total chlorophyll equation (Fig. 1) and ascribed to the sieve effect, was also evident for the total chlorophyll-SLN relationship (Fig. 3), as already pointed out in wheat by Evans (1983), and even more so for the SPAD-SLN relationship (Fig. 2). About the two relationships involving nitrogen, total chlorophyll-SLN and SPAD-SLN, the linear segment is related to mature, fully expanded leaves, whereas the curvilinear part, in the region of higher values, represent the younger leaves. The marked curvature of total chlorophyll-SLN relationship imply that younger leaves contain more N than expected from linearity. The lack of linearity in the relationship between chlorophyll measurement and total N leaf content at higher values, as already noticed by Porro et al. (2001) in a study on grapevines, should be related to the lack of a link between chlorophyll-pigments and N content. We hypothesized that the deviation from linearity in younger leaves could depend on the presence of a surplus of protein-N, in addition to chloroplast-N and the normal metabolic stuff. In fact, the younger leaves increase in size mostly through cellular expansion than through cell multiplication (Salisbury and Ross, 1978). For this, specific metabolic pathways involved in the synthesis of new cellular components would be active in the cells of those leaves and no longer in fully expanded, lower leaves. The even higher curvilinear component in the SPAD-SLN relationship, compared to the total chlorophyll-SLN, was supposedly attributed to the adding up of two causes, the sieve effect plus the extra N involved in leaf expansion, both acting on the younger leaves (higher leaf stalk positions in Fig. 2).

Validity of the relationships in different conditions

The two different varieties did not produce different relationships, nor did the year (results of the statistical analysis not shown). These results led to the conclusion that the highly significant relationships obtained are not sensitive to variable year, or to plant genotypes. DAT and leaf position were highly significant ($P \geq 0.001$) in influencing SPAD response to total chlorophyll (n = 294) and total N (n = 278) content. DAT effect was recognized as an important factor influencing the distribution of leaf status along the stalk positions, due to aging. The SPAD responses were therefore divided by single DAT.

SPAD as a N-status diagnostic tool

The reliability of polynomial functions relating chlorophyll meter values with total chlorophyll and total N leaf contents would allow the use of the SPAD instrument to estimate the total chlorophyll and N-status of the plant. Nevertheless, the complexity of procedures used in this work (measuring at least half of the leaves per plant) is time and labour consuming, resulting in an unfeasible diagnostic method. To reduce the number of observations, it was evaluated on whether a single leaf in a defined stalk position could represent the whole plant. However, it must be taken into account that during the rapid growth phase each leaf of the tobacco plant is at a different physiological age (McCants and Woltz, 1967) and then, at topping time, the basal leaves are approaching senescence whereas those in apical positions are still in a very active growth stage. The evaluation was performed using a set of orthogonal comparisons. Each single odd leaf was compared with all other odd leaves, a first time using the whole data set and then separately for each sampling date (i.e. DAT values). The analysis was repeated, with the same layout, for SPAD-SLN content relationship.

Table 1. Orthogonal comparisons of SPAD-total chlorophyll (mg dm^{-2}) content relation between each leaf stalk position, from bottom to top, and the whole set of leaves, at different sampling dates (in brackets, degrees of freedom of *F*-tests).

Leaf position	All data	Days after transplanting						
		1995		1996				
		41	57	26	33	40	47	50
1	NS (*191*)	NS (*29.2*)		NS (*18.5*)	NS (*17.9*)	NS (*24.7*)		
3	NS (*186*)	NS (*28.9*)	NS (*10.7*)	NS (*19.0*)	NS (*25.1*)	NS (*15.9*)		NS (*28.3*)
5	* (*202*)	** (*29.5*)	NS (*8.0*)	NS (*18.2*)	NS (*25.8*)	NS (*22.6*)	NS (*21.0*)	NS (*29.2*)
7	NS (*211*)	NS (*30.4*)	NS (*8.6*)	NS (*17.1*)	* (*25.8*)	NS (*27.5*)	NS (*22.0*)	NS (*31.4*)
9	**NS** (*221*)	**NS** (*30.7*)	**NS** (*10.2*)	**NS** (*18.1*)	**NS** (*23.9*)	**NS** (*22.7*)	**NS** (*23.9*)	**NS** (*31.5*)
11	**NS** (*228*)	* (*30.8*)	**NS** (*14.3*)	**NS** (*19.0*)	**NS** (*25.6*)	**NS** (*21.6*)	**NS** (*28.0*)	**NS** (*32.7*)
13	**NS** (*234*)	**NS** (*30.8*)	**NS** (*23.6*)	**NS** (*18.7*)	**NS** (*25.7*)	**NS** (*21.0*)	**NS** (*32.3*)	**NS** (*33.8*)
15	NS (*241*)	* (*30.7*)	NS (*25.9*)		NS (*25.9*)	NS (*20.4*)	NS (*34.3*)	NS (*33.6*)
17	NS (*236*)	NS (*30.1*)	NS (*20.8*)		NS (*24.6*)	NS (*21.1*)	NS (*34.9*)	NS (*33.5*)
19	NS (*251*)	NS (*27.2*)	NS (*17.5*)			* (*25.8*)	NS (*29.9*)	NS (*33.0*)
21	** (*246*)	NS (*26.5*)	NS (*13.9*)			NS (*21.8*)	* (*27.5*)	NS (*32.2*)
23	** (*262*)		NS (*12.1*)			NS (*25.8*)	* (*24.0*)	NS (*31.6*)
25	NS (*253*)		NS (*11.8*)				* (*23.1*)	NS (*27.8*)
27	NS (*236*)		NS (*12.2*)				NS (*24.4*)	NS (*25.0*)
29	NS (*247*)		NS (*10.6*)				* (*35.1*)	NS (*33.5*)
31	* (*267*)		NS (*14.0*)				* (*36.2*)	

*, ** Significant at the 0.05, 0.01 and 0.001 probability level, respectively. NS No significant differences at $P \leq 0.05$.

For the SPAD-total chlorophyll relationship, orthogonal comparisons showed that leaves from 9th to 13th positions were not significantly different from the whole subset of leaves at all dates, with the exception of the 11th leaf position at DAT 41 in 1995 (Tab. 1). Turning to the SPAD-SLN relationship, the 7th leaf partially meets an analogous condition, being not significantly different from the entire plant in the early growth stages (DAT 41 in 1995 and DAT 26, 33, and 40 in 1996), i.e. during the first six weeks after transplanting (Tab. 2). Thus, throughout this time, the 7th leaf could be considered as representative of the N-status of the whole plant. This period is crucial for tobacco flue-cured because any N shortage would have a very un-

Table 2. Orthogonal comparisons of SPAD-SLN (specific leaf N, mg dm^{-2}) content relation between each leaf stalk position, from bottom to top, and the whole set of leaves, at different sampling dates (in brackets, degrees of freedom of *F*-tests).

Leaf position	All data	Days after transplanting						
		1995		1996				
		41	57	26	33	40	47	50
1	*** (*212*)	*** (*21.2*)		NS (*15.6*)	** (*19.5*)	** (*27.9*)		
3	*** (*223*)	NS (*24.4*)	** (*30.8*)	* (*16.0*)	* (*21.2*)	** (*27.8*)		*** (*29.8*)
5	*** (*221*)	NS (*25.9*)	*** (*30.5*)	* (*15.4*)	NS (*21.8*)	* (*28.0*)	*** (*21.5*)	*** (*30.4*)
7	*** (*219*)	**NS** (*27.1*)	*** (*31.2*)	**NS** (*14.8*)	**NS** (*22.0*)	**NS** (*27.8*)	** (*23.2*)	*** (*32.3*)
9	* (*204*)	NS (*27.7*)	*** (*31.4*)	NS (*15.7*)	** (*21.9*)	*** (*27.9*)	*** (*31.1*)	* (*32.9*)
11	* (*197*)	* (*27.8*)	** (*31.6*)	NS (*15.9*)	* (*22.0*)	*** (*27.4*)	*** (*32.6*)	* (*32.7*)
13	NS (*202*)	* (*27.3*)	* (*31.7*)	* (*15.8*)	* (*21.8*)	*** (*27.9*)	*** (*32.4*)	NS (*31.3*)
15	NS (*207*)	** (*26.2*)	NS (*32.0*)		NS (*21.1*)	*** (*27.9*)	** (*33.0*)	NS (*30.4*)
17	NS (*220*)	* (*25.0*)	* (*31.6*)		NS (*21.6*)	** (*27.5*)	NS (*32.8*)	* (*31.4*)
19	NS (*229*)	NS (*26.9*)	* (*31.5*)			NS (*27.5*)	NS (*32.9*)	*** (*32.9*)
21	* (*241*)	NS (*27.4*)	*** (*32.0*)			* (*27.3*)	*** (*32.1*)	*** (*33.0*)
23	*** (*248*)		*** (*31.7*)				*** (*31.4*)	*** (*33.0*)
25	*** (*232*)		** (*31.1*)				*** (*31.8*)	** (*29.1*)
27	* (*234*)		** (*28.8*)				** (*32.4*)	NS (*32.2*)
29	* (*130*)		NS (*30.7*)					
31	* (*80.9*)		NS (*27.7*)					

*, **, *** Significant at the 0.05, 0.01 and 0.001 probability level, respectively. NS No significant differences at $P \leq 0.05$.

favourable effect on yield and quality (Miner and Tucker, 1990). Moreover, Raper and McCants (1967) reported that about 60% of N was absorbed during the three-week period following transplant recovery. SPAD readings on the 7th leaf for a representative number of plants would allow an easy way to control the N-status of the crop, in a period when an additional N supply is still profitable.

Conclusions

In this study, the distribution of total chlorophyll and total N contents in the leaves of flue-cured tobacco plants has been determined during the growth cycle. The two methods adopted for total chlorophyll content determination (*in vivo* by SPAD and *in vitro* by extraction) are well related to each other and the results are consistent with previous studies on tobacco and other species (Markwell et al., 1995; Castelli et al., 1996). Moreover, our data show that good relationships also exist between total chlorophyll measurements and total N content of tobacco leaf. In both relationships, SPAD-total chlorophyll and SPAD-SLN, the best fittings were obtained when total chlorophyll and total N were calculated on a leaf-area basis, as had already been observed by Peng et al. (1995).

All the three relationships examined showed an evident curvilinear trend, as a result of using second degree polynomial regressions. Variety and year did not affect either SPAD-total chlorophyll or SPAD-N relationships, at least during a period when a diagnostic tool is useful for corrective N side-dressing.

By splitting the analysis performed using the whole set of leaves into individual analyses for each DAT and each leaf stalk position, a strongly uniform pattern was revealed in the way the leaves behaved in the intermediate positions of the tobacco stalk. More precisely, for SPAD-total chlorophyll relationship in both years the leaves from the 9th to 13th position were not statistically different from all plant leaves considered as a set, without distinction between variety and during the entire growing season. The same analysis method applied to the SPAD-SLN relationship revealed less effective behaviour uniformity among leaves and DAT. Nevertheless, it was possible to find a specific leaf position, the 7th, as being representative of the whole set of leaves, even if only during the phase of active growth, i.e. during six weeks after transplant.

Notwithstanding these limitations, the use of SPAD instruments was revealed to be extremely practical, permitting the evaluation of the N-status of tobacco plant through the monitoring of only one single leaf per plant. SPAD intrinsically relative performances require repeated measurements not to discover point values, but trends; this objective is positively coherent with the reduced labour required, allowing the number of plants tested to be multiplied to cope with soil and crop heterogeneity.

A method of fine-tuning side-dressing N fertilization requirements based on the proposed criterion is effective during that particular period of plant growth, i.e. when N absorption reaches its maximum level and, mainly, when side-dressing is still useful for increasing quality and yield.

In conclusion, this study shows a new approach in tobacco N monitoring, based on a real-time diagnosis of current N-status of the tobacco plant during the vegetative growing season, by means of a non-destructive tool. Our data show the feasibility of using a chlorophyll meter on a single leaf per plant to asses plant N content. Therefore, SPAD could be applied by farmers and consultants in estimating N-status of the tobacco crop to improve N fertilization management in field conditions.

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References

Akehurst B.C. 1981. Tobacco. 2nd edition, Tropical Agriculture Series, Longman, London and New York.

Castelli F., Contillo R., Miceli F. 1996. Non-destructive determination of leaf chlorophyll content in four crop species. J. Agron. Crop Sci., 177:275-283.

Contillo R., Napolitano A., Raimo F., Cozzolino E., Postiglione C. 2001. Dynamics of mineral nitrogen in a volcanic soil in southern Italy. 7th International Meeting on Soils with Mediterranean Type of Climate, 23-28 September, Valenzano, Bari, Italy.

De Simone C., Lombardi D.A. 1982. Determinazione delle clorofille *a* e *b* in *Nicotiana tabacum* L. mediante la N,N-Dimetilformammide. Ann. Ist. Sper. Tabacco, 9:63-76.

Evans J.R. 1983. Nitrogen and photosynthesis in flag leaf of wheat. Plant Physiol., 72:297-302.

Farah S.M. 1974. Coefficients for assessing leaf area of flue-cured tobacco variety Kutsaga 51. Tob. Sci., 18:5-6.

Feibo W., Lianghuan W., Fuhua X. 1998. Chlorophyll meter to predict nitrogen sidedress requirements for short-season cotton (*Gossypium hirsutum* L.). Field Crops Res., 56:309-314.

Flower K.C. 1999. Agronomy and physiology. Field practices. In: Davis D.L., Nielsen M.T. (eds.): Tobacco: Production, Chemistry, and Technology, 76-103. Blackwell Publishing, Oxford, UK.

Fukshansky L. 1981. Optical properties of plants. In: Smith H. (ed.): Plants and Daylight Spectrum, 21-40. Academic Press, New York.

Goenaga R.J., Volk R.J., Long R.C. 1989. Uptake of nitrogen by flue-cured tobacco during maturation and senescence. I. Partitioning of nitrogen derived from soil and fertilizer sources. Plant Soil, 120:133-139.

Hussain F., Bronson K.F., Yadvinder-Singh, Bijay-Singh, Peng S. 2000. Use of chlorophyll meter sufficiency indices for nitrogen management of irrigated rice in Asia. Agron. J., 92:875-879.

Jones J.L., Reed T.D. 1991. Agronomic information. In: Jones J.L. et al. (eds.): 1992 Flue-cured Tobacco Production Guide, 1-26. Virginia Coop. Ext. Serv., Halifax, VA, USA.

Kelley W.T., Danehower D.A., Bowman D.T. 1990. A color scale for rapid measurement of a relative pigment concentration in burley tobacco leaves. Tob. Sci., 34:22-24.

Kowalczyk-Jusko A., Koscik B. 2000. Estimation of the state of nitrogen nourishment of tobacco using the N-tester. CORESTA Congress "Challenging the New Millenium", 15-19 October, Lisbon, Portugal.

Littell R..C., Milliken G.A., Stroup W.W., Wolfinger R.D. 1996. SAS System for Mixed Models. SAS Institute, Cary, NC, USA.

Makino A., Osmond B. 1991. Effects of nitrogen nutrition on nitrogen partitioning between chloroplasts and mitochondria in pea and wheat. Plant Physiol., 96:355-362.

Marchetti R., Castelli F., Gamba C., Spallacci P., Contillo R. 2001. Effetto della fertilizzazione azotata sulla dinamica dei nitrati in un suolo franco coltivato a tabacco di tipo flue-cured. Boll. Soc. It. Scienza del Suolo, Suppl., 50:131-139.

Markwell J., Osterman J.C., Mitchell J.L. 1995. Calibration of the Minolta SPAD-502 leaf chlorophyll meter. Photosynth. Res., 46:467-472.

McCants C.B., Woltz W.G. 1967. Growth and mineral nutrition of tobacco. Adv. Agron., Academic Press, New York and London, 19:211-265.

Miner G.S., Tucker M.R. 1990. Plant analysis as an aid in fertilizing tobacco, In: Westerman R.L. (ed.): Soil Testing and Plant Analysis. 3rd edition, SSSA Book Ser. 3. SSSA, Madison, WI, USA.

Minolta 1989. Chlorophyll Meter SPAD-502. Owner's Manual. Minolta Camera Co., Ltd., Osaka, Japan.

Moran R. 1982. Formulae for determination of chlorophyllous pigments extracted with N,N-dimethylformamide. Plant Physiol., 69:1376-1381.

Moran R., Porath D. 1980. Chlorophyll determination in intact tissues using N, N-dimethilformamide. Plant Physiol., 65:478-479.

Peedin G.F. 1999. Production practices. Flue-cured tobacco. In: Davis D.L., Nielsen M.T. (eds.): Tobacco: Production, Chemistry, and Technology, 104-142. Blackwell Publishing, Oxford, UK.

Peng S., Laza Ma.R.C., Garcia F.V., Cassman K.G. 1995. Chlorophyll meter estimates leaf area-based nitrogen concentration of rice. Commun. Soil Sci. Plant Anal., 26:927-935.

Piekielek W.P., Fox R.H. 1992. Use of a chlorophyll meter to predict sidedress nitrogen requirements for maize. Agron. J., 84:59-65.

Piekielek W.P., Fox R.H., Toth J.D., Macneal K.E. 1995. Use of a chlorophyll meter at the early dent stage of corn to evaluate nitrogen sufficiency. Agron. J., 87:403-408.

Porro D., Bertamini M., Dorigatti C., Stefanini M., Ceschini A. 2001. Lo SPAD nella diagnosi dello stato nutrizionale della vite. L'Inf. Agr., 26:49-55.

Raper C.D.Jr., McCants C.B. 1967. Nutrient accumulation in flue-cured tobacco. Tob. Sci., 11:190.

SAS Institute. 1989. SAS/STAT User's Guide, Version 6. 4th edition, Vol. 1 and 2. SAS Institute, Cary, NC, USA.

Salisbury F.B., Ross C.W. 1978. Plant Physiology. 2nd edition, Wadsworth Publishing Company Inc., Belmont, CA, USA.

Schepers J.S., Francis D.D., Vigil M., Below F.E. 1992. Comparison of corn leaf nitrogen concentration and chlorophyll meter reading. Commun. Soil Sci. Plant Anal., 23:2173-2187.

Singh B., Singh Y., Ladha J.K., Bronson K.F., Balasubramanian V., Singh J., Khind C.S. 2002. Chlorophyll meter- and leaf color chart-based nitrogen management for rice and wheat in northwestern India. Agron. J., 94:821-829.

Smith W.D., Wood S. 2005. 5. Nutrient management. In: Smith W.D. (ed.): 2005 Flue-cured Tobacco Information, 65-91. North Carolina State Univ. Coop. Ext. Serv., Raleigh, NC, USA.

Suggs C.W., Beeman J.F., Splinter W.E. 1960. Physical properties of green Virginia-type tobacco leaves. III. Relation of leaf length and width to leaf area. Tob. Sci., 4:194-197.

Turner F.T, Jund M.F. 1991. Chlorophyll meter to predict nitrogen topdress requirement for semidwarf rice. Agron. J., 83:926-928.

Vogelmann T.C. 1994. Light within the plant. In: Kendrick R.E., Kronenberg G.H.M. (eds.): Photomorphogenesis in Plants. 2nd edition, 491-535. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Yelverton F.H. 1995. A nondestructive method to predict nitrogen status and sucker growth in flue-cured tobacco. Proceedings 36th Tobacco Workers' Conference, 9-12 January, Tampa, FL, USA, 55-56.