

Estimation of leaf nitrogen concentrations in *Quercus robur* L. using the CCM-200 portable chlorophyll meter for different patterns of vegetative growth and acorn production

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Abstract The potential of the CCM-200 chlorophyll meter for providing accurate estimates of total nitrogen concentrations was studied from 2009 to 2011 using pedunculate oak (*Quercus robur* L.) leaves collected in a field trial near Zagreb, Croatia. A fertilization treatment carried out in early spring 2009 and in variable weather conditions caused between-year differences in acorn yield and production of second and third flush shoots. A total of 10 linear calibration equations were constructed based on data from leaves sampled in early July (first flush leaves), in mid-August (second flush leaves) and in mid-September (first and second flush leaves). These equations reliably described ($p < 0.001$) the relationships between chlorophyll content index values (CCI) measured by the chlorophyll meter and leaf nitrogen concentration (LNC) with respect to the year and month of sampling. The results suggest that variable seasonal dynamics of LNC are influenced by second and/or third flushes as well as by acorn yield. This finding has negative consequences for applying the chlorophyll meter as a suitable tool for LNC assessments during a growing season or over several growing seasons. However, the chlorophyll meter still proved to be a potentially useful tool for LNC estimations, but only when calibration equations were constructed based on the first flush leaves sampled in mid-September (in the last third of a growing season).

Keywords Chlorophyll meter · Calibration equation parameters · Leaf nitrogen dynamics · Second and third flush · Acorn yield

Introduction

Portable optical chlorophyll meters CCM-200 (Opti-Sciences, Tyngsboro, Massachusetts, USA) and SPAD-502 (Minolta Camera Co., Osaka, Japan) are constructed to allow for simple and rapid assessments of the total chlorophyll content in leaves. These chlorophyll

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meters have been described many times as potentially suitable tools for the assessment of the total nitrogen (N) content in the leaves of a large number of crops (Wood et al. 1992; Bullock and Anderson 1998; Rodriguez and Miller 2000; Jifon et al. 2005; Rostami et al. 2008) and for several forest tree species (Loh et al. 2002; Chang and Robison 2003; Van den Berg and Perkins 2004; Percival et al. 2008).

To use chlorophyll meters as a tool for estimating leaf nitrogen content, it is necessary to construct regression equations (calibration equations) that reliably describe the relationships between the relative index of the total chlorophyll content in leaves (CCI) and the total N amount in leaves determined by standard laboratory methods (Loh et al. 2002; Chang and Robison 2003; Van den Berg and Perkins 2004; Percival et al. 2008). In this case, a positive correlation between the CCI and the total N amount in leaves rests on the existence of a close relationship between the amount of chlorophyll in the leaves and N, which builds proteins that connect chlorophyll molecules with thylakoid membranes of chloroplasts (Jifon et al. 2005). However, in addition to N within chlorophyll-protein complexes, the total N in leaves includes N that builds other structural and metabolic components of leaves (Yasumura et al. 2006; Funk et al. 2013). Nevertheless, a regression analysis showed fairly strong relationships between the CCI and the total N amount in leaves of forest trees (Loh et al. 2002; Chang and Robison 2003; Van den Berg and Perkins 2004; Percival et al. 2008). Previous studies conducted using chlorophyll meters during a single growing season have shown that the total amount of N in leaves can be estimated. Solely based on these results, chlorophyll meters were deemed a potentially suitable tool for assessing the overall content and/or concentration of N in the leaves of forest trees (Loh et al. 2002; Chang and Robison 2003; Van den Berg and Perkins 2004; Percival et al. 2008).

However, it is still not clear whether, once constructed, calibration equations that describe the relationship between the CCI and the total N amount in leaves can be used successfully several times during the growing season and/or more times over a number of years. Deciduous forest trees have very efficient mechanisms of internal translocation of N during a growing season (Yasumura et al. 2002; Cantón et al. 2005; Millard and Grelet 2010; Ueda et al. 2011). The triggers for N translocation are often related to the formation of secondary shoots (Ueda et al. 2009) and fruit development (Han et al. 2008, 2011; Alla et al. 2012). For example, N is usually translocated during a growing season from older leaves (developed in spring) to newly formed leaves (Ueda et al. 2009), which can occur on successive flushes of shoots during the summer in response to favorable environmental conditions (Kozłowski and Pallardy 2008; Kuster et al. 2014), and/or to fruits that are developing and maturing during the summer (Han et al. 2011; Alla et al. 2012). The physiological processes of translocation do not equally affect N embedded in all leaf structures. For example, N in chlorophyll-protein complexes (which is closely associated with CCI readings) is more resistant to translocation than N embedded in other metabolic components of leaves (Mae 2004). Accordingly, it can be assumed that the relationship between the CCI and the total N content in leaves of forest trees changes from year to year depending on the translocation intensity of N, which depends on the successive production of shoots and/or fruits. In this case, for successful assessments of the total amount of N in leaves using a chlorophyll meter, it would be necessary to construct new calibration equations for each year. As a result, a chlorophyll meter might not be considered a useful tool for rapid, simple, inexpensive and non-destructive assessments of the total amount of N in leaves of forest trees, as indicated in Chang and Robison (2003) and Van den Berg and Perkins (2004).

Pedunculate oak may be an appropriate model species for testing these assumptions because it has the ability to successively produce shoots several times during a growing season (Le Hir et al. 2005; Spiess et al. 2012; Kuster et al. 2014) and is characterized by highly variable acorn yields from year to year (Koenig and Knops 2000). In addition, pedunculate oak is an important forest tree species (both ecologically and economically) that forms a series of forest communities in Europe (Maděra et al. 2008). Thus, considerable effort has been made to produce acorns and planting materials needed to successfully regenerate pedunculate oak stands (Matić et al. 2008; Schmal et al. 2011). According to Schmal et al. (2011), this production is highly dependent on nitrogen fertilization because N is the most important macronutrient necessary for all physiological processes that influence the growth and development of plants (Evans 1989; Evans and Poorter 2001; Lawlor 2001; Cantón et al. 2005; Han et al. 2008). A chlorophyll meter can provide a simple, rapid, inexpensive means of assessing plant nutritional status, which if proven reliable, could be used to monitor N levels in seed orchards in relation to acorn production and to optimise growing regimes in the production of planting stock in nurseries.

The main objective of this study was to determine whether it is possible to reliably estimate leaf nitrogen concentrations (LNC) of pedunculate oak several times during a growing season or over a number of years on the basis of only one calibration equation (an equation of the relationship between the CCI and the LNC). The calibration equations were constructed based on data collected during three consecutive growing seasons (2009, 2010 and 2011), which differed from one another with respect to acorn crops and the appearance of second and third flushes. Thus, the potential impact of successive flushes within a growing season (driven by mineral fertilization) and acorn crops on the homogeneity of calibration equation parameters (Y intercept and slope) were examined.

Materials and methods

Plant material and study site

The study was conducted during three consecutive growing seasons (in 2009, 2010 and 2011) on 30 pedunculate oak (*Quercus robur* L.) clones. Each clone was represented by a single ramet, which was originally produced by grafting mature trees over 80 years old (Vidaković 1996).

The ramets were planted in a field trial in spring 2008 near Zagreb, Croatia (45° 50' 20"N, 16° 06' 14"E; 128 m a. s. l.). The field trial was described in detail by Franjić et al. (2011) and Sever et al. (2012). The average plant height before the commencement of this study (spring 2009) was 79.2 ± 21.4 cm.

Climate of study site

According to Köppen's classification, the climate in the area of the field trial belongs to the 'Cfbwx' type. This is a moderately warm, rainy climate without a dry period and with precipitation uniformly distributed through the year. The mean temperature in the coldest month (January) does not drop below -0.4 °C, and the mean in the warmest month (July) usually does not exceed 21.4 °C. The annual precipitation amounts to 900 mm (up to 489 mm in the growing season), and mean air temperature in the growing season is 18.6 °C (Seletković 1996).

Plant fertilization and soil chemical properties

In the early spring of 2009, plants were fertilized with complex NPK 15-15-15 mineral fertilizer (Petrokemija Plc.—Fertilizer company, Kutina, Croatia) at 200 g per plant. The fertilizer was evenly distributed around each plant in a radius of approximately 70 cm and worked into the soil. The plants were not fertilized in 2010 and 2011. Soil samples for chemical analyses were collected at 0–30 cm depth at the end of each growing season. One soil sample consisted of three sub-samples that were taken at three positions at a circle placed 50 cm from the trunk of each plant. Soil reactions were determined with a HACH EC 30 pH meter in a suspension of soil and water. The humus concentration was determined according to Tjurin's method, and the total concentrations of nitrogen and carbon in the soil were determined with a CN analyzer (Carlo Erba FlashEA1112).

Plant development and meteorological conditions

Plant development was monitored based on phenological observations and by counting secondary shoots, which was carried out once per week each growing season. Plant heights were measured at the end of each growing season. A ratio between height (before the start of a growing season) and the number of second or third flush shoots was calculated for each plant. This ratio represents an index of shoots produced during the second and third flush. Acorns were collected in the autumn of 2010 and 2011 from each plant. The fresh collected acorns were weighed using analytical balances with an accuracy of 0.01 g.

Air temperature, precipitation and soil volumetric water content (SWC) were recorded by an automatic weather station (Spectrum Tehnologies, inc. 2007) installed at the field trial.

Leaf sampling and construction of calibration equations

Fully developed and healthy leaves (older than 50 days) were chosen for in situ measurements of CCI and sampling for laboratory analyses, i.e., for determining nitrogen concentrations in leaf dry matter.

Thus, leaves from the shoots formed during a first (spring) flush (first flush leaves) were sampled in early July and in mid-September. The leaves originating from the shoots formed during second flush (second flush leaves) were sampled in mid-August and in mid-September (Fig. 1).

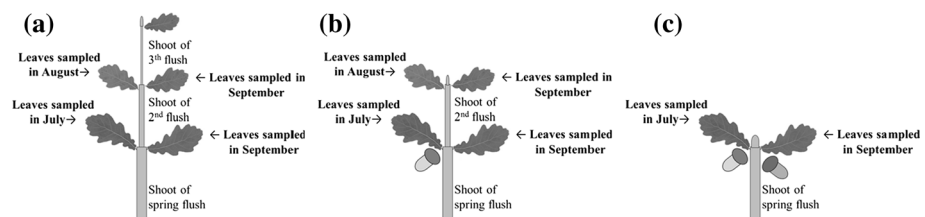


Fig. 1 Scheme of trees development and sampling method of leaves. In 2009, the trees produced shoots three times in growing season: during first (spring) flush, second flush and third flush whilst acorn crops were completely absent (a). In 2010, the trees produced shoots twice during the first (spring) and second flush of leaves, whilst acorn crops was poor (b). In 2011, trees produced shoots only once in growing season, during the first (spring) flush, whilst acorn crops was abundant (c). Accordingly, first- (spring) flush leaves were sampled in July and September in 2009, 2010 and 2011, whilst second-flush leaves were sampled in August and September in 2009 and 2010

A total of 10 calibration equations ($n = 30$) were calculated. These equations describe the relationship between the CCI and the LNC in the first-flush leaves sampled in July and September 2009, 2010 and 2011 and in the second-flush leaves sampled in August and September 2009 and 2010, respectively.

Measurements of CCI, LNC and LMA

CCI was measured using a CCM-200 portable optical chlorophyll meter (Opti-Sciences, Tyngsboro, MA). The working principles and basic features of this device were described in detail by Cate and Perkins (2003) and Silla et al. (2010). Five CCI readings were taken per leaf, and the average of these values represented a single CCI of an individual leaf. Subsequently, the leaves were excised from the shoot, placed in a cooler (at approximately 4 °C) and then dispatched to the laboratory for processing. Upon arrival in the laboratory, leaf area was measured using a software package WinFOLIA (WinFolia 2005b). The leaves were oven dried at 60 °C for 72 h, and their dry masses were measured with an analytical balance with an accuracy of 0.01 g. Leaf mass per area (LMA) was calculated based on dry mass and leaf surface area. Leaf nitrogen content as a percentage of dry weight was determined by combustion analysis using a CHN elemental analyzer (CEC Elemental Analyzer Model 440 with PC-compatible/CEC-490 Interface Unit; Leeman Labs. Inc., Lowell, MA).

Statistical analyses

All statistical tests were performed with the SAS statistical software package (SAS Institute Inc., Cary, NC, USA).

One-way ANOVA was performed to evaluate differences among years regardless of height growth increments and acorn yields. Two-way ANOVA was performed to evaluate the effects of year and month on CCI, LNC and LMA in each leaf flush type. In both cases, Tukey's post hoc test was performed to determine the significance of the differences ($p < 0.05$) between years and/or between months. Student's *t* test was performed to evaluate the differences ($p < 0.05$) between years regardless of size of the acorn crop.

Regression analysis was performed using proc REG in SAS/STAT 9.3 software (SAS Institute Inc., Cary, NC, USA). In linear regression equations (calibration equations) LNC was set as dependent variable and CCI as independent variable. Validation and comparison of results obtained by each linear regression equations was carried out graphically and analytically by Kolmogorov–Smirnov test.

ANCOVA was performed using the JMP 9.0 statistical software package (SAS Institute Inc., Cary, NC, USA) with interaction analysis to test for homogeneity of the Y intercepts and slopes of the calibration equations among years across leaf type and sampling month as well as between months across leaf type and sampling year.

Results

Soil chemical traits

The N concentration in the soil during the studied period varied between 0.12 and 0.15 %, indicating a sufficient nutrient supply. The ratio of C/N was quite narrow (12.2–16.2) and

stable. Significant differences among years regarding soil pH (5.47–5.54) and concentration of humus (1.76–1.83 %) were not determined despite the fertilization treatment in early spring 2009.

Meteorological conditions

Soil water content was quite high and did not fall below 22 % during the 2009 growing season. Similar SWC levels were found during the first half of the growing seasons of 2010 and 2011. However, SWC rapidly dropped to 13 % in the beginning of July 2010, then briefly recovered at the beginning of August, and then decreased again. In 2011, SWC rapidly decreased in mid-June and did not rise above 10 % by the end of September (Fig. 2).

Such dynamics of SWC were in accordance with evenly distributed precipitation during the growing season of 2009 (356 mm) and unevenly distributed rainfall in 2010 (508 mm) and in 2011 (212 mm). Average daily air temperatures during the growing season of 2009 (18.8 °C), 2010 (17.6 °C) and 2011 (19.0 °C) were similar and did not differ greatly from the 30-year average air temperature for the site (18.6 °C).

Vegetative growth and acorn yield

Vegetative growth and acorn yield differed significantly between years (Fig. 3). The spring flush appeared in all 3 years, second flush was recorded in 2009 and 2010, and a third flush was occurred only in 2009 (Fig. 3a). Accordingly, the largest plant height increments were in 2009, whereas the lowest were in 2011 (Fig. 3b). The spring flush began in early April in all 3 years, the second flush began in mid-June (both in 2009 and 2010), and the third flush began in mid-July of 2009 (Fig. 2). In 2009, trees produced significantly more shoots than in 2010, but a third flush did not occur in 2010 (Fig. 3a).

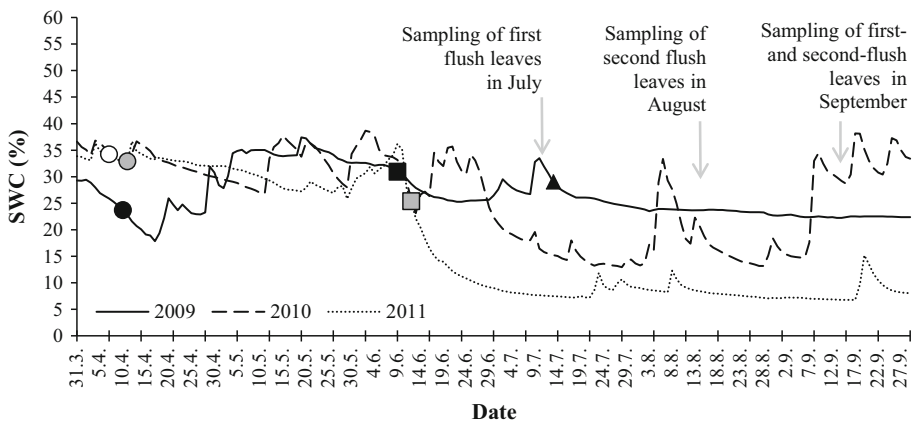


Fig. 2 Soil water content (SWC) during the three growing seasons (2009, 2010 and 2011). Circles indicate the average start of the first flush in 2009 (black circle), 2010 (grey circle) and 2011 (white circle). Squares indicate the average start of the second flush in 2009 (black square) and 2010 (grey square). The black triangle indicates the average start of the third flush in 2009. Gray arrows indicate the sampling dates of first-flush leaves in July and September and second-flush leaves in August and September

In 2009, the studied plants did not yield acorns. In 2010 and 2011, all plants yielded acorns. However, the crop was significantly more abundant in 2011 than in 2010 (Fig. 3c). In 2010 and 2011 a mean percentage of moisture content in the fresh collected acorns was 50 and 48 % respectively.

Effect of year and month on CCI, LNC and LMA

According to the ANOVA results, only CCI values of the first-flush leaves were significantly influenced by the year of sampling, whereas LNC values of both first- and second-flush leaves were significantly influenced by the year and month of sampling (Table 1).

Thus, CCI values of first- and second-flush leaves measured in July or August and September did not differ significantly in any year.

Therefore, the LNC of first-flush leaves decreased by 28.4 and 20.1 % in the period from July to September of 2009 and 2011, respectively. Additionally, the LNC of second-flush leaves decreased by 12.2 % in the period from August to September 2009. In 2010, the LNC of the leaves of both flushes was stable from July/August to September (Fig. 4).

The LMA values of first- and second-flush leaves were not significantly influenced either by the year or month of sampling (Table 1).

Relationship between CCI and LNC

Deviations of estimated LNC values by all 10 calibration equations in relation to the laboratory-determined values were normally distributed, as confirmed by the Kolmogorov–Smirnov test ($p > 0.05$), (result not shown).

A strong correlation ($p < 0.001$) between the CCI and the LNC was determined for all analyzed leaf flush types. The coefficients of determination (r^2) were high, except for first flush leaves sampled in July 2009 ($r^2 = 0.384$) and in September 2009 ($r^2 = 0.430$), as well as for second-flush leaves sampled in August 2010 ($r^2 = 0.435$) (Table 2).

The LMA of the leaves that were used for the construction of calibration equations was not affected by either the year effect or the month of sampling (Tables 1, 2).

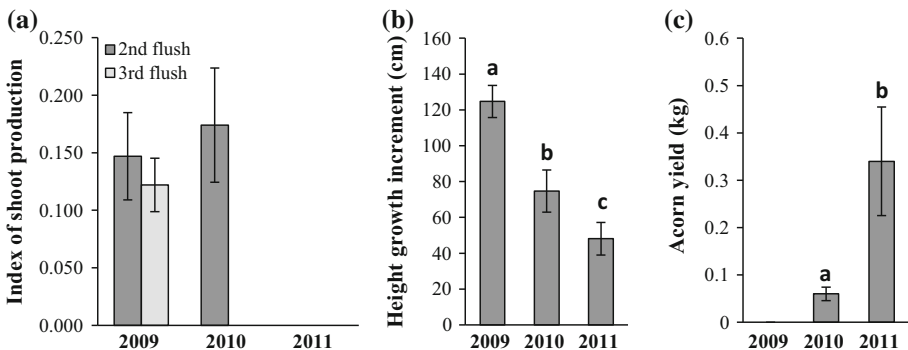


Fig. 3 Parameters, which describe trees development in 2009, 2010 and 2011. Index of shoot production represents mean ratio between height of trees at the beginning of the growing season and the number of second and/or third flush shoots produced during growing season (a), mean height growth increment (b) and mean acorn yield (c). Different letters indicate significant differences ($p < 0.05$) between years. Vertical bars indicate \pm SE

Table 1 ANOVA significance levels for year and month effect on chlorophyll content index (CCI), leaf nitrogen concentration (LNC) and leaf mass per area (LMA) in first- and second-flush leaves

Leaf flush type	Leaf trait	Year	Month	Year × month
First flush leaves	CCI	***	n.s.	n.s.
	LNC	***	***	***
	LMA	n.s.	n.s.	n.s.
Second flush leaves	CCI	n.s.	n.s.	n.s.
	LNC	*	*	*
	LMA	n.s.	n.s.	n.s.

Level of significance: * $p < 0.05$; *** $p < 0.001$; n.s. not significant

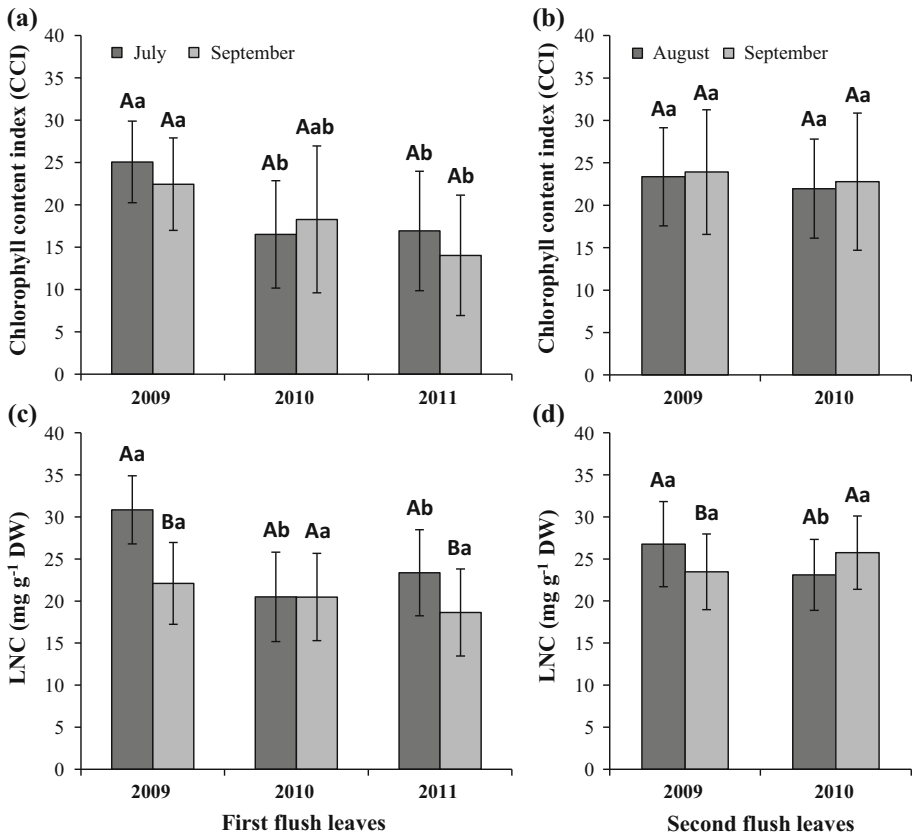


Fig. 4 Mean chlorophyll content index (CCI) and leaf nitrogen concentration (LNC) in 2009, 2010 and 2011 for first- and second-flush leaves regardless of sampling month (July/September and August/September, respectively). Different capital letters indicate significant differences ($p < 0.05$) between months within the same year, and different lowercase letters indicate significant differences ($p < 0.05$) between years for the same month. Vertical bars indicate \pm SD

Table 2 Calibration equations for three different years (2009, 2010 and 2011) for each leaf flush type (first and second flush) and sampling month (July, August and September), range of minimum and maximum values of leaf nitrogen concentrations (LNC) and mean values (\pm SD) of leaf mass per area (LMA)

Leaf flush type and sampling month	Year	n	Calibration equations	r^2	<i>p</i>	LNC (min–max)	LMA (g m ⁻²)
First flush leaves sampled in July	2009	30	LNC (mg g ⁻¹) = 17.28 + 0.52 × CCI	0.384	<0.00026	22.5–38.0	115.0 ± 13.1
	2010	30	LNC (mg g ⁻¹) = 8.87 + 0.70 × CCI	0.707	<0.00000	12.4–31.9	105.7 ± 13.0
	2011	30	LNC (mg g ⁻¹) = 12.04 + 0.67 × CCI	0.847	<0.00000	14.6–32.5	113.5 ± 13.2
First flush leaves sampled in September	2009	30	LNC (mg g ⁻¹) = 9.60 + 0.58 × CCI	0.430	<0.00009	13.2–30.0	119.4 ± 14.0
	2010	30	LNC (mg g ⁻¹) = 10.27 + 0.56 × CCI	0.870	<0.00000	9.6–31.5	115.2 ± 8.7
	2011	30	LNC (mg g ⁻¹) = 9.99 + 0.62 × CCI	0.720	<0.00000	10.7–29.7	111.3 ± 12.8
Second flush leaves sampled in August	2009	30	LNC (mg g ⁻¹) = 12.75 + 0.72 × CCI	0.681	<0.00000	17.5–40.0	126.4 ± 13.3
	2010	30	LNC (mg g ⁻¹) = 14.18 + 0.47 × CCI	0.435	<0.00007	15.2–34.6	127.0 ± 17.2
Second flush leaves sampled in September	2009	30	LNC (mg g ⁻¹) = 8.74 + 0.61 × CCI	0.640	<0.00000	15.9–31.7	129.4 ± 13.5
	2010	30	LNC (mg g ⁻¹) = 16.26 + 0.41 × CCI	0.594	<0.00000	14.6–33.3	127.1 ± 14.3

The equations convert the chlorophyll content index (CCI) into the estimated leaf nitrogen concentration (LNC)

Effect of month and year on calibration equation parameters

According to the ANCOVA results, the slopes of the calibration equations were not significantly influenced by the month (Table 3) or by the year effects (Table 4).

The month effect was not significant for the Y intercepts when calibration equations constructed on the basis of first- and second-flush leaves in 2010 were compared. However, the month effect was significant for the Y intercepts of equations constructed on the basis of first-flush leaves sampled in 2009 and 2011 as well as for the Y intercepts of equations constructed on the basis of second flush leaves sampled in 2009 (Table 3).

The year effect was not significant for the Y intercepts when calibration equations constructed on the basis of first-flush leaves sampled in September were compared. However, the year effect was significant for the Y intercepts of equations constructed on the basis of first-flush leaves sampled in July as well as for equations constructed for second-flush leaves sampled in August and September (Table 4).

Discussion

Nutritional status, meteorological conditions and plant development

Thanks to favorable chemical conditions of the soil, N nutritional status of the plants was quite good. This status was confirmed by LNC values of first- and second- flush leaves, which did not significantly fall drop below $20 \text{ mg g}^{-1} \text{ DW}$ (Fig. 4c, d). An LNC level of $20 \text{ mg g}^{-1} \text{ DW}$ indicates a satisfactory N nutritional status for pedunculate oak (Bergmann 1993). Overall, the leaves of the first flush sampled in July 2009 had significantly higher LNC compared to samples from 2010 and 2011, but the LNC levels decreased significantly until September 2009 (Fig. 4c, d). This result indicates a gradual weakening of the influence of mineral fertilization (carried out in early spring 2009) on plant N nutritional status from July/August until September 2009 as well as during the same period in 2009–2010.

Good N nutritional status and favourable meteorological conditions that are believed to stimulate second and third flush of shoots (Spiess et al. 2012; Kuster et al. 2014). The oak trees in this study had the highest amount of vegetative growth in 2009, a little less in 2010 and the least in 2011 (Fig. 3a, b). According to Spiess et al. (2012) exposure of

Table 3 ANCOVA significance values (*p*) for differences in the Y intercept and slope of the equation constructed for first- and second-flush leaves in different months (July, August and September)

Leaf flush type and sampling year	Effect of month	Effect of CCI	Month × CCI
First flush leaves sampled in 2009	<0.0001	<0.0001	0.7355
Second flush leaves sampled in 2009	<0.0001	<0.0001	0.4040
First flush leaves sampled in 2010	0.0808	<0.0001	0.1046
Second flush leaves sampled in 2010	0.3203	<0.0001	0.6593
First flush leaves sampled in 2011	<0.0001	<0.0001	0.5690

n = 30 leaves per leaf-flush type and sampling year

A significant interaction (Month × CCI) indicates differences in slope

Values in italics indicate significant effects; *p* < 0.05

Table 4 ANCOVA significance values (*p*) for differences in the Y intercept and the slope of the equation constructed for first- and second-flush leaves in different years (2009, 2010 and 2011)

Leaf flush type and sampling month	Effect of year	Effect of CCI	Year × CCI
First flush leaves sampled in July	<i><0.0001</i>	<i><0.0001</i>	0.3720
First flush leaves sampled in September	0.2200	<i><0.0001</i>	0.8358
Second flush leaves sampled in August	<i><0.01</i>	<i><0.0001</i>	0.1815
Second flush leaves sampled in September	<i><0.001</i>	<i><0.0001</i>	0.0735

n = 30 leaves per leaf-flush type and sampling month

A significant interaction (Year × CCI) indicates differences in slope

Values in italics indicate significant effects; *p* < 0.05

pedunculate oak to low SWC of 10 % causes severe drought stress that may have prevented a second flush of shoots, significantly reducing the total amount of vegetative growth that might be expected. In accordance with these observations, a severe drought occurred in 2010 and 2011, when soil water content during the summer months was extremely low (<13 %), which could have reduced the frequency of additional shoot flushes in those years (Fig. 2).

Relationship between CCI and LNC

Considering the working principle of the CCM-200 chlorophyll meter (Cate and Perkins 2003; Silla et al. 2010), it is clear that CCI readings are related only to the N that builds chlorophyll-protein complexes. According to Hikosaka (2003) N that builds chlorophyll-protein complexes is much more stable during a growing season than N embedded in other metabolic leaf compounds (Calvin-cycle enzymes, amino acids, nucleic acids, secondary defense compounds and inorganic N) which represents mobile fraction of N in leaves (Hörtensteiner and Feller 2002; Yasumura et al. 2005; Funk et al. 2013). Besides the N embedded into the chlorophyll-protein complexes, stable fraction of N in the leaves also represents N embedded into the plasma membrane and the cell walls; this content does not significantly change in leaves during a growing season (Ueda et al. 2011). Accordingly, the CCI readings (which represents only one part of stable N fraction in leaves) did not significantly change from July/August to September (Fig. 4a, b), with the result that CCI was more stable than total LNC, which includes both fractions of N in leaves (stable and mobile) (Fig. 4c, d).

Despite CCI readings being closely related only to N that builds chlorophyll-protein complexes, the results indicate the strong relationships between CCI and the LNC in leaves of pedunculate oak (Table 2). According to these results, 38–87 % of the total variation of LNC of first- and second-flush leaves was explained using CCI as an independent variable (Table 2). This finding is consistent with the results of earlier research conducted on forest trees (Chang and Robison 2003; Van den Berg and Perkins 2004; Percival et al. 2008; Salifu et al. 2008; Ghasemi et al. 2011).

Influence of month and year on the slopes and Y intercepts

The slopes of the calibration equations that describe the relationships between CCI readings and LNC in first- and second-flush leaves were quite similar with respect to sampling month and year (Tables 3, 4). This finding suggests that the relationship between LNC and

CCI was stable within the same leaf-flush type for leaves sampled in the same month of any particular growing season (Tables 3, 4).

However, variations in the Y intercepts limit the possibility of using constructed calibration equations that accurately describe the relationships between CCI readings and LNC several times during a growing season and/or more times over a number of years (Tables 3, 4). According to Chang and Robison (2003), variations in Y intercepts directly depend on the LNC used for equation construction. The LNC used depends on a number of internal and external factors (Campbell et al. 1990).

The results of this study showed that fertilization treatment and weather conditions (external factors) greatly influenced the formation of shoots by the second and third flush (internal factors), and it also increased acorn yields (internal factor). It seems that these external and internal factors directly affected LNC in the first- and second-flush leaves in each of the three growing seasons, resulting in considerable variation in LNC values.

Accordingly, in 2009 and in 2011, N content decreased in first-flush leaves (from July to September) and second-flush leaves (from August to September), whereas in 2010, it was stable (Fig. 4c, d). In 2010, there was no third flush of shoots and acorn yields were very low, unlike in 2009 and 2011 (Fig. 3a, c). Environmental factors during the July to September period 2010 appeared to be unfavourable, so the translocation of N from leaves was not initiated, with the result that N content levels were stable that year. For this reason, it can be assumed that shoot production during the third flush in 2009 and the development of acorns in 2011 triggered the translocation of N from first- and second-flush leaves. This assumption agrees with the results from earlier studies that showed that the translocation of N in the canopy of deciduous trees during a growing season is controlled by the second- and/or third-flush (Ueda et al. 2009, 2011) and by fruit development (Kawada and Maruyama 1986; Han et al. 2008, 2011; Alla et al. 2012). Second- and third-flush leaves and fruits are the main sink organs of N translocation from first-flush leaves in addition to storage tissues inside trunks and roots (Millard and Grelet 2010). However, intensive N translocation from leaves into storage tissues in temperate deciduous trees occurs just before the end of the growing season (usually in mid-October) (Staa and Stjernquist 1986). This phenomenon further supports the assumption that the development of the third-flush and acorn yields directly resulted in a decrease of LNC for first- (sampled from July to September) and second-flush leaves (sampled from August to September) in 2009 and 2011.

Accordingly, the impact of the month effect on the Y intercepts and slopes of the calibration equations constructed on the basis of first- and second-flush leaves was non-significant only in 2010 (Table 3). Therefore, it was possible to accurately use a calibration equation based on first- and second-flush leaves several times during a growing season only in one (2010) of the 3 years studied.

Using a single calibration equation over a number of years for accurate estimation of the total LNC using the CCM-200 chlorophyll meter was possible only if the equation was constructed using data from leaves with fairly equal N concentrations. These conditions occurred for first-flush leaves sampled in mid-September in this study (Table 4; Fig. 4c). The first-flush leaves are the oldest leaves in a canopy of deciduous trees, and these are a major source of N, which is translocated during summer into shoots of second and/or third flushes as well as into developing fruits (Kawada and Maruyama 1986; Ueda et al. 2009, 2011; Han et al. 2011; Alla et al. 2012). In deciduous trees, it is mainly the mobile N fraction that is intensively translocated from leaves, resulting in changes in stable/mobile N ratios toward the end of the growing season (Onoda et al. 2004; Takashima et al. 2004; Ueda et al. 2011). As a result, the total N content in leaves gradually decreases and

becomes stable toward the end of the growing season (Migita et al. 2007; Koyama et al. 2008). For this reason, the stable N fraction represents the majority of the LNC by the end of a growing season (Ueda et al. 2011). The translocation of N from first- to second-flush leaves probably occurred from July to September in 2009 and 2010 and to third-flush leaves in 2009, as well as to developing acorns in 2011. This process may have been completed towards the end of the growing season, resulting in similar LNC values by mid-September. Additionally, the majority of the total LNC of the first flush leaves sampled in mid-September was the stable N fraction that did not significantly vary among years (Fig. 4c). For these reasons, the year did not significantly affect the Y intercepts, except in those calibration equations that were constructed on the basis of first-flush leaves sampled in mid-September (Table 4).

Conclusions

Fertiliser application and weather conditions during the growing season affected the number of shoot flushes and acorn production. Consequently, the LNC values were highly variable and the CCM-200 chlorophyll meter did not accurately detect some of these changes. This finding suggests that environmental factors may also indirectly affect the estimation of LNC using the chlorophyll meter. However, the CCM-200 chlorophyll meter still proved to be a potentially useful tool for LNC estimations, but only in cases in which the calibration equations were constructed based on data collected from the oldest leaves in a tree canopy (first-flush leaves) sampled in the last third of the growing season (in mid-September).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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