Indicators of nitrogen status for ornamental woody plants based on optical measurements of leaf epidermal polyphenol and chlorophyll contents

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Received 30 November 2006; received in revised form 24 July 2007; accepted 4 October 2007

Abstract

Indicators of plant nitrogen (N) status adapted to woody ornamental plants are essential for the adjustment of fertilization practices in nurseries. The objective of this study was to investigate whether optical measurements of leaf epidermal polyphenol (EPhen) and chlorophyll (Chl) contents could be used as N status indicators for woody deciduous and evergreen ornamental plants. One-year-old plants of Lagerstroemia indica, Callicarpa bodinieri and Viburnum tinus were grown outdoors in containers. They received low (TN1, 4 mg L⁻¹) or high (TN2, 105 mg L⁻¹) levels of N during 2 months in spring and summer. TN1 treatment limited shoot growth from 28 to 37 days after treatment initiation in Lagerstroemia and Callicarpa, respectively. Shoot growth was unaffected until day 176 in Viburnum. The mass-based leaf N content (NM) of a sample of young expanded leaves exposed to direct sunlight was tightly correlated with shoot N content and differentiated treatments several weeks before shoot growth reduction for the three species. NM was therefore used as an index of plant N status. EPhen and Chl contents were recorded with DualexTM and SPAD-502 leaf-clip meters, respectively. Dualex values were strongly and negatively correlated with NM, and differentiated the treatments early in the experiment, in all three species. SPAD values were positively correlated with NM for Lagerstroemia and Callicarpa, but not for Viburnum, because large variations in leaf mass per area (LMA) in this species compensated for variations in leaf dry mass invested in Chl. The SPAD/Dualex ratio was used to assess changes in the proportion of leaf dry mass allocated to proteins and polyphenols in response to fertilization. It differentiated between the treatments early in the experiment and was correlated with NM in all three species.

Keywords: Nitrogen nutrition indicator; SPAD; Dualex; Ornamental plants; Lagerstroemia indica; Callicarpa bodinieri; Viburnum tinus

1. Introduction

In woody plants, nitrogen (N) nutrition is known to influence growth (Millard and Proe, 1991; Cabrera, 2003) and criteria of major importance for ornamental plant quality, such as architecture (Fustec and Beaujard, 2000), susceptibility to diseases and insects (Daane et al., 1995), cold hardiness (Pellet and Carter, 1981) and shoot/root balance (Macfarlane et al., 2005). Overfertilization may also lead to water pollution, due to nitrate leaching (Alt et al., 1989). Fertilization is often excessive in ornamental plant nurseries (Charpentier, 1995; Alt, 1998), because it is difficult to determine N fertilizer requirement. Many crops display variability in N nutrition with climate (e.g. Dong et al., 2001; McDonald et al., 2002), but ornamental plants are subject to additional sources of variability, due to the diversity of genera, species and cultivars, plant ages and production systems. Moreover, the amount of stored N available for remobilization is very difficult to measure. It is therefore important to check plant N status. Indicators of N status for use in ornamental plant nurseries must be sensitive (i.e. detecting changes in N status early), appropriate for use in a wide range of taxa, rapid, easy to use, inexpensive and non-destructive. The chemical determination of plant N content is a reliable method, as long as thresholds are determined in advance (see Olfs et al., 2005 for a review), but is time-consuming, destructive and expensive. Plant-sap or petiole nitrate-N concentration measurements are rapid and sensitive in various species (Olfs et al., 2005), but are...
invasive and can be used only in species in which nitrate is the principal form of N in the sap. Methods based on the optical properties of chlorophyll (Chl) within the leaf, such as canopy reflectance (Offs et al., 2005), leaf transmittance (Offs et al., 2005) and leaf fluorescence (Cartelat et al., 2005; Corp et al., 2003), have provided new non-destructive, rapid and easy-to-monitor N indicators.

Leaf Chl content is closely related to leaf N content because photosynthetic proteins account for more than half of the N in a leaf (Evans, 1989). Leaf Chl content increases with N supply and is low when N is limiting (e.g. Chang and Robison, 2003 for woody species). N supply influences the synthesis of both proteins and polyphenols, because the biosynthetic pathways of these two classes of compounds share a common precursor, the amino acid l-phenylalanine (PHE) (Jones and Hartley, 1999). Following N fertilization, the amount of PHE allocated to polyphenols decreases or remains constant, whereas the amount allocated to proteins increases (Koricheva et al., 1998; Jones and Hartley, 1999). In contrast, N shortage increases PHE allocation to polyphenols, as growth protein demand is lower. A high polyphenol content and a low protein/polyphenol ratio are therefore potential indicators of low N status. Polyphenols are carbon-based compounds, whereas proteins are nitrogen-based compounds. The relative amounts of these two types of compounds can be used as a signature of the carbon–nitrogen balance within the leaf. This may be useful to assess the phenotypic plasticity induced by N availability in plants (Meyer et al., 2006). There is currently no optical method for assessing total leaf protein and polyphenol contents in situ. However, recent studies have shown that Chl and epidermal polyphenol (EPhen) contents can be used to represent these two classes of compounds. This makes it possible to use optical techniques to assess these signatures in the field (Cartelat et al., 2005; Meyer et al., 2006).

Both leaf Chl and EPhen contents can be assessed with optical leaf-clip devices. Chl content can be estimated by the sequential measurement of transmission through leaves of red (650 nm) and infrared (940 nm) light, using a portable Minolta SPAD-502 chlorophyll meter (hereafter referred to as SPAD). The difference in transmission at these two wavelengths is an indicator of Chl content per unit leaf area (Markwell et al., 1995). EPhen can be assessed by the sequential measurement of leaf Chl fluorescence excited by UV (375 nm) and red (650 nm) light, using a FORCE-A Dualex™ meter (hereafter referred to as Dualex) (Goulas et al., 2004). EPhen content is deduced, based on the UV-absorbing properties of the leaf, as first described by Bilger et al. (1997, 2001). EPhen are mainly water-soluble glycosylated flavonoids stored in vacuoles and hydroxycinnamic acids bound to cell walls. They absorb UV radiation only. Red light is used as a reference, as it crosses the epidermis without being absorbed to reach the Chl in the mesophyll. The ratio of Chl emission (from 695 nm) under excitation by UV and red light is an index of EPhen content per unit leaf area.

Chl and EPhen, estimated by SPAD and Dualex, respectively, are area-based variables. SPAD and Dualex values therefore incorporate two variables: (i) the proportion of the leaf dry mass invested in Chl or EPhen, corresponding to PHE allocation to protein or polyphenol biosynthesis and (ii) dry mass accumulation per unit leaf area – LMA – in which the amounts of Chl and EPhen are diluted (Meyer et al., 2006). PHE allocation affects the leaf dry mass invested in Chl and EPhen differently, but LMA variations affects the leaf dry mass invested in these two types of compound similarly. A signature of PHE allocation to protein or polyphenols within leaves can be obtained by calculating the ratio of SPAD and Dualex values successively recorded on the same leaf. This promising method was first used to estimate N nutrition index in wheat crops (Cartelat et al., 2005), and needs to be tested in woody plants.

The aim of this study was to test the three available optical indicators of N status – SPAD, Dualex and SPAD/Dualex – for assessing the effect of fertilizer applications on ornamental woody plants grown in nurseries. An experimental design generating a wide range of plant and leaf N contents was used. The effects of contrasting N treatments on plant growth and leaf traits were assessed in two deciduous and one evergreen species with different strategies of N storage and use. The correlations between leaf N content and optical measurements of Chl and EPhen, expressed on a per area or per mass basis, and the SPAD/Dualex ratio were investigated.

2. Materials and methods

2.1. Plant material, growth conditions and experimental design

One-year-old plants of Lagerstroemia indica cv. Red Emperor (deciduous, Lythraceae), Callicarpa bodinieri cv. Profusion (deciduous, Verbenaceae) and Viburnum tinus cv. Macrocarpa (evergreen, Caprifoliaceae) were transplanted in March 2002 into 4 L containers filled with a peat/perlite mixture (50/50, v/v, pH 6.4). The plants were placed outdoors at Angers (France, latitude 47 30'N, longitude 00 35'W, altitude 56 m) at a density of 6 plants m⁻², appropriate for their development. Insects were controlled by pesticide applications on all plants of Lagerstroemia on 2 May and 3 June; no pesticide applications were required on the other species. The plants were drip irrigated as a function of evaporative demand, calculated on an hourly basis. From 30 April to 2 June 2002, all the plants received a complete nutrient solution (78, 107, 47, 93, 29, 154 and 18 mg L⁻¹ for N–NO₃, P, S, Ca, Mg, K and Na, respectively). N was supplied exclusively as nitrate, to prevent N adsorption onto soil particles by cation exchange. From 3 June to 31 July 2002, the plants received either a nutrient solution with a low N concentration—treatment TN₁ (4, 107, 90, 94, 93, 29, 154 and 18 mg L⁻¹ for N–NO₃, P, S, Ca, Mg, K and Na, respectively), or a nutrient solution with a high N concentration – treatment TN₂ (88, 17, 121, 47, 93, 29, 151 and 18 mg L⁻¹ for N–NO₃, N–NH₄, P, S, Ca, Mg, K and Na, respectively). Sulfate, chloride and, to a lesser extent, phosphate concentrations varied between the nutrient solutions to ensure that calcium and potassium (both anions supplied with the NO₃⁻ in treatment TN₁) concentrations were the same.
in both solutions. From 1 August to 31 October 2002, all plants were irrigated with tap water only, and irrigation was then stopped. The experimental treatments began on 3 June 2002, referred to as day zero. Each species was grown on an individual plot of 12 rows, the spacing between pots was 0.50 m between rows and 0.33 m within a row. The border rows were avoided when sampling to minimize edge effects. For each species, a complete randomized design with three replicates was used. Two plants per replicate were sampled each week in June and July 2002. Ten leaves per plant were used for analysis. Leaf age at sampling was kept as uniform as possible by sampling the youngest leaves that had reached at least 75% of their final size. Only leaves exposed to direct sunlight (grown at the top of the canopy) were harvested. In Viburnum, only new leaves (developed in 2002) were selected. Plant age varied with their final size. Only leaves exposed to direct sunlight (grown at the top of the canopy) were harvested. In Viburnum, only new leaves (developed in 2002) were selected. Plant age varied with sampling date, but leaf age did not.

2.2. Optical measurements of leaf Chl and EPheN contents

Aera-based Chl content (ChlA) was estimated with a SPAD-502 chlorophyll meter (Minolta, Carrière-sur-Seine, France). Two measurements were taken in the middle of each sampled leaf, on the adaxial side, avoiding the midrib (Cartelat et al., 2005). Aera-based EPheN (EPheNA) content was estimated using Dualex (FORCE-A, Orsay, France). Four Dualex readings were taken per leaf, two on the adaxial and two on the abaxial side. The Dualex readings of each side of the leaf were summed to estimate the EPheN content of the epidermis on both sides of the leaf (Cartelat et al., 2005). The Dualex measurements were made at approximately the same location as the SPAD readings. SPAD and Dualex values have no units. Mass-based Chl (ChlM) and EPheN (EPheNM) contents were estimated by dividing SPAD and Dualex values by LMA. The values obtained represent the proportion of leaf dry mass allocated to Chl and EPheN, respectively. The ratio between SPAD and Dualex values (the SPAD/Dualex ratio) was calculated and corresponds to the relative proportion of leaf dry mass invested in Chl and EPheN. This ratio was used as a signature of the change in carbon–nitrogen balance within the leaf.

2.3. Measurements of plant growth and N content

Leaf area was measured with a Delta-T Image Analysis System (Cambridge, England). The leaves were then dried at 60 °C and weighed. LMA was calculated as the ratio of leaf dry mass to leaf area. The remaining shoots were dried at 60 °C and dry mass was measured. Shoot dry mass was also measured on 26 November 2002 in Viburnum, a slow-growing species, N content was measured as described by Dumas (1831). The samples were dried at 60 °C, weighed and ground. An aliquot of the powder was combusted in an elemental analyzer (Flash EA 1112, Thermo Electron S.A., Courtaboutef, France). The N concentration of the leaves used for optical measurements was recorded at each sampling date, and shoot N concentration was measured for several sampling dates. Both were expressed on a per mass basis. Leaf mass-based N content (NM) was converted into area-based N content (NA) by multiplying NM by LMA (NA = NM LMA).

2.4. Statistical analysis

Statistical analysis was carried out with Statgraphics (Statistical Graphics Corp., Statgraphics Plus for Windows 3.1, 1994–1997) and S-Plus (Insightful Corp., S-Plus 6 Professional Release 1, 1998–2001). The significance of differences between means was assessed by F-tests in ANOVA, equivalent to t-tests, on observations made on the same date: three replicates per treatment, each replicate corresponding to two plants. The normality assumption of the ANOVA was tested with a Shapiro–Wilk test on the residuals and an F-test was used to check the homoscedasticity assumption. In some cases, normality or homoscedasticity was severely violated, and in such cases, the two treatments were compared using a Wilcoxon test (Sokal and Rohlf, 1981). Correlation and regression techniques were used to analyze the relationships between LMA and NA, shoot and leaf N contents, and optical measurements and leaf N, using all the sampling dates and replicates for the species considered.

3. Results

3.1. Changes in plant dry matter, leaf N content and optical measurements during the treatments

Shoot dry mass increased as the plant aged. Fertilization significantly decreased shoot dry mass accumulation from 28 and 37 days after the start of treatment in Lagerstroemia and Callicarpa, respectively (data not shown). At the end of the treatments (days 49–51), shoot dry mass was 16.6 and 37.9 g/plant in Lagerstroemia for the TN1 and TN2 treatments, respectively, and 14.1 and 26.1 g/plant in Callicarpa for the TN1 and TN2 treatments, respectively. In Viburnum, shoot dry mass did not differ significantly between the TN1 and TN2 treatments over 50 days following the start of treatment, but was significantly lower for TN1 than for TN2 on day 176: 45.4 g/plant for TN1 and 73.3 g/plant for TN2 treatments (data not shown). In Lagerstroemia and Callicarpa, treatments affected leaf N content 2–3 weeks earlier than shoot dry mass (Fig. 1a and b). The effect of fertilizer application persisted throughout the experiment. In Viburnum, NM differed between treatments from day 15 onwards. Differences in NA were significant from day 8 onwards, but were no longer significant on days 22, 43 and 50 (Fig. 1c). In all three species, NA was less sensitive to fertilization than NM (Fig. 1), because N fertilization decreased LMA (Fig. 2), and this partly compensated for the increase in NM. Similarly, the general increase in LMA throughout the experiment as plant aged (Fig. 2) had a dilution effect on NM (Fig. 1).

SPAD values increased and Dualex values decreased in response to N fertilization in the three species (Fig. 3). The differences in SPAD values between the TN1 and TN2 treatments first became significant on days 15 and 23 in Lagerstroemia and Callicarpa, respectively (Fig. 3a and b). These differences remained significant throughout the experiment, with the
exception of day 51 for *Callicarpa*. In *Viburnum*, TN2 treatment significantly increased SPAD values from day 8, but values were no longer significantly higher on days 22, 43 and 50 (Fig. 3c). The lower SPAD values obtained for TN2 on days 43 and 50 were attributed to a sharp decrease in LMA (Fig. 2c). Dualex values differed significantly between TN1 and TN2 treatments from day 15, 23 and 22 onwards in *Lagerstroemia*, *Callicarpa* and *Viburnum*, respectively (Fig. 3). These differences then remained significant throughout the experiment. Dualex values remained more stable with the TN2 treatment than with the TN1 treatment. In *Viburnum*, on days 43 and 50, Dualex values had decreased strongly for the TN2 treatment. Like SPAD values, Dualex values were influenced by a decrease in LMA (Fig. 2c). In all three species, SPAD/Dualex ratio increased significantly in response to N fertilization from day 15, 23 and 29 onwards in *Lagerstroemia*, *Callicarpa* and *Viburnum*, respectively (Fig. 4).
treatment, the slope of the regression line of SPAD/Dualex ratio against time was not significantly different from zero (at 5% level, \( p \)-values, not shown), indicating that the SPAD/Dualex ratio remained stable throughout the experiment. For TN\(_1\) treatment, a similar analysis indicated that the ratio was stable from day 15, 23 and 29 onwards in *Lagerstroemia*, *Callicarpa* and *Viburnum*, respectively. This shows that N fertilization affected the allocation of leaf dry mass to proteins and polyphenols earlier in deciduous than in evergreen species. Dry mass allocation remained constant thereafter. One month after the start of the treatments, the difference in SPAD/Dualex ratio between treatments was more than 30% higher than the differences in SPAD values or Dualex values considered separately for the three species (Figs. 3 and 4).

3.2. Relationships between N, Chl and EPhen contents in young expanded leaves

Considering all sampling dates together for each species, the correlations between LMA, \( N_M \) and shoot dry mass as well as...
the correlations between optical measurements and leaf N content, expressed on a per unit area or mass basis were investigated (Table 1). This allows estimating the most reliable N indicators.

LMA and NM were strongly negatively correlated in the three species, with the highest correlation coefficient obtained for *Viburnum* ($r = -0.71$; Table 1). Thus the leaves with the highest LMA – thick leaves with dense tissues – had the lowest protein content, particularly in evergreen species, consistent with the universal leaf economic spectrum (Wright et al., 2004).

Leaf N content was strongly correlated with shoot N content in each species. This correlation was stronger if leaf N was expressed on a per unit mass basis (NM) rather than on a per unit area basis (NA). The relationship between NM and shoot N content was linear and similar in all three species (Fig. 5).

The correlation between SPAD and NM was strong and positive for the deciduous species but low and even negative for *Viburnum* (Table 1), as LMA strongly influenced SPAD values in *Viburnum*. There was also a strong positive correlation between SPAD and NM in all three species. Mass-based SPAD values (SPAD/LMA) were strongly correlated with NM in all three species, confirming the allocation of leaf N principally to photosynthetic proteins. Measurements of leaf Chl content by a wet chemistry method (data not shown) gave similar correlations to SPAD values. Dualex measurements were strongly negatively correlated with NM in all three species (Table 1). The relationship between the dependent variable, Dualex value and the predictor variable, NM, was species-dependent and did not give a straight line (Fig. 6). As suggested by this figure and using the basic transformations generally used in linear models (Draper and Smith, 1981), the best fit for the three species was obtained with logarithmic transformation of the predictor variable. The lower determination coefficient

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**Table 1**

<table>
<thead>
<tr>
<th>Y vs. X relationships</th>
<th>Lagerstroemia</th>
<th>Callicarpa</th>
<th>Viburnum</th>
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<tbody>
<tr>
<td>LMA vs. NM</td>
<td>$-0.487 (36)^{**}$</td>
<td>$-0.545 (42)^{***}$</td>
<td>$-0.708 (48)^{***}$</td>
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<tr>
<td>N&lt;sub&gt;4&lt;/sub&gt; vs. shoot N</td>
<td>0.942 (36)&lt;sup&gt;***&lt;/sup&gt;</td>
<td>0.878 (18)&lt;sup&gt;***&lt;/sup&gt;</td>
<td>0.939 (18)&lt;sup&gt;***&lt;/sup&gt;</td>
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<tr>
<td>N&lt;sub&gt;A&lt;/sub&gt; vs. shoot N</td>
<td>0.465 (36)&lt;sup&gt;***&lt;/sup&gt;</td>
<td>0.837 (18)&lt;sup&gt;***&lt;/sup&gt;</td>
<td>0.679 (18)&lt;sup&gt;***&lt;/sup&gt;</td>
</tr>
<tr>
<td>SPAD values vs. NM</td>
<td>0.847 (36)&lt;sup&gt;***&lt;/sup&gt;</td>
<td>0.441 (42)&lt;sup&gt;**&lt;/sup&gt;</td>
<td>$-0.311 (48)^*$</td>
</tr>
<tr>
<td>SPAD values vs. N&lt;sub&gt;A&lt;/sub&gt;</td>
<td>0.720 (36)&lt;sup&gt;***&lt;/sup&gt;</td>
<td>0.458 (42)&lt;sup&gt;**&lt;/sup&gt;</td>
<td>0.522 (48)&lt;sup&gt;***&lt;/sup&gt;</td>
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<tr>
<td>(SPAD values/LMA) vs. NM</td>
<td>0.565 (36)&lt;sup&gt;***&lt;/sup&gt;</td>
<td>0.658 (42)&lt;sup&gt;***&lt;/sup&gt;</td>
<td>0.720 (48)&lt;sup&gt;***&lt;/sup&gt;</td>
</tr>
<tr>
<td>Dualex values vs. NM</td>
<td>$-0.917 (30)^{***}$</td>
<td>$-0.695 (30)^{***}$</td>
<td>$-0.916 (48)^{***}$</td>
</tr>
<tr>
<td>Dualex values vs. N&lt;sub&gt;A&lt;/sub&gt;</td>
<td>$-0.378 (30)^*$</td>
<td>$-0.097 (30)$ ns</td>
<td>$-0.209 (48)$ ns</td>
</tr>
<tr>
<td>(Dualex values/LMA) vs. NM</td>
<td>0.179 (30) ns</td>
<td>0.183 (30) ns</td>
<td>0.394 (48)&lt;sup&gt;***&lt;/sup&gt;</td>
</tr>
<tr>
<td>(Dualex/Dualex ratio) vs. NM</td>
<td>0.918 (30)&lt;sup&gt;***&lt;/sup&gt;</td>
<td>0.648 (30) ns</td>
<td>0.680 (48)&lt;sup&gt;***&lt;/sup&gt;</td>
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Abbreviations: LMA, leaf mass per unit area (g cm$^{-2}$); N<sub>4</sub>, leaf area-based N content (mg cm$^{-2}$); N<sub>A</sub>, leaf mass-based N content (%); shoot N, shoot mass-based N content (%). SPAD values, Dualex values and SPAD/Dualex ratio have no units. SPAD/LMA and Dualex/LMA are mass-based expressions of SPAD and Dualex values, respectively. For each species, all the sampling dates (from 3 June to 31 July 2002) and replicates (three replicates, each consisting of two plants and 10 leaves per plant) were used to test for correlations. Significance: ns, non-significant ($P \geq 0.05$), *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

*a* In parentheses: sample size.

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**Fig. 5.** Relationship between the N content of the shoots and that of the young leaves expressed per unit mass (NM). The regression equation is: $y = 1.260 x$ ($R^2 = 0.88$). Each point represents one replicate (two plants, 10 leaves per plant).

**Fig. 6.** Relationship between the N content of young leaves expressed per unit mass (NM) and Dualex values. The regression equations are: *Lagerstroemia*: $y = -9.21n (x) + 36.6$ ($R^2 = 0.85$); *Callicarpa*: $y = -10.21n (x) + 38.7$ ($R^2 = 0.53$); *Viburnum*: $y = -14.21n (x) + 38.1$ ($R^2 = 0.83$). Each point corresponds to one replicate (two plants, 10 leaves per plant).
for Callicarpa ($R^2 = 0.53$) than for Lagerstroemia ($R^2 = 0.85$) and Viburnum ($R^2 = 0.83$) was due to a scattering of the points for $N_M$ exceeding 2.7%. Dualex values were correlated with $N_A$ in Lagerstroemia ($r = -0.38$; Table 1) only, but the correlation was weak. Dualex measurements divided by LMA, to obtain mass-based units, were not correlated with $N_M$ in the two deciduous species and were only weakly positively correlated with $N_M$ in Viburnum ($r = 0.39$; Table 1). Thus, the strong correlation between Dualex values and $N_M$ was influenced principally by LMA. The SPAD/Dualex ratio and $N_M$ were positively and tightly correlated in all three species (Table 1). This shows that $N_M$ was allocated to leaf proteins (represented by Chl) in a constant proportion that differed between the species (Fig. 3).

4. Discussion

N fertilization increased shoot growth and foliar N content ($N_M$), consistent with the results obtained by Cabrera (2003). The response to fertilization was slower in Viburnum than in the other two species. Internal N remobilization must have supplied demands for the growth of new shoots at the start of the growing season in this slow-growing species (Millard, 1996; Bollmark et al., 1999). N fertilization affected both the anatomy and the chemical composition of leaves. It decreased LMA, confirming the frequently described decrease in leaf dry mass in response to N fertilization (Witkowski and Lamont, 1991). It increased mass-based N and Chl contents but had little effect on mass-based EPhen content. Thus, fertilization increased the N and carbon demand for protein synthesis for growth and photosynthesis, as described by Jones and Hartley (1999). As TN2 treated plants experienced no environmental stress (high irradiance, high UV levels, injury) that was not experienced by TN1 treated plants, the carbon demand for polyphenol synthesis did not increase with N fertilization. The leaf carbon/nitrogen ratio therefore decreased in response to fertilization as suggested by the positive correlation between SPAD/Dualex ratio and $N_M$. The slight decrease in LMA in N fertilized plants should lead to the slight, passive concentration of polyphenols and proteins. However, LMA increased during the course of the experiment, and the main passive mass effect was therefore a dilution of leaf N, Chl and EPhen amounts due to leaf dry mass accumulation as the plant aged. These results are generally consistent with models predicting the influence of N availability on leaf polyphenol content (Koricheva et al., 1998; Jones and Hartley, 1999), making it possible to define optical signatures of EPhen and carbon–nitrogen balance within the leaf as indicators of $N_M$. As leaf and shoot N expressed on a per unit mass basis were strongly and similarly correlated in all species, optical signatures of $N_M$ can be considered as indicators of shoot N for these species.

The best indicator would be non-invasive, rapid, sensitive, specific for plant N status and suitable for use with a wide range of species (Meynard et al., 1997). In this study (i) Dualex value was the optical measurement most strongly correlated with $N_M$, which was strongly correlated with shoot N content in all three species; (ii) the SPAD/Dualex ratio could be used to detect the change in leaf dry mass allocation to proteins and polyphenols in response to fertilization in all three species. SPAD measurements could also be used as an indicator of N nutrition, but only for the two deciduous species (early diagnosis of N deficiency, consistency of the response over time). In Viburnum, SPAD values were not consistent over time because of large LMA variations. LMA and Chl$_{M}$, the two components of area-based Chl measurements by SPAD, are negatively and positively correlated with $N_M$, respectively. For this reason, SPAD values are only moderately correlated with $N_M$ and cannot be used to predict $N_M$ accurately in Viburnum and other woody species (Chang and Robison, 2003; Ifon et al., 2005), restricting the domain of application of SPAD for woody plants. Unfortunately, no non-destructive technique for monitoring LMA in the field is currently available. In contrast, LMA and EPhen$_{M}$, the two components of area-based EPhen, recorded by Dualex, are both negatively correlated with $N_M$ (Table 1; Meyer et al., 2006). However, Dualex values varied more strongly with leaf dry mass than with dry mass allocation to polyphenols, as shown by the strong negative correlation between Dualex values and $N_M$ and the absence of correlation or slight positive correlation between Dualex/LMA and $N_M$. The relationship between Dualex values and $N_M$ was driven by that between LMA and $N_M$ in the three species (Meyner et al., 2006). The relationship between LMA and $N_M$ has been demonstrated in a very wide range of species (Wright et al., 2004), and the relationship between Dualex values and $N_M$ is therefore also likely to be reliable in other ornamental species. The SPAD/Dualex ratio provides another indicator of $N_M$. This ratio probes changes in leaf chemical composition rather than the dry mass dilution effect with plant aging or treatments. It may therefore be only weakly correlated with $N_M$ in slow-growing species such as Viburnum, which displays a delayed response to fertilization. Measurements establishing this relationship in Viburnum should have been spread over a longer period of time. The main advantage of the SPAD/Dualex ratio is the amplitude of its response to fertilization and its physiological significance.

The plant response to N nutrition was detected early and large amounts of data were rapidly monitored using three indicators: SPAD, Dualex and SPAD/Dualex values. All detected N fertilizer requirement several weeks before the decrease in shoot dry mass accumulation, and probably before any change in total dry mass accumulation, as shoot growth is more sensitive to N deficit than root growth (Millard and Proe, 1991, 1993). Dualex and SPAD are portable leaf-clip devices that are easy to use and generate immediate non-destructive measurements. Dualex measurements took longer than SPAD measurements, because one measurement was made on each side of the leaf, but this was probably not entirely necessary as the values for the adaxial and abaxial surfaces were strongly correlated (Lagerstroemia: $r = 0.86, n = 30, P < 0.001$; Callicarpa: $r = 0.80, n = 30, P < 0.001$; Viburnum: $r = 0.88, n = 48, P < 0.001$). Similar results have also been obtained with Acer and Fraxinus saplings (Barthod et al., 2007). Furthermore, the time course over which indicators should be monitored and the frequency of recordings should be adapted to the growth rate of the species considered.
Limitations to the use of optical signatures include the effects on SPAD and Dualex values of other factors that affect Chl and/or EPhen content and leaf structure, such as injury, high light intensity, water stress, leaf age and senescence (e.g. Long et al., 2001; Martinez and Guiamet, 2004; Barthod et al., 2007). This may limit the specificity of optical indicators, and seems to be an inherent restriction of methods based on the optical properties of Chl (Richardson et al., 2002). Water stress is generally avoided in nurseries growing ornamental plants in containers. Variation due to leaf age can be limited by selecting the youngest leaves that have already reached 75% of their final size. This age criterion is less accurate than real leaf age, but is simple and suitable for routine recordings in ornamental plant nurseries—a major issue if these results are to be transferred into horticultural practice. Only leaves exposed to direct sunlight (at the top of the canopy) were sampled, limiting variation in SPAD and Dualex values, and NSD due to leaf incident radiation. Another limitation is the species-dependence of SPAD and Dualex values. If the indicators are to be used routinely in ornamental nurseries, specific calibration equations relating indicators to N status will need to be established in advance. N fertilizer recommendations may also be based on a relative approach, with growers using a few plants as overfertilized controls.

In conclusion, Dualex measurements and the SPAD/Dualex ratio are more promising methods than SPAD measurements alone for the assessment of N status in situ. This should make it possible to avoid the overfertilization of woody ornamental crops. The SPAD/Dualex ratio also provides a means of assessing in situ the change in carbon/nitrogen economy within leaf in response to fertilization. Studies of these indicators should be carried out on other species, with the building of a standard scale and definition of the threshold values useful for managing fertilizer applications for given ornamental woody species and other crops.

Acknowledgments

We would like to thank L. Bidel (INRA, UMR SAGAH) for helpful discussions during the setting up of the protocols and for assistance in running the experiments. G. Guillermian, G. Céral (INRA, UMR SAGAH) and Benoît Le Gac (trainee INRA, UMR SAGAH) were responsible for the experimental measurements, R. Guisnel, J.-N. Reynaud and G. Sintès (INRA, UMR SAGAH) were responsible for maintaining growth conditions. Y. Baraud-Roussel, E. Billaud (INH UMR SAGAH) and M. Sigogne (INRA, UMR SAGAH) carried out the chemical analyses. J. Sappa (scientific translator, Alex Edelman & Associates) reviewed the paper for English usage. This work was supported by the CNRS through the GDR 1536 ‘FLUOVEG’.

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