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Regular paper

Visible foliar injury caused by ozone alters the relationship between SPAD meter readings and chlorophyll concentrations in cutleaf coneflower

Howard S. Neufeld[1,*], Arthur H. Chappelka[2], Greg L. Somers[2], Kent O. Burkey[3], Alan W. Davison[4] & Peter L. Finkelstein[5]

*[1]Department of Biology, Appalachian State University, 572 Rivers St., Boone, NC 28608, USA; [2]School of Forestry and Wildlife Sciences, Auburn University, 108 M. White-Smith Hall, Auburn, AL 36849, USA; [3]USDA-ARS Plant Science Research Unit, 3127 Ligon Street, Raleigh, NC 27607, USA; [4]Department of Biological Sciences, University of Newcastle, Newcastle, NE1 7RU, UK; [5]Atmospheric Modeling Division, NOAA: on assignment to US EPA, Research Triangle Park, NC 27711, USA; *Author for correspondence (e-mail: neufeldhs@appstate.edu; fax: +1-828-262-2127)*

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Abstract

The ability of the SPAD-502 chlorophyll meter to quantify chlorophyll amounts in ozone-affected leaves of cutleaf coneflower (*Rudbeckia laciniata* var. *digitata*) was assessed in this study. When relatively uninjured leaves were measured (percent leaf area affected by stipple less than 6%), SPAD meter readings were linearly related to total chlorophyll with an adjusted R^2 of 0.84. However, when leaves with foliar injury (characterized as a purple to brownish stipple on the upper leaf surface affecting more than 6% of the leaf area) were added, likelihood ratio tests showed that it was no longer possible to use the same equation to obtain chlorophyll estimations for both classes of leaves. Either an equation with a common slope or a common intercept was necessary. We suspect several factors are involved in altering the calibration of the SPAD meter for measuring chlorophyll amounts in visibly ozone-injured leaves, with the most likely being changes in either light absorption or scattering resulting from tissue necrosis.

Introduction

The ability to detect subtle changes in chlorophyll in response to stress has been greatly enhanced by the development of the SPAD-502 chlorophyll meter (Konica-Minolta Corporation, Ramsey, NJ), which can non-destructively estimate total chlorophyll amounts in leaves of a variety of species with a high degree of accuracy (Gratani 1992; Finnan et al. 1997; Samdur et al. 2000; Azia and Stewart 2001). It has also been successfully used to estimate foliar N amounts (Chang and Robison 2003; Young et al. 2003), leaf absorptances in the field (Earl and Tollenaar1997) and photosynthetic rates (Ma et al. 1995). The meter works by measuring the ratio of the transmittance to light at 920 nm, which is essentially unaffected by the leaf chlorophyll, to that at 650 nm, which is strongly absorbed by the chlorophyll. Generally, this results in a near-linear species-specific relationship between the ratio of the absorbances and leaf chlorophyll amount.

The chlorophyll content of leaves can be a useful diagnostic indicator of the health and potential physiological performance of a plant (Kumar et al. 2002). Chlorophyll amounts change during leaf development (Costa et al. 2001), and can be altered in response to a wide variety of environmental stresses (Fanizza et al. 1991;

Samdur et al. 2000; Lawson et al. 2001), including air pollution (Carter et al. 1995). Ozone can induce the loss of chlorophyll and cause premature leaf senescence in a variety of plant species (Tenga and Ormrod 1990; Ommen et al. 1999), but only a few studies have used the SPAD meter to monitor changes in chlorophyll content after ozone exposure. Bindi et al. (2002) and Lawson et al. (2001), using data derived from a SPAD-502 meter, reported decreases of up to 12% in chlorophyll content following exposure to either elevated CO_2 or ozone in potatoes. Ommen et al. (1999) also found similar results with wheat, while Tenga and Ormrod (1990) discovered large decreases in chlorophyll in tomato leaves exposed to ozone for four consecutive days, a characteristic that was also evident in leaves that did not show any signs of visible foliar injury.

While these studies illustrate the efficacy of using the SPAD meter to estimate chlorophyll amounts in plants during the early stages of injury, none have reported using the instrument on leaves with extensive visible injury. Ozone induces a response known as foliar stipple, which is the discoloration of small patches of cells on the adaxial leaf surface, caused by either the production of colored pigments, such as anthocyanins or phenols (Krupa et al. 2000), or by the loss of chlorophyll (Tonneijck and van Dijk 2002). Prolonged exposure eventually leads to cell death (Evans et al. 1996), with necrotic lesions often coalescing into larger patches of dead tissue. This combination of chlorophyll loss, synthesis of colored pigments, and necrosis also leads to large changes in the spectral qualities of ozone stressed leaves (Carter et al. 1992; 1995; Neufeld, unpublished data).

We report in this study a reduced ability of the SPAD meter to adequately estimate chlorophyll concentrations in leaves that exhibit significant amounts of ozone-induced foliar stipple. The reasons for this failure are discussed, and precautions for using this meter are prescribed if leaves that are to be measured have significant amounts of foliar stipple.

Materials and methods

We chose to measure chlorophyll amounts in leaves of the ozone-sensitive plant cutleaf coneflower *(Rudbeckia laciniata var. digitata*). Our group has previously demonstrated that this plant is extremely sensitive to ambient levels of ozone in Great Smoky Mountains National Park (Chappelka et al. 2003; Davison et al. 2003; Finkelstein et al. 2004). Plants were sampled from a large population growing along a forest edge at the Appalachian Highlands Science Learning Center at Purchase Knob, Great Smoky Mountains National Park, North Carolina (Lat 35°35′14″ N, Long 83°04′31″ W, elevation 1494 m).

Some individuals of cutleaf coneflower are more sensitive to ozone than others and exhibit symptoms to a greater extent than neighboring plants (Chappelka et al. 2003). We sampled leaves on the flowering stems from both sensitive and insensitive individuals, deliberately selecting leaves with a range of visible injury from 0 to $>75\%$ of the surface area. Leaves were rated for the amount of stipple using a modified Horsfall-Barratt rating scale (Horsfall and Barratt 1945; 1 = 0%, 2 = 1–6% injured, 3 = 7–25%, 4 = 26–50%, 5 = 51–75%, 6 > 75%).

A SPAD-502 meter was used to obtain a measure of the chlorophyll content of each leaf. Three readings per leaf were averaged. Afterwards, 1.0 cm^2 of tissue was removed from each leaf near the location where the SPAD meter reading was obtained, immediately immersed in 3 ml of *N*,*N*′-dimethylformamide and allowed to extract in the dark for 24 h. A Shimadzu UV-1201 spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD) was used to read absorbances at 647 and 664 nm after zeroing at 750 nm; chl *a*, chl *b*, and total chl amounts were calculated according to the equations in Porra et al. (1989) and Welburn (1994). Various regression models were used to relate the SPAD readings to the chlorophyll amounts, and likelihood ratio tests were used to determine differences in slopes and intercepts between regressions for uninjured and injured leaves (Neter et al. 1990; SAS Institute 1999).

Results

After testing a variety of models, including logarithmic, exponential and quadratic, the simplest, best-fit model for the coneflowers in this study was linear. While a single equation was significant ($P < 0.001$ and adjusted $R^2 = 0.84$) the relationship between the SPAD readings and total

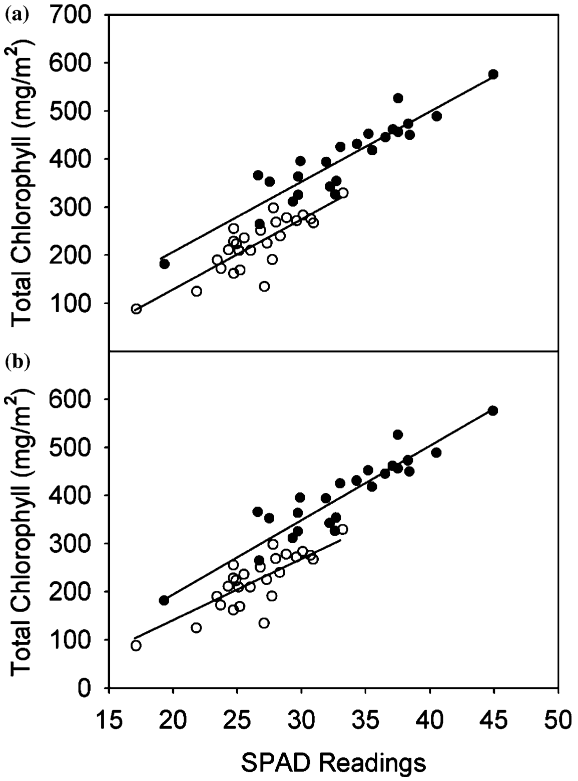

Figure 1. Total chlorophyll vs SPAD meter readings for injured and uninjured leaves. Closed circles, uninjured leaves ($N = 24$); open circles, injured leaves ($N = 26$). (a) separate intercepts and common slope; (b) common intercept and separate slopes. See Table 1 for equations.

chlorophyll appeared to change as foliar injury increased (Figure 1). The data were divided into two groups of relatively uninjured leaves (rating < 3, less than 6% leaf area affected) and injured leaves (more than 6% leaf area affected). Likelihood ratio tests rejected the null hypothesis that a single equation with common intercepts and slopes was the same as separate equations for each group ($P < 0.001$). Further tests accepted the null hypothesis of either a common slope ($P = 0.916$, adjusted $R^2 = 0.92$) or a common intercept ($P = 0.318$, adjusted $R^2 = 0.91$). This implies that while a single equation is not sufficient, either separate slopes or separate intercepts are all that are necessary. Both sets of coefficients are provided in Table 1 and shown in Figure 1a for common slope and Figure 1b for common intercept.

Using analysis of variance and reducing the leaf injury classes from six to three by combining classes 1 and 2 (low injury), 3 and 4 (moderate injury), and 5 and 6 (severe injury), showed that leaves with low injury had significantly more total chlorophyll than either moderately or severely injured leaves (Table 2). The same pattern was found for the chlorophyll *a*:*b* ratio, which decreased monotonically from low to severely injured leaves (Table 2).

Interestingly, despite the shift in the chlorophyll *a*:*b* ratio with increasing injury, chlorophyll a and chlorophyll b were highly and positively correlated with each other ($r = 0.99$) with a positive slope (data not shown). Even though both chl a and chl b decreased as foliar injury increased, the chlorophyll *a*:*b* ratio also significantly decreased with increasing injury, primarily due to a greater loss of chlorophyll a (Figure 2). Because the relationship between the SPAD readings and chlorophyll *a*:*b* ratio depends on the amount of foliar injury, separate equations (Table 1) were also necessary for injured and relatively uninjured leaves ($P = 0.010$, adjusted $R^2 = 0.54$).

Table 1. Equations to predict total chlorophyll and chlorophyll *a*:*b* ratio from SPAD meter readings

Leaf type	Parameter		Adjusted R^2	Mean square error
	Intercept	Slope		
Total chlorophyll – common slope				
Injured	−161.8	14.548	0.918	1117.5
Uninjured	−83.86	14.548		
Total chlorophyll – common intercept				
Injured	−113.56	12.747	0.916	1141.9
Uninjured	−113.56	15.422		
Chlorophyll *a*:*b* ratio				
Injured	1.3957	0.034885	0.541	0.020441
Uninjured	2.1795	0.011276		

Table 2. Total chlorophyll and the chlorophyll *a:b* ratio as a function of leaf injury class.* Values followed by same letter are not different at $P < 0.05$. $N = 24$, 13, and 13 for low, moderate and severe injury classes, respectively. *Note:* $P < 0.001$ for ANOVA significance for both parameters

Parameter	Injury class		
	Low	Moderate	Severe
Total chlorophyll (mg/m^2)	399.1 ± 17.61^a	249.9 ± 11.66^b	199.7 ± 13.06^b
Chlorophyll *a:b* ratio	2.55 ± 0.030^a	2.43 ± 0.030^b	2.20 ± 0.034^c

* Injury classes are: Low ≤ 6%, Moderate = 7 to 50%, Severe > 50% leaf area stippled.

Discussion

The ability of the SPAD meter to estimate chlorophyll amounts in cutleaf coneflower leaves was quite satisfactory as long as the leaves did not exhibit extensive foliar injury from ozone. When the injury rating was less than 3 (corresponding to less than 6% of the leaf area stippled) a linear relationship provided a good fit for total chlorophyll amount, similar to that of other researchers (Gratani 1992; Castelli et al. 1996; Finnan et al. 1997; Azia and Stewart 2001). Some studies have found quadratic models to work best (Castelli et al. 1996; Finnan et al. 1997; Azia and Stewart 2001), but the exact fit seems to depend on the species used, as both linear (Samdur et al. 2000) and exponential models have proved successful (Markwell et al. 1995).

When leaves had an injury ranking of 3 or greater (>6% leaf area injured), a separate intercept was required to adjust the chlorophyll estimates for the injury. This shows that the SPAD meter becomes less reliable for estimating leaf chlorophyll amounts when leaves are moderately to severely injured by ozone in this species.

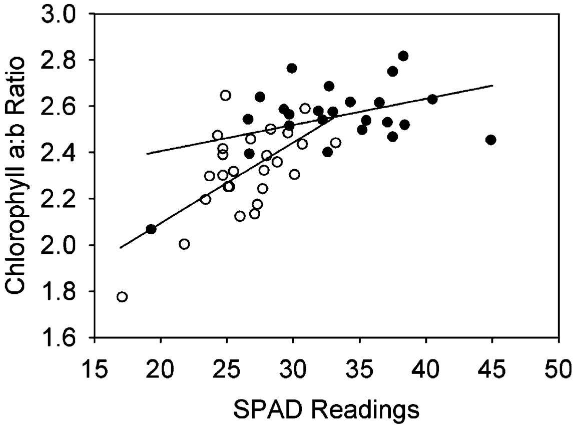

Figure 2. Chlorophyll *a:b* ratio vs SPAD meter reading with separate slopes and intercepts for injured and uninjured leaves. Closed circles, uninjured leaves ($N = 24$); open circles, injured leaves ($N = 26$). See Table 1 for equations.

Why the meter becomes unreliable in the face of large amounts of stipple is not yet fully understood. However, there are several possible causes. First, coneflower leaves produce brown, water soluble phenolic compounds in the epidermal cells upon exposure to ozone (Neufeld, unpublished data). They may also produce anthocyanins. However, Manetas et al. (1998) demonstrated that the calibration of the SPAD-502 was not affected by differing amounts of anthocyanins in leaves, which have their primary absorption peak at 520 nm. Although most phenolics preferentially absorb in the UV-B range, which theoretically should not interfere with the operation of the SPAD meter, Peñuelas and Filella (1998) have shown that brown, water soluble pigments decrease reflectance of long-wave radiation in ozone-injured as compared to uninjured leaves, beginning at about 750 nm and carrying on through to at least 1000 nm. In fact, Peñuelas et al. (2004) have proposed using a brown pigment index based on spectral reflectances as an indicator of oxidative stress in plants. This decrease in reflectance may occur close enough to the 920 nm reference wavelength of the meter to alter its calibration. If the decrease in reflectance results in a uniform increase in transmittance at 920 nm in injured leaves, then this could explain the pattern shown in Figure 1b, where the slopes for injured and uninjured leaves are similar, but the intercepts are offset. However, a lower reflectance may indicate increased absorption, and *less* transmission, so the role of phenolics is still unclear.

Another possibility is that severely injured leaves may simply have too little chlorophyll for the SPAD meter to read accurately. The SPAD-502 becomes less accurate when chlorophyll amounts are low (Gratani 1992), especially if reduced below 100 mg/m^2 (Monje and Bugbee 1992). However, chlorophyll amounts for injured leaves, with the exception of one leaf, were consistently above the 100 mg/m^2 minimum, and some values were in the same range as uninjured leaves (Figure 1). Thus for the most part, both sets of leaves were within the operational range of the meter, suggesting that chlorophyll deficiency was not the primary factor. Alternatively, perhaps ozone injury leads to greater spatial variation of chlorophyll within the leaf, which in turn, introduces additional scatter in the SPAD/total chlorophyll relationship. To cope with this latter possibility, we did try to sample the chlorophyll from the same area where we obtained the SPAD readings (which were an average of readings from three different locations on the leaf), but it is still possible that we did not get the exact same area.

The most plausible explanation is that the presence of necrotic tissue probably changes the amount of light transmitted through the leaf. The loss of cytoplasm and pigments in the dead cells would reduce absorption and thus enhance light transmission, whereas the collapse of affected cells would alter light scattering properties. In reality, light transmission through injured leaves is probably determined by a combination of absorption and scattering changes that could vary with overall foliar injury. It is known that variation in specific leaf mass affects the calibration of the meter (Yamamoto et al. 2002) as do differences in light scattering and chlorophyll distribution within the leaf (Monje and Bugbee 1992; Castelli et al. 1996). Severely stippled leaves eventually contain many dead cells in the upper epidermis and mesophyll layers, which would alter the leaf specific weight and spectral properties. Perhaps more light is transmitted through injured leaves at the 920 nm wavelength, which would increase the ratio of transmittances (as mentioned above), and explain why the SPAD readings are higher in injured leaves at a given chlorophyll content than in uninjured leaves (see Figure 1b). Further studies with the SPAD meter should be conducted to assess the role of necrotic lesions in affecting light scattering and transmission. Finally, the weaker predictive relationship for the injured leaves may be due to a combination of all the above factors, leading to a reduced ability of the SPAD-502 to accurately assess chlorophyll in ozone-injured leaves.

As a result of our findings, we caution that even though the SPAD-502 meter can be used to assess the early loss of chlorophyll in ozone-affected leaves, it may become unreliable if ozone injury levels reach above a certain species-specific threshold. Researchers wanting to follow ozone-induced losses of chlorophyll in species that exhibit appreciable foliar stipple are advised to perform separate calibrations for injured and uninjured leaves to assure themselves that the meter can reliably measure chlorophyll over the observed range of injury. Increasing the sample sizes can help to overcome some of the problems associated with the larger variability in injured leaves. Further studies should be conducted to determine whether this is a general problem with the SPAD meter for estimating chlorophyll amounts in other ozone sensitive species.

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