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# *In situ* estimates of waterhyacinth leaf tissue nitrogen using a SPAD-502 chlorophyll meter☆

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ABSTRACT

Previous investigations with *Eichhornia crassipes* and its biological control agents *Neochetina bruchi* and *N. eichhorniae* identified leaf tissue nitrogen (N) as a driver in their interactions. However, traditional methods for determining plant tissue N content are cumbersome, time-consuming, and destructive–and thus unsuited for rapid *in situ* evaluations. We therefore tested the utility of a hand-held chlorophyll meter as a means of producing *in situ* estimates of N in the leaves of this floating aquatic weed. The Minolta SPAD-502 chlorophyll meter provided excellent estimates ($F = 385.96$, $P < 0.0001$) of leaf tissue N levels. SPAD readings varied within leaves ($F = 78.66$, $P < 0.0001$), so average readings per leaf were used. The relationship between SPAD readings and tissue N levels was affected by the phenological stage of the leaf ($F = 102.79$, $P < 0.0001$), but not leaf size ($F = 0.75$, $P = 0.3867$). The estimates were also unaffected by fertilizer level ($F = 0.95$, $P = 0.4354$), but were marginally affected by herbivory ($F = 3.86$, $P = 0.0505$). Thus, with suitable calibration (e.g., different field sites, presence and type of herbivory) output from the SPAD-502 could be used to provide consistent estimates for the nitrogen content of *E. crassipes* leaves.
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## 1. Introduction

Plant nutritional quality, especially tissue nitrogen concentrations, can profoundly affect the outcome of insect–plant interactions (White, 1993; Larsson et al., 2000) and thereby determine the success or failure of biological control of invasive weeds programs. We have been studying the interactions between *Eichhornia crassipes* (Mart.) Solms and two biological control agents, the weevils *Neochetina bruchi* Hustache and *N. eichhorniae* Warner, over the past several years in order to understand why biological control outcomes have been so variable (Center and Dray, 1992; Center et al., 1999a,b; Center and Dray, 2010). These studies have shown that leaf tissue nitrogen levels (1) drive weevil population dynamics by directly affecting reproductive capacities (Center and Dray, 2010), and (2) may thus be key determinants to successful biological control. The associated studies required production of waterhyacinth plants with predictable nutritional quality, and the ability to monitor *in situ* the nitrogen concentrations available to the insects.

Assessment of tissue nitrogen requires time consuming analyses using expensive equipment which precludes *in situ* measurements (Richardson et al., 2002). It also requires destruction of the tissue which precludes repeated assessments of changes over time (Murillo-Amador et al., 2004). There is a close correlation between chlorophyll and nitrogen in leaf tissue content (Evans, 1989). Thus, hand-held meters used to estimate chlorophyll content offer an inexpensive, non-destructive means of estimating leaf nitrogen levels. These meters have proven useful for detecting nutrient deficiencies in a variety of agricultural commodities (e.g., Richardson et al., 2002; Murillo-Amador et al., 2004; Spaner et al., 2005; Uddling et al., 2007). A few such studies have focused on weeds (e.g., Kapotis et al., 2003) and/or aquatic vegetation (e.g., Biber, 2007; Spencer et al., 2007).

Recently, this technique has begun to be used to explore plant–insect interactions relating to biological control of weeds projects (e.g., Coetzee et al., 2007; Goolsby et al., 2009). Careful review of studies employing hand-held chlorophyll meters suggests results are species specific and affected by environmental variables. Therefore, we wished to determine if a hand-held chlorophyll meter could provide consistent, efficient, and non-destructive estimates of nitrogen levels in *E. crassipes* leaves, and thereby facilitate investigations of interactions between this weed and the two plant-feeding insects *N. bruchi* and *N. eichhorniae*. To do so, we compared leaf tissue nitrogen levels from plants grown under various fertilizer regimes with readings from a SPAD-502 chlorophyll meter to answer a series of questions: (1) does location sampled on the leaf, or the size or phenological stage of the leaf, affect SPAD readings; (2) are differences in fertilizer level reflected in either leaf tissue nitrogen or SPAD readings, or both; (3) do SPAD

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**Table 1**
Summary of the four experiments comprising this investigation.

| Exp. | Dates | Fertilizer levels (no.) | Herbivory | Leaves sampled[a] | Data[b] |
|---|---|---|---|---|---|
| 1 | August–September 2007 | 16 | No | M | SPAD, N, & A[c] |
| 2 | November–December 2007 | 16 | No | M | SPAD |
| 3 | February–August 2008 | 16 | No | M & C | SPAD & N |
| 4 | June–September 2008 | 5 | Yes | M & C | SPAD, N, & A |

[a] M, youngest mature leaf; C, unfurling central leaf.
[b] SPAD, relative chlorophyll content; N, leaf nitrogen content (% dry wgt); A, leaf area ($cm^2$).
[c] For experiment 1 nitrogen and leaf area were only measured once, on the last sampling date.

readings consistently reflect leaf tissue nitrogen levels and does this relationship change across fertilizer regimes, and (4) does feeding by the two waterhyacinth weevils affect the SPAD-leaf nitrogen relationship.

## 2. Methods and materials

### 2.1. Experimental set-up

The research described herein represents a series of four experiments conducted over a thirteen month period in 2007 and 2008. Table 1 summarizes the experimental treatments and types of data collected. Experiments 1–3 were carried out in four concrete tanks with a capacity of 1144 L and measuring 0.8 m wide × 2.2 m long × 0.65 m deep. Each tank was covered with a screened (mesh size 8 × 6.5 strands/cm) cage measuring 0.8 m wide × 2.2 m long × 0.75 m high to exclude herbivores. Sixteen plastic tubs filled with 16.6 L of tap water were placed in each tank. The sixteen tubs were each treated with a different level of fertilizer, and tubs were assigned random positions within the tank. Each tank thus constituted a randomized block. Slow-release fertilizer (Osmocote® Plus, Scotts Miracle-Gro Company, Marysville, Ohio, USA; see also Husby, 2000) was added to each tub once at the beginning of the experiment at rates ranging from 0.055 to 0.886 g $L^{-1}$ in 0.055 g $L^{-1}$ increments. Five *Eichhornia crassipes* (waterhyacinth) plants with bulbous petioles and roots ranging in length from 10 cm to 40 cm were placed in each tub at the beginning of each experiment.

Experiment 4 was conducted in concrete tanks similar to those described above, but without the small tubs. Each of these tanks was filled with 880 L of well water and inoculated with twenty *E. crassipes*. Fertilizer was applied once at the beginning of the experiment at rates of 0.034 to 0.578 g $L^{-1}$ in 0.136 g $L^{-1}$ increments of Osmocote® Plus (the same formulation as above), together with corresponding levels of iron (0.001, 0.006, 0.011, 0.016, and 0.020 g $L^{-1}$) in the form of Miller® Iron Chelate DP (10% Fe). One of four weevil treatments (both *Neochetina* species together, *N. bruchi* alone, *N. eichhorniae* alone, or no weevils) was applied to each tank, as well. Each weevil treatment consisted of a pair of weevils (one ♂ and one ♀) $plant^{-1}$, with the mixed treatment containing half *N. bruchi*, and half *N. eichhorniae*.

### 2.2. Analyses

The SPAD-502 chlorophyll meter (Konica-Minolta Sensing, Inc.: Osaka, Japan) measures the absorbance of two distinct wavelength regions in a 2 × 3 mm area of the leaf and uses these data to generate a (unit-less) value representing the relative amount of chlorophyll present in the leaf. Initial readings were collected one week after fertilizer was added. Three readings were taken on undamaged portions of the adaxial surface of each sampled leaf: 15 mm from the edge of the lamina on the right and left sides at the widest part of the leaf, and 15 mm from the apex along the leaf midline. The unfurling central leaf and youngest mature leaf was sampled on each of three plants per tub (experiments 1–3) or tank (experiment 4). Preliminary trials (experiments 1 and 2) involved collecting weekly SPAD readings, to determine whether the meter could differentiate between fertilizer levels (Table 1). In later trials (experiments 3 and 4), the SPAD readings were recorded opportunistically and then the sample leaves were excised, placed in labeled bags, and dried at 50 °C to constant weight. The dry samples were weighed, ground in a Wiley mill to pass a 40-mesh screen, and then analyzed using a C-H-N analyzer (Perkin-Elmer® Series II CHNS/O Analyzer Model 2400; PerkinElmer Life and Analytical Sciences: Shelton, Connecticut, USA) to measure percentage nitrogen content for comparison with the SPAD readings.

All statistical analyses were conducted using the GLM procedure in SAS® 9.1 (SAS Institute Inc., Cary, NC, USA). Variability in SPAD readings at different positions on a leaf, and in SPAD readings and N levels both among leaves of different age and among weevil treatments were examined by ANOVA with the Student-Newman-Keuls means separation test. Regression analyses were used to investigate the relationship between SPAD and leaf N across studies, across dates within studies, across fertilizer treatments, and across herbivory treatments. Type III Sum of Squares interaction terms identified regressions in which group slopes differed. Individual slopes were then compared using the contrast statement in GLM. To reduce the number of contrasts in comparisons across fertilizer treatments, data from experiment 3 were restricted to the five fertilizer treatments most closely approximating the five fertilizer levels from experiment 4. SigmaPlot® 11.0 (Systat Software Inc., San Jose, CA, USA) was used to generate graphical representations of the data, including calculations of the regression coefficients presented in the equations.

## 3. Results

Tissue N can vary within a leaf, so we compared SPAD readings taken at three different locations on the leaf in all four experiments. Potential sampling locations were constrained to within 15 mm of a leaf edge by the design of the meter. SPAD readings taken at the tips of the first fully mature leaves were consistently higher than those near the edges of the widest portion of the leaves (44.7 at tip vs 40.9 and 40.5 on the left and right edges, respectively; $F_{[2,5955]} = 78.66$, $P < 0.0001$). Given that leaf tissue nitrogen is calculated from whole leaf blades, these results suggested that the most accurate method for estimating leaf N would be to average multiple SPAD readings from each leaf. We therefore used average readings in the remainder of our analyses.

Adult *Neochetina* spp. weevils feed upon the tissues of fully mature leaves but are also frequently abundant within the unfurling apical bud leaves. The nutritive content of waterhyacinth leaves varies with leaf position (Center and Wright, 1991), so we used the data from experiments 3 and 4 to compare the utility of SPAD measurements for determining N levels in leaves at these two phenological stages. Leaf N was greater in unfurling leaves versus youngest mature leaves (lsmeans = 3.2 vs. 2.9%; $t_{[796]} = 5.33$, $P < 0.0001$). In contrast, SPAD readings were lower on unfurling central leaves versus youngest mature leaves (lsmeans = 34.2 vs. 47.6; $t_{[796]} = 25.94$, $P < 0.0001$). Thus, the SPAD versus N relationship