NRC Research Press

SHORT COMMUNICATION

Microplate assay for boron analysis in soil and plant tissue

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Abstract: The boron concentration in soil extracts and ashed plant tissue was quantified with a rapid and reproducible microplate assay. The microsized azomethine-H method required adjustment to reduce pH and chemical interferences in soil and plant tissue samples. Microplate spectrophotometry permits replication and quality control and is suitable for high-throughput analysis.

Key words: ashing, boron, hot-water extractable, microassay, plant tissue analysis, soil nutrient testing.

Résumé : Les auteurs ont dosé la concentration de bore dans des extraits de sol et les cendres de tissus végétaux au moyen d'une méthode rapide et reproductible recourant à des microplaques. Le microdosage à l'azomethine-H exige une correction pour réduire le pH et les interférences chimiques entre les échantillons de sol et de tissus végétaux. La spectrophotométrie sur microplaque convient à l'analyse à fort débit, car elle peut être répétée et autorise un contrôle de la qualité. [Traduit par la Rédaction]

Mots-clés : incinération, bore, extraction à l'eau chaude, microdosage, analyse des tissus végétaux, dosage des oligoéléments dans le sol.

Introduction

Boron (B) is an essential plant micronutrient that is implicated in cell wall formation and stabilization, as well as lignification, xylem differentiation, pollen germination, and growth. Sufficient levels of B in plant tissue are approximately 10–100 μg B g^{-1} dry weight, with lower B concentration in monocots than dicots (Welch and Shuman 1995). Excessive B inputs can lead to toxicity, especially for crops growing in acid soils (Nable et al. 1997). Boron deficiency is more common in field crops (e.g., canola, corn, soybean, small grains, and potatoes) than tree crops because field crops generally cannot mobilize B reserves through the phloem, and require a soluble source of B that is transported through the xylem to support the growth of younger tissues (Dell and Huang 1997). Soluble B may be obtained from the soil, which contains plant-available B in the form of boric acid (H_3BO_3), or from boric acid-based fertilizer that may be applied to soil or foliage. Fertilizer recommendations are often based on soil test analysis, in which a hot-water extractable B concentration in soil of 1.1–1.5 mg B kg^{-1} soil is adequate for most crops (Centre de référence en agriculture et agroalimentaire du Québec 2010).

Soil testing and plant analysis for B are important to rationalize fertilizer applications and to assess the nutritional status of crops during the growing season. Direct measurement is the only way to correctly diagnose a B deficiency, as visual symptoms are similar to plant responses to stresses such as low or high moisture, abnormally low or high temperatures, insects, and diseases (Hughes-Games 1991). Soil test results may not be as definitive as plant analysis in verifying B-deficient soils, because the efficacy of nutrient extraction is strongly affected by soil pH, organic matter content, and texture (Matula 2009). However, soil tests can confirm plant analysis results, so both diagnostic methods should be used together to evaluate the soil–plant B status and thereby confirm the sufficiency or insufficiency of B for a given crop.

Several methods are used for estimating the B concentration in soil and plant samples based on photometry. The photometric methods rely on color development with dyes such as azomethine-H, curcumin, carminsaure,

Received 9 October 2016. Accepted 1 April 2017.

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crystal violet, and carminic acid. Azomethine-H hydrolyzes in buffered solutions, and when those solutions contain B, the hydrolysis products recondense to form a Schiff base (yellow-colored reaction product) in proportion to the concentration of B in the sample (Harp 1997). Although the azomethine-H method is relatively cheap and sensitive, it suffers from numerous interferences. For example, charcoal must be added in the hot-water extraction procedure to remove interfering organic carbon from soil extracts prior to B analysis (Hettiarachchi and Gupta 2007). Ascorbic acid is added to stabilize the azomethine-H solution, whereas ethylenediaminetetraacetic acid (EDTA) is often used to bind and chelate interfering cations that inhibit the formation of the colored reaction product (Gupta 1993). Nevertheless, the azomethine-H procedure has potential as a quantification method and is widely used in soil testing laboratories that have not yet acquired an inductively coupled plasma (ICP) spectrometer (Sahrawat et al. 2012). Kartal and Green (2002) provided a method for quantitative assessment of B in water-based leachates of treated wood after microsizing the azomethine-H method for a colorimetric microplate assay. It is doubtful that the method of Kartal and Green (2002) is suitable for complex matrices such as soil extracts that contain interfering compounds (Hettiarachchi and Gupta 2007) or for acidic solutions such as plant tissue digests because they require pH adjustment to avoid deprotonation of azomethine-H, which affects the colorimetric reaction (Ozaki et al. 2013). The objective of this study was to adjust the microplate assay for colorimetric determination of the B concentration in soil (hot-water extracts) and plant tissues (ashed and dissolved in dilute acid) based on the azomethine-H method.

Reagents and Solutions

Analytical-grade chemicals (azomethine-H, ascorbic acid, ammonium acetate, and sulfuric acid) were purchased from Sigma–Aldrich Canada (Oakville, ON, Canada). Reagent solutions were filtered through Whatman No. 40 filter paper before use and stored in plastic containers to avoid possible contamination due to the contact with borosilicate glass. Ultrapure deionized water (18 MΩ water from a Milli-Q deionized water system) was used for all reagent and standard preparations, dilutions, and as a blank in all procedures. Standards containing 0–10 μg B mL^{-1} were prepared from analytical grade boric acid.

The azomethine-H solution was prepared by dissolving 0.225 g of azomethine-H and 0.5 g of ascorbic acid in 50 mL of water with gentle heating (50 °C) and stirring. The azomethine-H solution was stored in an opaque, acid-washed plastic container and prepared fresh daily. During the assay, the azomethine-H solution was kept in an ice-water bath to minimize hydrolysis. Buffer solution was prepared by dissolving 25 g of ammonium acetate ($NH_4C_2H_3O_2$) in 40 mL of deionized water, then adding 1.5 g of EDTA and 12.5 mL of glacial acetic acid. The buffer solution was stored in an opaque, acid-washed plastic container and kept in the refrigerator and used within 24 h.

Soil and Plant Tissue Samples

Samples analyzed in this study were soil and plant tissue samples collected from field experiments investigating canola responses to nitrogen, sulfur, and B fertilization, conducted in 2011 and 2012 at six locations in eastern Canada (Ma et al. 2015). Soil B was extracted in plastic centrifuge tubes using hot water with charcoal (0.4 g) added to remove interference from organic carbon compounds (Hettiarachchi and Gupta 2007). Canola tissue samples (collected during vegetative growth, at pod-filling and maturity stages, from oilseed and straw) were ashed in ceramic crucibles at 500 °C for 6 h, resolubilized in dilute acid mixture (300 mL of HCl plus 100 mL of HNO_3 diluted to 1 L with deionized water; Jones and Case 1990) and neutralized with ammonium hydroxide. A plant tissue standard (peach leaves containing 29.00 μg B g^{-1}, SRM 1547, NIST, Gaithersburg, MD, USA) underwent the same procedure for quality control purposes.

It is important to ensure neutral pH conditions in solutions prepared for B analysis because absorbance of the colored azomethine-H–B complex is greatest in solutions with pH 7.0–7.5 (Ozaki et al. 2013). This was the rationale for neutralizing (pH 7.0) the dilute acid solutions containing plant tissue B with ammonium hydroxide. Furthermore, the volume of ammonium acetate buffer in the mixed sample – buffer – azomethine-H solution (see microplate assay below) was selected to achieve the neutral pH in the reaction mixture, confirmed by a semi-quantitative pH paper.

Boron Microplate Assay

The assay was conducted in a 96-well microplate (clear nontreated polystyrene surface, clear flat-bottom wells, nonsterile; Corning Inc., Corning, NY, USA). The proportions of sample, buffer, and azomethine-H solution were based on Kartal and Green (2002). In each well, a 50 μL aliquot of sample or standard was added and the plate was agitated on low speed for 10 s on a vortex mixer equipped with a microplate holder. Then, 50 μL of buffer solution was added and the plate was agitated at low speed for 10 s on the vortex mixer. Finally, 50 μL of the azomethine-H solution was added, the plate was agitated at low speed for 10 s on the vortex mixer, then covered and incubated 35 min at 25 °C for color development following Kartal and Green (2002). Absorbance was read at 420 nm using UQuant Microplate reader (BioTek® Instruments Inc., Winooski, VT, USA).

Quantifying Boron Concentration with the Microplate Assay

Initial testing of the microplate assay was done with B standards (0–10 μg B mL^{-1}); after color development, these generated a standard curve (R^2 = 0.99) with absorbance values from approximately 0.15 to 1.8. The absorbance of standards was measured at wavelengths of 416 and 424 nm to determine the deviation in absorbance compared with 420 nm, the wavelength selected for the microplate assay by Kartal and Green (2002).

Repeatability of the microplate assay (intra-day precision) was evaluated by measuring the absorbance of a 0.1 μg B mL^{-1} standard (in triplicate) in five separate microplates on the same day. The 0.1 μg B mL^{-1} standard was chosen because its absorbance reading (approximately 0.18) is near the lower limit of detection for the azomethine-H method determined with our microplate reader. This is consistent with the lower limit of detection of 0.0514 μg B mL^{-1} on a UV–visible (UV–vis) spectrometer reported by Mohammed et al. (2014). Consistency of the microplate assay (inter-day precision) was assessed by comparing the absorbance values of the 0.1 μg B mL^{-1} standard (in triplicate) in microplates prepared on 2 d (n = 4 on each day) in a 1 wk period.

The B concentration was quantified in soil extracts and ashed plant tissues using the microplate assay and the conventional UV–vis spectrometer procedure (i.e., reaction occurs in test tubes, thereafter transferred to cuvettes for colorimetric determination by UV–vis spectrometer; Gupta 1993). Seven soil samples were extracted with hot water in two batches, in which the first batch was analyzed directly (unspiked) and the second batch was analyzed after adding 0.25 μg B mL^{-1} to the hot-water extract (spiked). Eight plant tissue samples plus peach leaf standard were ashed in two batches, and one batch was analyzed directly after the solubilization–neutralization step (unspiked) whereas the second batch was spiked with 0.1 μg B mL^{-1} after solubilization–neutralization step. Furthermore, we measured the B concentration of the peach leaf standard that was included as a quality control with seven separate batches of plant tissue samples. Every soil extract and plant acid solution were analyzed in triplicate, and absorbance of samples was measured at wavelengths of 416, 420, and 424 nm to determine how the variation in the wavelength would affect absorbance of the colorimetric reaction product.

Results of the Boron Microplate Assay

The B microplate assay gave intra-day repeatability of 114.9% and inter-day consistency of 115.5%. This may suggest that color development increases slightly with time due to hydrolysis of the azomethine-H solution, which supports the recommendation of Hettiarachchi and Gupta (2007) to prepare fresh color development solution each day. We also recommend to keep the azomethine-H solution in an ice-water bath to minimize hydrolysis when multiple microplates are being prepared for analysis. Absorbance readings of the colorimetric reaction product were evaluated at 416 and 424 nm, which is ±4 nm of 420 nm wavelength suggested for the azomethine-H method (Harp 1997; Kartal and Green 2002). Absorbance readings of standards and samples were, on average, 1.2% higher at 416 nm and 7.1% lower at 424 nm, which is an acceptable degree of variation that allows for small deviations in the internal calibration of microplate readers used for the B microplate assay. This also confirms that our procedure provides adequate buffering of the mixed sample – buffer – azomethine-H solution to near-neutral pH. According to Ozaki et al. (2013), the maximum absorption of the colorimetric product occurs at 413 nm when the reaction mixture pH is 6.5, and an increase in pH results in the maximum absorption increasing to 425 nm (reaction mixture pH 7.0) and 434 nm (reaction mixture pH 7.5).

The B concentration was compared in spiked (+0.1 μg B mL^{-1}) and unspiked samples, giving recoveries of 89%–113% in plant tissue samples and 79%–97% in soil samples (Table 1). The microplate reader and conventional UV–vis spectrometer gave similar B concentrations (within 5%) in unspiked soil samples (Table 2). Peach leaf standard containing 29.00 μg B g^{-1} was analyzed repeatedly, with seven separate batches of ashed plant tissue samples, and gave an average concentration of 28.55 ± 0.34 μg B g^{-1} with 98.4% accuracy using the microplate assay. The lower recovery of B in spiked soil samples could be related to chemical interference or inadequate buffering of the hot-water extracts, suggesting that pH measurement and neutralization of soil extracts could be warranted. Ideally, this would be ascertained by evaluating the performance of soil reference material under the same conditions. Reference soils could be obtained from SCP Science (www.scpscience.com) or the North American Proficiency Testing Program (www.naptprogram.org/samples).

Boron Microplate Assay for Agricultural Soil and Plant Tissue Samples

Colorimetric methods of B analysis are gradually being replaced by ICP spectrometry, which has an advantage of detecting B concentrations at part per billion levels, regardless of pH and chemical compounds that interfere with the azomethine-H reaction. Still, many testing laboratories worldwide have not yet acquired ICP systems. For instance, Sahrawat et al. (2012) reported that almost all soil testing laboratories of the national programs in India still use colorimetric methods, commonly the azomethine-H method, to determine extractable B in soils. For such laboratories, the

Table 1. Quantification of boron (B) in unspiked and spiked samples.

B concentration in ashed plant tissue			B concentration in soil hot-water extracts		
Unspiked plant ($\mu g\ B\ mL^{-1}$)	Spiked plant ($\mu g\ B\ mL^{-1}$)	Recovery (%)	Unspiked soil ($\mu g\ B\ mL^{-1}$)	Spiked soil ($\mu g\ B\ mL^{-1}$)	Recovery (%)
0.223 (±0.004)	0.329 (±0.003)	106.5	0.397 (±0.002)	0.605 (±0.014)	82.9
0.190 (±0.007)	0.303 (±0.016)	113.3	0.255 (±0.002)	0.469 (±0.014)	85.6
0.201 (±0.004)	0.297 (±0.005)	96.0	0.374 (±0.007)	0.591 (±0.014)	87.0
0.162 (±0.001)	0.251 (±0.006)	89.2	0.197 (±0.007)	0.394 (±0.007)	78.8
0.190 (±0.021)	0.288 (±0.029)	98.2	0.194 (±0.005)	0.435 (±0.048)	96.5
0.180 (±0.010)	0.272 (±0.009)	91.9	0.313 (±0.003)	0.503 (±0.007)	76.1

Note: Ashed plant tissue samples (canola straw) were resolubilized in dilute HCl/HNO_3 acid mixture, neutralized with NH_4OH and analyzed directly or spiked with 0.1 $\mu g\ B\ mL^{-1}$ before analysis. Mineral soils from canola field experiments were extracted with hot water and then analyzed directly or spiked with 0.25 $\mu g\ B\ mL^{-1}$ prior to analysis. Recovery (%) was calculated as (spiked sample − spike)/unspiked sample for each replicate, and the mean recovery (%) is presented. Values are the mean ± standard error ($n = 3$).

Table 2. Boron (B) determination in soil hot-water extracts by the B microplate assay and the standard UV–visible (UV–vis) spectrometer analysis.

Soil sample No.	B microplate assay ($\mu g\ B\ g^{-1}$ soil)	UV–vis analysis ($\mu g\ B\ g^{-1}$ soil)	Similarity (%)
1	0.36 (±0.051)	0.38 (±0.015)	95
2	0.25 (±0.046)	0.26 (±0.016)	96
3	0.37 (±0.004)	0.37 (±0.002)	100
4	0.47 (±0.120)	0.47 (±0.093)	100
5	0.28 (±0.007)	0.27 (±0.002)	96
6	0.48 (±0.030)	0.46 (±0.133)	96
7	1.03 (±0.006)	1.02 (±0.050)	99

Note: Values are the mean ± standard error ($n = 3$).

advantage of this B microplate assay, compared with conventional spectrometry, is that it resulted in the analysis of hundreds of samples per day (we routinely analyzed more than 600 samples per day) and used 40-fold less reagent volume to analyze each sample. A microplate reader is relatively inexpensive and requires little formal training to operate, compared with an ICP system. However, the B microplate assay developed by Kartal and Green (2002) requires adjustment to quantify B in soil and plant tissue.

Hettiarachchi and Gupta (2007) recommend adding charcoal to soil during the hot-water extraction step to remove organic carbon and some interfering cations. Our experience was consistent with the recommendation of Hettiarachchi and Gupta (2007), in that it was necessary to add enough charcoal to completely decolorize the extract, to avoid interference with the yellow azomethine-H – B complex, before the hot-water extract was ready for the B microassay. Plant tissue samples needed more attention. Our first attempts to measure B in plant tissue following acid digestion (e.g., H_2SO_4, H_2SO_4/H_2O_2, and HNO_3) in acid-washed borosilicate glass tubes were a failure due to the high concentrations of soluble cations that interfered with B quantification. The interference problem was not resolved by repeating the acid digestion in propylene tubes, by adding more chelating agents such as EDTA and ascorbic acid in the assay, or by using a standard B addition technique (i.e., to determine the B concentration in the sample by difference). The B analysis in plant tissue must follow the standard method of first ashing the plant material and then resolubilizing the ashes in dilute acid (Gupta 1993), followed by a neutralization step to prepare the plant tissue digest for the B microassay. Adequate buffering of solutions is essential, as the azomethine-H method is highly sensitive to the pH in the reaction mixture (Harp 1997; Ozaki et al. 2013; Mohammed et al. 2014). We conclude that our adjustments to the B microplate assay improved the quantification of B in soil and plant tissue samples. As the B microplate assay uses a smaller (40 times less) volume of chemical reagents per sample than conventional spectrometry and microplates permit the simultaneous analysis of samples, quality controls, and standards, it is cost-effective and suitable for high-throughput analysis in research and commercial laboratories that have not yet acquired an ICP system for multi-elemental analysis.

Acknowledgements

We thank three anonymous reviewers for helpful comments that improved this manuscript. This study received partial financial support from the Eastern Canada Oilseed Development Alliance (ECODA) through the Developing Innovative Agri-Products Initiative (DIAP), the Agri-Innovation Project (AIP-P025) of Agriculture, and Agri-Food Canada Growing Forward I and II.

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