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Using Microplates to Test Boron in *Zea mays* Leaf Plant and the Surrounding Soil

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ABSTRACT

Background: Boron plays a crucial role in cell wall construction in plants and also facilitates the transfer of carbohydrates, which are essential for various physiological processes. The objective was to measure element concentrations in soil, water extracts and plant tissues (*Zea mays*) using Azomethine-H and ICP-MS within a controlled microplate assay.

Methods: A microscopic plate repeat test was used to analyze the concentration of boron in soil extracts and plant tissue ash from local *Zea mays* leaves, considering the global trend in environmental sustainability. Tests aimed to modify the azomethine-H microscopic approach to eliminate pH and chemical interferences in soil samples and plant tissues. Due to their ability to ensure sample repeatability and quality control, micro spectrophotometers are ideal for high-throughput analysis.

Result: The microplate test has enhanced boron measurement in soil and plant tissue samples. Our improvements to the microplate B assay have resulted in more accurate boron measurements in these samples. Microplate B testing is effective and suitable analysis in modern research and laboratories, using 40 times less chemical reagent per sample than traditional spectrophotometry. Additionally, microplates enable simultaneous analysis and calibration of samples.

The modified plate test significantly improves boron measurement in soil and plant tissue samples, using 40 times fewer reagents than traditional spectrophotometry. This method is cost-effective, ideal research , commercial analysis and making it a valuable tool for modern laboratories.

Key words: Boron, Soil contamination, *Zea mays* analysis.

INTRODUCTION

Boron is found in plants and aids in constructing the cell wall. It also facilitates the transfer of carbohydrates necessary for cell division, bark formation, hormone transmission and pollen germination. Research indicates that boron influences the rate of nutrient absorption in plants. Its presence enhances the plant's tolerance to drought. Boron is crucial for plant hormones that affect the growth of the stem and root tips. Additionally, it plays a role in regulating calcium absorption and is absorbed in the forms BO_3^{3-}, HBO_3^{2-}, $H_2BO_3^-$ and $B_4O_7^{2}$ (APHA 2023).

Once absorbed, boron becomes fixed in the tissues it reaches and does not migrate. As an immobile element, signs of boron deficiency first appear on young leaves. Symptoms of boron deficiency start with the collapse of meristematic tissue cells, which are actively dividing, particularly in the developing apices and cambium zones. The vascular bundles of the roots and stems are affected, disrupting water transport and causing wilting, which is usually the first indication of a deficiency. This can lead to the death of the terminal bud of the stems (Abad-Segura *et al.,* (2019).

In plants experiencing boron deficiency, glucose content in the roots and stems is low due to impaired glucose transport and increased concentrations in the leaves. Boron deficiency causes developing shoots to die, new leaves to become distorted and brown or black corky patches to form in the storage organs of roots and tubers (Haile and Tadele, 2023). It can also lead to yellowing or browning of leaves, though the most common symptom is the curling of the edges of new leaves. Additionally, a pale yellow tint with uneven distribution may appear on the leaves of root vegetables. Boron-deficient plants are typically smaller and the growth tips of roots and stems die. Low ground humidity, high temperatures and intense light worsen the symptoms of boron deficiency, as these conditions hinder the movement of boron from the leaves to other plant organs (Beebe *et al.,* 2000; Ayvaz *et al.,* 2016).

Boron deficiency causes the formation of brown or black corky areas on the surface of beet roots or along their growth rings. In turnips, large brown spots develop toward the center of the root. Cauliflower discs turn brown and broccoli blossom buds also become brown. Watery spots appear on the stems of cauliflower, broccoli and cabbage, eventually developing into horizontal fissures.

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Brown, decaying lines are seen on the petioles of celery leaves, with epidermal cell disintegration occurring inside. Sometimes, pale stripes with notches form on the inner side of the leaf petioles. In grapes, terminal buds fail to develop, numerous side branches form and yellow patches and holes appear on the leaves, especially along the edges (Ati *et al.,* 2023). According to Rékási *et al.* (2021), vegetables are classified into three groups based on their boron needs:

- According to their boron needs, include beets, cabbage, broccoli, cauliflower, asparagus, radish, Brussels sprouts, celery and turnips.
- Vegetables with a modest boron requirement can tolerate moderate boron concentrations in soil and irrigation water. The element concentration in the ground solution must be between 0.5 and 0.1 parts per million. In descending order of boron requirement, these vegetables include tomatoes, lettuce, potatoes, carrots and onions.
- Excess boron in the soil and irrigation water is toxic to vegetables with low boron demands. The boron concentration in the ground solution should not exceed 0.1 parts per million. Listed in ascending order of boron sensitivity, these include sugar corn, peas, fava beans, fruits, olives and crops such as wheat, barley, rice, corn and sugar beets, which are among the crops most vulnerable to boron deficiency (Kopecká *et al.,* 2023).
- The goal of this work was to calorimetrically assess the concentration of this element in the soil, water extracts, plant tissues (ash and dissolved in dilute acid) of *Zea mays,* using the Azomethine-H and ICP-MS controlled microplate assay techniques.

MATERIALS AND METHODS

Solutions and reagents

Chemicals for analysis, including Azomethine-H, ammonium acetate, ascorbic acid and sulfuric acid, were filtered through Whatman No. 40 paper and stored in plastic containers to prevent contamination from borosilicate glass. All reagent and standard preparations, dilutions and procedures used high-purity deionized water (approximately 18 MΩcm) from the Milli-Q deionized water system. To develop standards, 0-10 g of analytical-grade boric acid was used. A solution was prepared daily by gradually heating to about 50°C and stirring 0.225 g of Azomethine-H and 0.5 g of ascorbic acid in 50 mL of water in an opaque container. During the test, the solution was kept in a cold water bath to reduce hydrolysis. A buffer solution was made by dissolving 25 g of ammonium acetate ($NH_4C_2H_3O_2$) in 40 mL of deionized water, then adding 1.4 g of EDTA and 11.5 mL of acetic acid. This buffer solution was refrigerated and stored in an opaque, acid-washed plastic container.

Plant tissue and soil samples

This study collected soil and *Zea mays* leaf tissue samples from randomly selected farms to examine responses to boron fertilizer at five agricultural sites. Soil samples were extracted in polypropylene centrifuge tubes with hot water and 0.4 g of charcoal to remove interfering organic carbon components, according to Zeki *et al.* (2019).

Boron microplate testing

The experiment was carried out in a microplate, with sample and solution proportions determined using Hettiarachchi *et al.* (2008). The plate was agitated at low speed in a mixer for 10-12 seconds before being stirred at low speed in a vortex mixer for 10 seconds after adding the buffer. Finally, 50 μL of H-azomethine solution was added to the plate, which was swirled at low speed for 10 seconds with a vortex mixer before covering and incubating at 25°C for 35 minutes to wait for color change. The same quality control measures were applied using standard tissue culture papers (30.00g/g) [SRM ″1547″ ″NIST″ Gaithersburg, MD, USA (QA, QC)].

According to Hettiarachchi and Gupta (2008), the absorption of the colored Azomethine complex is stronger in solutions with a pH of 7.0-7.5. To maintain the neutral pH required for analysis, the solutions were stabilized at pH 7.0. Ammonium hydroxide was used to dilute acidic solutions containing plant tissue. The volume of ammonium acetate buffer solution was selected to achieve the neutral pH in the reaction mixture for the microplate test. Color change was monitored using semi-quantitative pH paper. The experiment was conducted in a 95-well microplate, with solution proportions determined as described by Hettiarachchi and Gupta (2008). Each well received a 60 μL aliquot of the standard. The plate was agitated at low speed for 10 seconds using a vortex mixer with a microplate holder, then spun for 10-12 seconds at low speed before being covered and incubated at 25°C for 35 minutes to allow for color development, following the protocol in Maret and Sandstead (2006).

Microplate test for boron concentration measurement

The initial microplate screening tests were conducted using boron standards (1-10 g B/mL). After color development, a standard curve ($R^2 = 0.97$) was constructed with absorbance values ranging from 0.18 to 1.9. The absorbance of the standards was measured at wavelengths of 417 nm and 425 nm to determine the deviation from 421 nm, the wavelength used by Kartal and Green for microplate screening (Hettiarachchi and Gupta, 2008).

The reproducibility of the microplate test and intra-day accuracy were determined by measuring the absorbance of a 0.1 g B/mL standard in five different microplates, each in duplicate. Since the absorbance value of about 0.21 is close to the lower limit of the azomethine-H technique established using our microplate results, the 0.1 g B/mL standard was chosen. The lower limit of about 0.14 g B/mL agrees with Kartal and Green (2002) for ultraviolet-visible (UV-vis) spectrometry. The consistency of microplate assay accuracy was evaluated by comparing absorbance readings for the 0.1 g B/mL standard (in triplicate) in

microplates prepared on two different days (n= four on each day) over a one-week period (Ozaki *et al.,* 2013).

Boron concentrations in soil extracts and plant tissue ash were determined using a microplate assay and measured with UV spectrometry Ajmi *et al.* (2018). Soil samples were extracted in two batches: the first batch directly examined and the second batch evaluated after adding 0.27 g B/mL to the hot extract. *Zea mays* leaf tissue samples were processed in two batches: one tested immediately after dissolution-neutralization and the second batch with 0.01 g B/mL added post-dissolution-neutralization. Plant ash standards were also analyzed for boron concentration as quality control. Each soil extract and plant acid solution was tested in triplicate, measuring absorbance at wavelengths 417, 421 and 425 nm to assess wavelength effects on colorimetric product absorbance and concentration-related color changes, following Mohammed *et al.* (2014).

RESULTS AND DISCUSSION

The plate B test exhibited an intraday repeatability of 9.6% and an interday consistency of 5%. This suggests that color change increases slightly over time due to hydrolysis of the H-azomethene solution, supporting the recommendation in Zeki *et al.* (2019) change solution daily when preparing multiple microplates for analysis and to use a cold water bath to minimize hydrolysis. The colorimetric reaction measured the absorbance of products at 417 nm and 425 nm, slightly deviating from the 421 nm wavelength proposed for the azomethine-H technique, as noted in Hettiarachchi and Gupta (2008). Absorbance readings for standards showed an average deviation of 2.7% higher at 417 nm and 9.1% lower at 425 nm, which is an acceptable level of variance considering the internal calibration of the microplate and the composition of the sample-buffer-azomethene-H solution at near-neutral pH.

According to Kartal and Green (2002), the absorption maximum of chromatographic products occurs at 412 nm when the reaction mixture pH is 6.4. Increasing the pH results in absorption maxima at 427 nm for reaction mixture pH 7.0 and 436 nm for pH in the range of 7.0-7.5. This yields recoveries between 99% to 133% in plant tissue samples and 88% to 99% in soil samples, as illustrated in Fig 1 and 2, which depict the final quantitative measurement of boron (B) in soil samples and plant tissues.

The findings of microplate and conventional UV spectroscopy showed that the soil samples had similar B contents (within 5%), (Fig 3).

Boron poisoning indirectly impacts fruit yield by accelerating the degradation of leaf tissue. Although the rate of leaf tissue breakdown is moderate, it can lead to a slight reduction in fruit output under specific conditions. Some fruits may experience direct consequences. For instance, peach fruits exhibit symptoms of boron toxicity, including the development of dark brown woody patches that extend into the core World Health Organization (2013).

Using the microplate assay, a standard *Zea mays* leaf containing 33.00 μg Bg1 was evaluated repeatedly across seven consecutive batches of ash plant tissue samples. The analysis revealed an average concentration of (44.58±0.53 μg) Bg1, achieving a high accuracy rate of 98%. However, the observed lower recovery of B in spiked soil samples points to potential chemical interference or insufficient buffering of hot water extracts. This suggests that monitoring pH levels and neutralizing soil extracts might be necessary. Validation of these findings could be enhanced by evaluating the performance of soil reference materials under conditions described in Ajmi *et al.,* (2018B) and Abishek *et al., (*2024).

Colorimetric methods for boron analysis have gradually been replaced by ICP-MS spectrophotometry, which offers the advantage of detecting boron quantities at the ppb level. According to findings reported in [9], virtually all soil testing laboratories in national programs in India still utilize colorimetric methods, specifically the azomethine-H

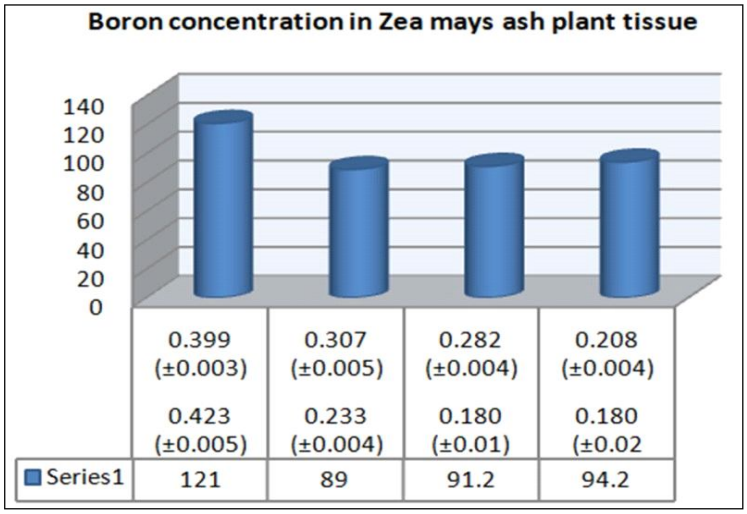

Fig 1: Boron (B) quantification in ash soil samples.

method, for direct estimation of extractable potassium in soil. Compared to conventional spectrometry, microplate testing offers the advantage of analyzing hundreds of samples per day (consistently examining over 600 samples per day) and uses 40 times less reagent volume per sample. In contrast to an ICP-MS system, a microplate reader is less expensive and requires less formal training to operate. However, the microplate B assay requires adaptation to measure the quantity of boron in soil and plant tissues, especially in plant parts with tough cell walls and in older trees (Mohan and Jones, 2018; Xiao *et al.,* 2019).

Boron poisoning indirectly affects fruit yield through the degradation of leaf tissue. While the rate of leaf tissue breakdown is moderate, it can slightly decrease fruit output in certain instances. Certain fruits may be directly impacted. For example, peach fruits show symptoms of boron toxicity, such as dark brown woody patches that extend to the core World Health Organization (2013).

Adding charcoal to the soil during the hot water extraction step, consistent with previous studies on cucumber and cauliflower plants, serves to remove organic carbon and certain intervening cations to prevent interference with the yellow azomethine-H-B complex. We added sufficient charcoal to thoroughly decolorize the extract before conducting the hot water extraction, following the recommendation in Ajmi *et al.* (2018B). Boron is considered impactful when its concentration exceeds 0.5 ppm in water or 190 parts per million in leaf tissue, affecting sensitive plant species Brdar-Jokanović (2020). Soil testing and monitoring boron levels in plants and soil are crucial for optimizing fertilizer use and assessing crop nutrition throughout the growing season, with the aim of promoting environmental sustainability and safeguarding national agricultural yields.

Boron plays a critical role in plant metabolism, impacting cell wall formation, pollen development and carbohydrate transport (Lahane *et al.,* 1995) . Variations in

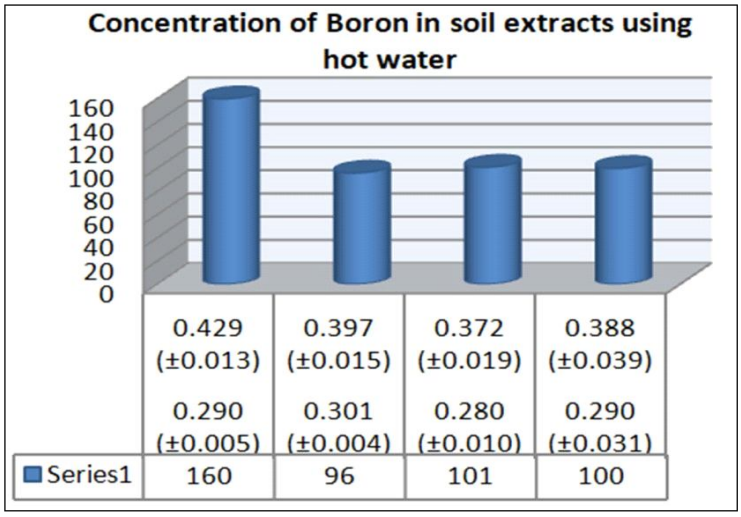

Fig 2: Boron (B) quantification in ash plant samples.

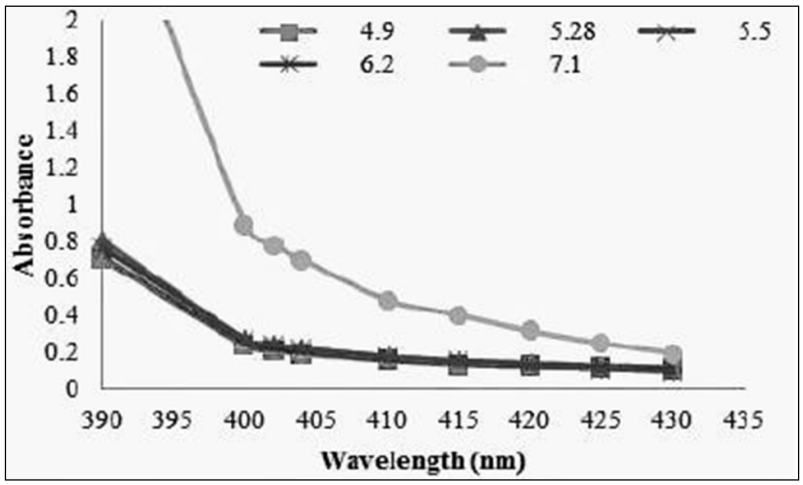

Fig 3: Boron (B) determination in soil extracts extracted with hot water Microplate B test and standard UV-vis Spectroscopic analysis (UV-Vis).

boron levels can significantly impact both crop yield and quality, highlighting the importance of precise assessment in sustainable agriculture. Traditional methods for analyzing boron are often labor-intensive, with limited capacity and sensitivity. The emergence of microplate assays, however, has revolutionized this field, enabling efficient, high-throughput and precise measurement of boron concentrations in plant tissues and soil samples alike (Pereira *et al*., 2021). Because the functions of boron in the plant are important, it controls the percentage of water inside the plant as well as the absorption of water from the soil. It is also related to the movement of sugars to their storage places, as it affects the absorption of some elements such as nitrogen, potash and calcium. It is necessary for the formation of hormones in the plant and is very important in the process of forming proteins in the plant and amino acid tryptophan (Long and Peng, 2021).

Symptoms of boron deficiency on leaves also appear on large leaves if the deficiency continues and worsens. The most important symptoms of boron deficiency are the death of buds and growing tops, the death of root tips and the branches and leaves break easily Amanda Sarfo Boateng *et al.* (2023). There are special symptoms that differ depending on the crop, the most important of which is, for example, in almonds, the buds do not open, in barley, kernels do not form in ears and in citrus fruits, water spots appear on the leaves, then they become transparent, then they fall and the branch is exposed from top to bottom and in fruits, brown spots appear on the thickness of the peel increases and it does not form. The seeds and fruits are dry and hard and the juice is low, as is the sugar content. This is consistent with our study on the yellow corn plant, as in cases of severe deficiency, corn bushes take a tangled shape due to the short distances between the nodes and the meristematic tissue dies and the leaves become thick and breakable and the pink buds also fall and a dark ring swelling is observed, equipped with dense hairs on the leaf petioles (Ajmi *et al.,* 2018B).

CONCLUSION

- Determining boron in plant tissue after acid digestion in acid-washed borosilicate glass tubes is challenging due to high levels of soluble cations that interfere with boron quantification.
- The conventional method for extracting plant tissue involves ashing the material first, followed by acid dissolution, dilution and subsequent neutralization. Proper buffering of these solutions is crucial, as the H-azomethene method is highly sensitive to the pH of the reaction mixture.
- Enhancements to the plate test have improved the measurement of boron in soil and plant tissue samples. Microplate B testing uses significantly less chemical reagent per sample (40 times less) compared to conventional spectrophotometry, making it more cost-effective and suitable for high-throughput analysis in both research and commercial settings.
- Microplates facilitate simultaneous analysis of samples alongside quality control and standards. They have been employed for testing boron in *Zea mays* leaves and surrounding soil using the azomethine-H microscopic approach, allowing for sample replication and quality assurance are instrumental in yield analysis and have advanced the measurement of boron in soil extracts obtained through the hot water Microplate B test, complemented by standard ultraviolet (UV-Vis) spectroscopy.

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Conflict of interest

No conflict of interest.

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