AGRICULTURAL MATERIALS

Continuing Assessment of the 5-Day Sodium Carbonate-Ammonium Nitrate Extraction Assay as an Indicator Test for Silicon Fertilizers

Wendy Zellner

U.S. Department of Agriculture, Agricultural Research Service, Application Technology Research Unit, Greenhouse Production Research Group, 2801 W. Bancroft St, Mail Stop 604, Toledo, OH

Russell L. Friedrich

U.S. Department of Agriculture, Agricultural Research Service, Application Technology Research Unit, 27 Horticultural Insects Research Laboratory, 1680 Madison Ave, Wooster, OH

Sujin Kim and Douglas Sturtz

U.S. Department of Agriculture, Agricultural Research Service, Application Technology Research Unit, Greenhouse Production Research Group, 2801 W. Bancroft St, Mail Stop 604, Toledo, OH

Jonathan Frantz

DuPont Pioneer Hi-Bred, Applied Technology Systems, 7305 NW 62nd Ave, Johnston, IA

James Altland and Charles Krause

U.S. Department of Agriculture, Agricultural Research Service, Application Technology Research Unit, 27 Horticultural Insects Research Laboratory, 1680 Madison Ave, Wooster, OH

The 5-day sodium carbonate-ammonium nitrate extraction assay (5-day method) has been recognized by the American Association of Plant Food Control Officials as a validated test method to identify fertilizers or beneficial substances that provide plant-available silicon (Si). The test method used the molybdenum blue colorimetric assay to quantify percentage Si; however, laboratories may use inductively coupled plasma optical emission spectroscopy (ICP-OES) for elemental analysis. To examine the use of either colorimetric or ICP-OES methods for Si determination, the 5-day method was performed on the following Si-containing compounds; wollastonite, sand, biochar, and a basic oven furnace (BOF) slag. Grow-out studies using *Zinnia elegans* were also performed using varying rates of the wollastonite, biochar, and BOF slag. Our results show using the 5-day method, wollastonite had the highest extracted amounts of silicic acid (H_4SiO_4) at 4% followed by biochar (2%), BOF slag (1%), and sand (0%). Extraction values calculated using either the molybdenum blue colorimetric assay or ICP-OES for detection of the H_4SiO_4 had a significant correlation, supporting the application of either detection method for this type of analysis. However, when extracted values were compared to amounts of Si taken up by the plants, the 5-day method overestimated both wollastonite and biochar. While this method is a valid indicator test for determining a soluble Si source, other plant species and methods should be perused to potentially provide more quantitative analyses for plant-available Si content of all materials.

Silicon (Si) is the second most-abundant element in the lithosphere, and it has the ability to accumulate in many grass species, such as rice (*Oryza sativa*), and other dicotyledonous plants, such as zinnia, well above the macronutrient level of 5%, accounting for 10% of plant dry weight (1). Specialized aquaporins have been identified in higher accumulators that have the ability to transport silicic acid (H_4SiO_4) across a membrane both passively and actively within the plants (2). Nutrient uptake is a dynamic process in that multiple factors including time of year, plant age, and especially in the case of Si, stress influence these processes (3–5). Few studies have attempted to analyze the regulation of both the acquisition and accumulation of Si within plants, making the task of chemically determining the plant availability of Si from a potential Si source challenging. Most notably, Yamaji and Ma (6) have identified a signaling hub in node 1 of rice plants to be crucial to the trafficking of H_4SiO_4 out of the xylem and into the rice grain, diverting the element from deposition into the flag leaves. Researchers have demonstrated the involvement of both passive and active transporters in this regulation. Even though most plants accumulate foliar levels of Si well below 10%, the literature is inundated with studies showing the beneficial effects under various abiotic and biotic stresses (5, 7). Si-fed potatoes had an increase in tuber yield and decrease in stem lodging under drought stress when compared to nonamended controls (8). The low accumulator, *Nicotiana tabacum*, had a reduction in *Tobacco ringspot virus* symptoms with an increase in foliar Si levels of Si-treated plants compared to controls (9). While these data have not been sufficient enough to include Si as an essential element at the present time, the American Association of Plant Food Control Officials

(AAPFCO) has recognized Si as a beneficial substance and has developed guidelines for validation and marketing of fertilizers or beneficial compounds containing the element.

The use of a 5-day sodium carbonate-ammonium nitrate extraction method as a standardized protocol to identify potential fertilizer sources of Si has been proposed and accepted as a standard test method for detection of H_4SiO_4 (10). This proposal was based on the notion that H_4SiO_4 is the form of Si available to plants and is deemed the form of plant "available" Si by AAPFCO terminology. In this specific extraction method, the sodium carbonate solubilizes the test material, while the ammonium nitrate supplies protons to the reaction, maintaining Si as uncharged H_4SiO_4 (11). While this test was shown to work well as an indication of plant-available Si with solid fertilizers tested in this study, there were still some limitations to this test method. The 5-day extraction values from liquid fertilizers did not correlate to plant grow-out studies (12). In addition, Pereira et al. (13) showed a heavy bias towards Linz and Donawitz (LD) slags versus blast furnace (BF) slags and clay materials when this test method was used to determine plant-available Si. While this extraction has been an accepted standard method for determining plant-available Si, the use of this assay to determine the validity of other proposed procedures should be done with caution, due to the tendency of the test to favor certain materials.

Si-containing molecules come in many shapes and forms (14). While the 5-day sodium carbonate-ammonium nitrate extraction is used to detect the amount of H_4SiO_4 present at the time of the digestion and was developed for calcium-silicate materials, it has not been investigated for all potential types of Si fertilizers. Wollastonite ($CaSiO_3$) is considered an excellent source for plant-available Si and in many studies is used as a positive control (15). On the other hand, slag-based materials, a secondary product of the steel industry, have been used for decades in Japan as a source of plant-available Si (16). Calcium-based material, such as limestone and dolomite, are often used in the production of steel to pull out elemental impurities, including Si, manganese, and aluminum (17). In addition to calcium silicates, there are multiple Si-containing compounds within these slags, which vary greatly depending on how the slag was produced (heat, flux, etc.; 18). Biochar in the form of gasified rice hulls (amorphous reactive SiO_2), has been used for studies analyzing the effect of the material on improving plant growth (19–21). Other Si-containing materials used to provide plant-available Si include recycled glass and nanoparticles. These novel materials when applied to plants result in increased foliar levels of Si, qualifying them as Si-fertilizers. But how they respond to the 5-day extraction method has not been determined. Sand, on the other hand, is mainly composed of quartz or nonavailable silica (SiO_2) and can be used as a negative control for extraction methods detecting plant-available Si. Quartz sand has been used for germination of seedlings for decades, and in studies where Si levels were examined, no additional accumulation was observed in plants grown in the material, unless available Si was supplied (22).

While the 5-day sodium carbonate-ammonium nitrate extraction has been validated as an indicator test in a single-laboratory study (23), there were limited grow-out studies provided with this improved method to correlate extraction methods in detecting potential plant uptake. In addition, previous work done in developing the test only used a single concentration of the fertilizers, which could result in misleading conclusions since Si accumulation in plants is a complex and regulated process (2). Because calibration curves require more than one data point to construct, grow-out studies testing the validity of an extraction test should be performed at varying concentrations of the specific fertilizer in order to accurately represent plant uptake and ultimately plant "availability" of Si.

These studies were performed to further analyze the accuracy of the 5-day extraction method. The tests also were designed to compare the use of molybdenum blue and inductively coupled plasma optical emission spectrometry (ICP-OES) as a means of Si detection in the extraction solution, in addition to comparing results to a grow-out study in a high-accumulator plant, other than a member of the *Poaceae* family, at varying fertilizer concentrations.

Experimental

5-Day Sodium Carbonate-Ammonium Nitrate Extraction Technique

Si-containing materials used in these studies were wollastonite (R.T. Vanderbilt, Norwalk, CT) as a positive control, sand as a negative control, a basic oxygen furnace (BOF) slag (Plant Tuff, Edward C. Levy Co., Dearborn, MI), and gasified rice hull ash (biochar, Charsil, Scott-Glenn Co., Birmingham, AL) as potential Si fertilizers. The 5-day extraction procedure was followed (24). Material was ground with a mortar and pestle and passed through a 300 μm sieve (Fisher Scientific, Pittsburgh, PA) prior to extraction. Briefly, 100 mL 0.094 M sodium carbonate and 100 mL 0.20 M ammonium nitrate were added to 0.2 g of each test material in a polypropylene flask. The flasks were agitated at 140 rpm for 1 h and then left undisturbed for 5 days, all at room temperature. Following incubation, the top portion of the solutions was carefully transferred to a 50 mL falcon tube (Fisher Scientific). The extracts were diluted by transferring 2 mL extraction solution to 198 mL 18 MΩ deionized (Milli-Q; EMD Millipore, Billerica, MA) water in a 250 mL polypropylene flask. The solutions were mixed by inverting 10 times. Three extractions/treatment were performed in Experiment 1, followed by six extractions/treatment for both Experiments 2 and 3.

Analysis of Si Concentrations

The molybdenum blue colorimetric analysis was performed on the solutions within 24 h. The volume analyzed was reduced by a factor of 10 from the documented procedure (24). Briefly, standards were diluted from an Assurance Grade Si standard (SPEX CertiPrep, Fisher Scientific) PLS19-2T, 100 mg/L to 0, 0.25, 0.5, 1.0, and 2.0 mg/L Si using Milli-Q water. To 15 mL culture tubes (Fisher Scientific), 2 mL of each standard or extraction solution and 0.2 mL ammonium molybdate solution [0.06 M $(NH4)_6 \cdot Mo_7O_{24}$, 1.8 M H_2SO_4] were added. Tubes were vortexed for 10 s followed by a 10 min rest. Next, 0.2 mL 1.33 M tartaric acid solution was added to each sample and tubes were vortexed for 10 s. After the tubes were at rest for 5 min, 0.2 mL 0.071 M ascorbic acid was added. Again, the tubes were vortexed for 10 s and then left undisturbed for 60 min to allow the reaction to take place. The absorbance was measured at 660 nm with a spectrophotometer (Genesys 6,

Table 1. Total estimated Si concentration in a Sunshine Mix No. 2 (medium) as a function of varying application rates from three sources of Si fertilizers[a]

Application rates, g/L	Amount of Si in medium, mg/L		
	Wollastonite	Biochar	BOF Slag
0.225	27.5	32.0	11.0
0.45	54.9	64.0	22.1
0.9	110.0	128.0	44.1

[a] Calculated amounts of Si in the medium were derived from total Si values obtained from Brookside Labs using HF digestion on the material. (The amount of residual Si provided from the peat was not included in these calculations).

Thermo Scientific, Waltham, MA). A standard curve was used to determine the percentage Si in the solution. The colorimetric analysis was performed in triplicate.

ICP-OES was performed on the same extract as used for the colorimetric assay to quantify total Si (24). Briefly, the solutions produced by the 5-day sodium carbonate-ammonium nitrate extraction were analyzed using a matrix-matched standard to determine Si concentrations. A 1:1 standard was made using 0.094 M sodium carbonate and 0.20 M ammonium nitrate. The digested extraction solution samples were diluted by adding 1 mL sample to 9 mL Milli-Q water and analyzed using ICP-OES (iCAP 6300 spectrometer; Thermo Scientific) at a wavelength of 212.412 nm.

Correlation analysis was performed in R using the cor.test function (www.r-tutor.com). Data were subjected to analysis of variance (ANOVA), and means were separated with Tukey's honestly significantly different (HSD) test using the R program (25, 26).

Zinnia Grow-Out Studies

Zinnia elegans var. Oklahoma white were sown in oasis cubes (Smithers-Oasis) and placed in a growth chamber with 16:8 h light:dark and 23:21°C day:night temperature with a constant 85% relative humidity. Seedlings were watered with tap water as needed and supplemented with a half Hoagland's solution made with Milli-Q water (7.5 mM N, 2 mM P, 8.5 mM K, 2.5 mM Ca, 1 mM Mg, 1 mM S, 71 μM Fe, 9 μM Mn, 1.5 μM Cu, 1.5 μM Zn, 45 μM Mo, 24 μM Cl, 0.2 μM Na, and 3 mM S) once green cotyledons were present. Plants were transplanted to 15 cm wide pots containing Sunshine Mix No. 2 soilless media (SunGro Horticulture, Quincy, MI) with a bulk density of 0.1 g/cm and 2.7 mg/kg 0.5 M acetic acid extractable Si. The medium was amended with 0, 0.225, 0.45, or 0.9 g/L fertilizer (Table 1). The last three are equivalent to 2.25, 4.5, and 9.0 g/kg application rates, respectively. For field soil applications, assuming a depth of incorporation of 15 cm, the rates used in this study would be equivalent to 337.5 to 1350 kg/ha. The amendments used were Wollastonite, biochar made from gasified rice hulls (CharSil), and a BOF slag (Plant Tuff, Levy Co.). Fertilizers were analyzed for total Si with HF digestion (Brookside Laboratories, New Bremen, OH; Method 6010B) and contained 122, 142, and 49 g/kg Si fertilizer for wollastonite, biochar, and BOF slag, respectively. Pots were arranged in a completely randomized design with six replications/treatment. Plants were grown in the same growth chambers under the same conditions, and pots were watered

twice a week with 250 mL complete fertilizer to reduce leaching of the material from the substrate. After 3 weeks, leaf tissue was collected, washed in 0.1 M HCl, and rinsed with Milli-Q water. The tissue was then placed in paper bags and dried at 60°C in a forced-air drying oven (Binder) for 72 h. Dry weights were collected prior to grinding with a stainless-steel coffee grinder (KitchenAid). Tissue was digested for total Si analysis via ICP-OES.

Si concentrations were determined using ICP-OES on foliar and root tissue. Briefly, 0.1 g tissue was digested in a 55 mL Teflon® vessel (CEM Corp., Matthews, NC) with 3.0 mL 7.5 M KOH. The solutions were heated via ramping the temperature over a 15 min interval to 200°C and maintaining the temperature for an additional 15 min in a programmable microwave oven (MARS 6 Xpress, CEM Corp.). The digest was then allowed to cool to room temperature at which time 2 mL 30% H_2O_2 was added and solutions were again heated by ramping to 200°C for 15 min and holding the temperature for 5 min. After the solutions cooled to room temperature, they were filtered (Whatman No. 2 paper; Fisher Scientific). The solutions were diluted 1:10 with Milli-Q water and analyzed by ICP-OES at a wavelength of 212.412 nm. Data were subjected to ANOVA and means were separated with Tukey's HSD test using the R program. Regression analysis was conducted in Sigma Plot 12.5 where the value of estimated available Si was fitted against total Si uptake in zinnia tissue. Si uptake was fitted to a quadratic function:

$$y = c + bx + ax^2$$

where y = Si uptake, x = estimated available Si, and the parameters a, b, and c were calculated. The estimated available Si was determined by multiplying the amendment rate by the percentage Si obtained from the 5-day extraction assay.

Results and Discussion

There were no differences between colorimetric and ICP-OES values within the same experiments as determined by the 5-day extraction procedure (Figure 1). The correlation coefficient was significant at 0.99 with a *P*-value <0.001. However, there were differences among experiments for all materials tested, except
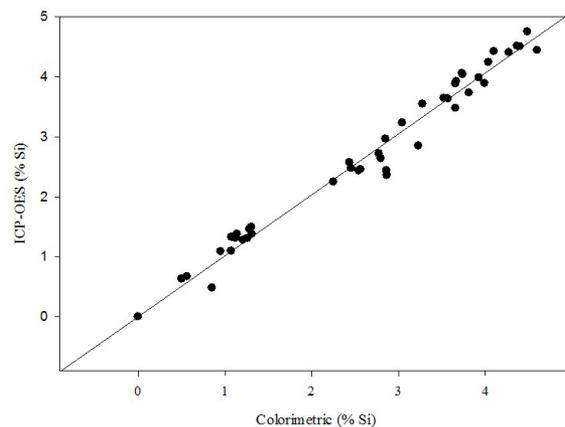
Figure 1. Scatterplot of % Si of fertilizer detected by ICP-OES versus molybdenum blue colorimetric analysis. The relationship between ICP and the colorimetric analysis is y = 1.0163x + 0.0008, correlation coefficient = 0.9934, with a *P*-value <0.0001.

Table 2.   Average percentage Si detected via molybdenum blue colorimetric (color) and ICP-OES (ICP) analyses following the 5-day extraction assay[a]

Material	Si, % Experiment 1		Si, % Experiment 2		Si, % Experiment 3		ANOVA P-values		
	Color	ICP	Color	ICP	Color	ICP	Method (M)	Experiment (E)	M*E
Wollastonite	3.85	4.17	3.77	3.77	4.36	4.48	0.6200	0.0031	0.4500
Biochar	2.98	2.55	2.56	2.50	3.13	3.31	0.7900	0.0064	0.1400
BOF Slag	0.85	0.48	0.98	1.06	1.15	1.34	0.4900	0.0001	0.0480
Sand	NA	NA	0	0	0	0	0.3300	0.3300	0.3300

[a]   P-values from ANOVA analysis on the effect of the method and experiment for each individual material are listed. Sand was not included in the first experiment (NA).

for sand (Table 2). There was a significant interaction between method and experiment for percentage Si in the slag material only. Despite the significant experimental effect on extractable Si, the magnitude of the differences from each experiment was relatively small compared to the magnitude of the differences between fertilizer sources within an experiment. Therefore, averaging across the three experiments, wollastonite had the highest amount of extractable Si at 4.07%, followed by biochar and slag at 2.84 and 0.98%, respectively. Sand had no detectable levels of Si for both colorimetric and ICP-OES analyses.

These data suggest the use of either the colorimetric or ICP-OES method as a final analysis of the 5-day extraction procedure will yield similar results. Variations between Experiments 2 and 3 were less than 1%. Since this test method is an indicator test, the variability did not alter the final conclusion that wollastonite, biochar, and slag all provide plant-available Si, while sand does not.

Grow-out studies using the high-accumulator, *Z. elegans*, were performed to analyze the accuracy of the 5-day extraction values for determining plant-available Si. An initial grow-out study in zinnia was performed with wollastonite and BOF slag at varying concentrations. At values of 1.8 and 3.6 g/L for both wollastonite and slag, Si uptake was saturated in the zinnia after 4 weeks of growth (data not shown). Because we wanted to analyze the amount of available Si supplied from each fertilizer source within the linear range of plant uptake, fertilizer concentrations were lowered. Sand was also omitted from grow-out studies, since the literature convincingly demonstrates that it does not supply additional Si to plants. Analysis of foliar tissue showed Si concentrations had an increasing trend with significant differences only observed in wollastonite-treated plants between 0.225 and 0.9 g treatments (Figure 2). Regression analysis resulted in no significant increase in Si concentration with fertilizer treatment for all materials (P-values >0.06).

The calculated available Si from the 5-day method was plotted against the total uptake of Si in zinnia tissue to analyze their relationship. When all data were included in the analysis, the R^2 value of the quadratic relationship was 0.46 (Figure 3A). To further analyze the data, each treatment was also graphed independently (Figure 3B–D). Wollastonite had the highest R^2 value of 0.77, followed by biochar and BOF slag with 0.42 and 0.39, respectively. Interestingly, BOF slag and wollastonite both had negative calculated parameters, while biochar had a positive value. This could suggest in our system with zinnia and a peat-based medium, the Si had reached or was reaching its saturation point with respect to BOF slag and wollastonite, while the slower-release nature of biochar was allowing for a

steady release of Si at a concentration below the Si uptake rate of zinnia.

Even though the 5-day extraction method was a valid indicator test that could detect the presence or absence of plant-available Si, it did not accurately quantify the plant-available Si from the solid fertilizers. A number of factors could contribute to the differences, including uptake in the roots and stems that was not accounted for, leaching or polymerization of the released Si from the material resulting in less available Si, or simply that the root system of the plants did not come into contact with all the material applied. However, if we compare data between biochar and BOF slag, both fertilizers had similar leaf levels but differed significantly in extracted H_4SiO_4 levels, suggesting that a material bias may be involved in the overestimation of plant-available Si in reference to the biochar. This was reported earlier by Pereira et al. (13), who found that the 5-day extraction biased LD slags compared to BF slags. Not only did this method bias against types of slag, but also against other silica (SiO_2) compounds such as silicate clay and schist, both of which provided higher Si to rice (*O. sativa* L.) compared to the slag material but were ranked lowest in quantity of measurable Si of the 19 fertilizers tested when the 5-day extractions were used to categorize them. In addition, the data strongly support the importance of using multiple application rates to calculate predicted available Si from fertilizers. Previous grow-out studies used to correlate chemical extractions used limited rates of Si ranging from 200 to 400 g/L (12, 13). In our studies with zinnia, we found that above 25 mg/L Si, plant Si uptake did not continue to increase linearly, resulting in differences between the extracted and calculated percentages of Si. This difference needs to be factored in when trying to correlate extraction procedures to grow-out studies.
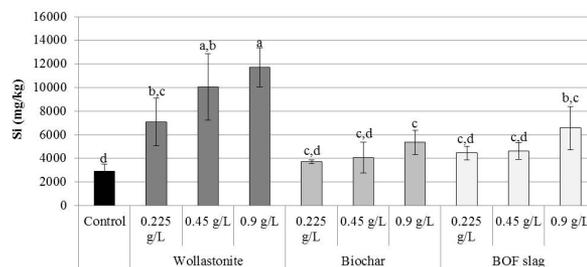
Figure 2.   Si in *Z. elegans* leaves grown for 4 weeks in 15 cm wide pots containing Sunshine Mix #2 (control) amended with wollastonite, rice hull biochar, or BOF slag at varying rates. ICP-OES was performed on dry tissue to determine the amount of Si present.
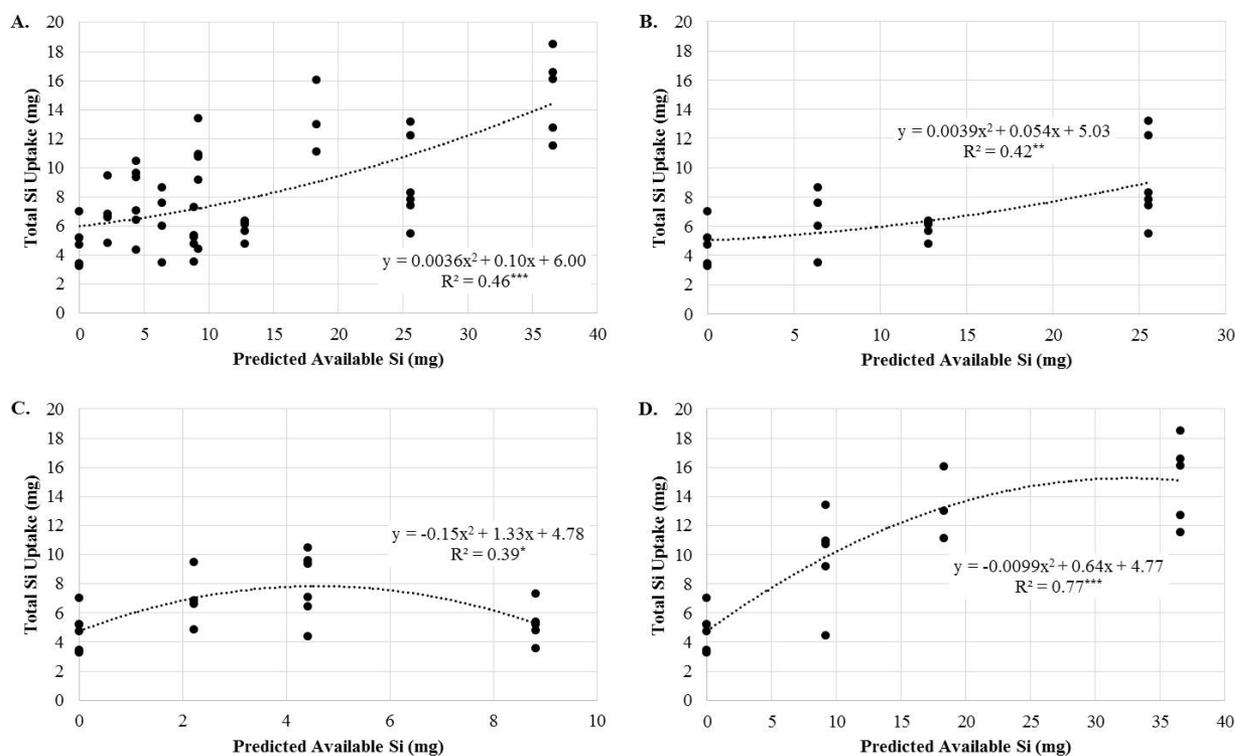
Figure 3. The relationship between 5-day extraction values ('Predicted Available Si', x-axis) and zinnia grow-out data (Total Si uptake, y-axis). The predicted-available Si was calculated and plotted against the total Si uptake in zinnia from all amendments (A), or biochar (B), BOF slag (C), and wollastonite (D) independently. Both the quadratic equation and R^2 values are indicted in each graph. Regression analysis was performed in SigmaPlot 12.5 with *P*-values < 0.05, 0.01, and 0.001 indicated as *, **, and ***, respectively.

While H_4SiO_4 has been shown to be the dominant form of Si within the vasculature of the plants, new studies on the uptake of nanosilica particles in plants suggest there may be other plant-available forms of the element. Siddiqui and Al-Whaibi (25) found that treating tomatoes with 12 nm nanosilica at a concentration of 8 g/L significantly enhanced germination of seedlings. Not only are plants influenced by these nanoparticles, but uptake of the molecules has also been demonstrated. Exposure of Arabidopsis, lupine, maize, and wheat to mesoporous nanoparticles (20 nm) resulted in uptake into both apoplastic and symplastic tissues of the leaves and roots (26). In a follow-up study by Sun et al. (27), the uptake of nanosilica particles and systemic movement was concluded by an observation of the particles in foliar tissue of root-fed plants. Considering these studies, it could be argued that chemical extractants that favor H_4SiO_4 detection would fail to detect other SiO_2 compounds that may truly be plant-available forms of Si.

Conclusions

The 5-day extraction method is a valid indicator test for the presence of Si. Our data support the use of either ICP-OES or colorimetric analyses to determine the amount of Si in the extraction solutions. However, the quantities obtained from the analysis do not predict potential uptake by *Z. elegans*, merely that it is present or not. While this method tests for the presence of H_4SiO_4 in potential Si fertilizers or beneficial substances, there still may be other forms of plant-available Si that have not been studied, thus accounting for the variation between chemical extractions and plant grow-out studies.

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