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What Constitutes Plant-Available Molybdenum in Sandy Acidic Soils?

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Molybdenum (Mo) is critical for the function of enzymes related to nitrogen cycling. Concentrations of Mo are very low in sandy, acidic soils, and biologically available Mo is only a small fraction of the total pool. While several methods have been proposed to measure plant-available Mo, there has not been a recent comprehensive analytical study that compares soil extraction methods as predictors of plant Mo uptake. A suite of five assays [total acid microwave digestion, ethylenediamenetetraaacetic acid (EDTA) extraction, Environmental Protection Agency (EPA) protocol 3050B, ammonium oxalate extraction, and pressurized hot water] was employed, followed by the determination of soil Mo concentrations via inductively coupled mass spectroscopy. The concentrations of soil Mo determined from these assays and their relationships as predictors of plant Mo concentration were compared. The assays yielded different concentrations of Mo: total digest > EPA > ammonium oxalate ≥ EDTA > pressurized hot water. Legume foliar Mo concentrations were most strongly correlated with ammonium oxalate–extractable Mo from soils, but an oak species showed no relationship with any soil Mo fraction and foliar Mo. Bulk fine roots in the 10- to 30-cm soil horizon were significantly correlated with the ammonium oxalate Mo fraction. There were significant correlations between ammonium oxalate Mo and the oxides of iron (Fe), manganese (Mn), and aluminum (Al). Results suggest that the ammonium oxalate extraction for soil Mo is the best predictor of plant-available Mo for species with high Mo requirements such as legumes and that plant-available Mo tracks strongly with other metal oxides in sandy, acidic soils.

Keywords Acidic soils, ammonium oxalate, ICP-MS, metal oxides, molybdenum

Introduction

Molybdenum (Mo) is an element vital to the global nitrogen (N) cycle because of its necessity for the function of the enzymes nitrate reductase (plant utilization of nitrate; NO_3^-) and nitrogenase [fixation of atmospheric nitrogen (N_2) to ammonium (NH_4)] (Williams and da Silva 2002). Molybdenum has been experimentally demonstrated to limit N_2 fixation, the only biological means for new N import in ecosystems (Silvester 1989; Vitousek and Howarth 1991). Observations also suggest that Mo can limit N_2 fixation in systems

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exposed to elevated carbon dioxide (CO_2) (Hungate et al. 2004). Therefore, understanding the behavior of Mo in soils is crucial to understanding plant uptake of Mo and its role as a potentially limiting factor in N conversion reactions.

Much attention has been given to estimating plant-available Mo for the reasons stated previously (Sims 1996). Water-soluble Mo may be less than 1% of the total soil Mo pool (Adriano 2001). However, the strongest correlations between available or labile Mo with plant uptake are at pH levels above 5.6 (Liu et al. 1996). Extracting soils with ammonium carbonate [$(NH_4)_2CO_3$] provides excellent predictive power ($r^2 = 0.98$) for plant Mo uptake of soil Mo, but this extraction is carried out at pH 9.0 and perhaps less useful in determining Mo in acidic soils (Vlek and Lindsay 1977).

The most important factor influencing Mo availability is soil pH. Unlike most metal nutrients, Mo is less bioavailable under acidic conditions (Kabata-Pendias 2001). Molybdate (MoO_4^{2-}) has greater activity/solubility under alkaline conditions, and soluble thiomolybdates (MoS_4^{2-} and $MoO_2S_2^{2-}$) form under reducing conditions (Kabata-Pendias 2001). Furthermore, Mo is recalcitrant at low pH because of the ligand exchange mechanism:

$$S_u\mathrm{OH} + \mathrm{Mo}^{2-} + \mathrm{H}^{+} \leftrightarrow S_u\mathrm{Mo}^{-} + \mathrm{H_2O}$$

where S_u is the soil substrate (Sposito 1984). From this model it is shown that as pH decreases (and proton concentration, $[H^+]$, increases), more Mo is bound to the soil substrate. Adsorption of Mo to soil particles becomes near 0% at pH 7.0 (Goldberg, Forster, and Godfrey 1996). Therefore, in acidic soils, the labile Mo fraction is considerably less than it would be under more alkaline conditions, and soil extractions carried out at pH greater than the soil substrate certainly overestimate the amount of labile Mo in acidic soils.

Another consideration of Mo cycling is that it is strongly fixed by the oxides of iron (Fe), aluminum (Al), and manganese (Mn), which serve as binding agents of free Mo, and the concentration of these compounds in soils should be considered when estimating Mo pools (Bibak and Borggaard 1994; Carter 1993).

The goal of this study is to report on the yield of five Mo extraction techniques analyzed with quadrupole inductively coupled mass spectroscopy (ICP-MS) and multicollector ICP-MS to determine the relative pools of Mo in a sandy acidic spodosol and the correlations between these pools and plant Mo concentrations and Mo-use efficiency. The different extractions were used to elucidate the total and four different labile Mo pools fractions, and the correlation of these methods with legume and oak Mo content as a proxy for plant uptake. The results are reported to direct future studies of Mo concentrations in soils towards analytically tractable methods for estimating plant-available Mo in acidic soils.

Materials and Methods

Soils were collected in August and October 2006 and June 2007 from the Smithsonian Environmental Center's Elevated CO_2 experiment, Kennedy Space Center, Florida (28° 38′ N, 80° 42′ W). Full details of the experiment can be found in Hymus et al. (2002). Five soil cores were systematically collected within the experimental chambers to create a composite sample. Soils at the SERC are well drained, acidic Pomello (Arenic Haplahumods) and Poala (Spodic Quartzipsamments) sands (Johnson et al. 2003). Soil moisture content

was determined gravimetrically. Soil pH was determined by adding 25 ml deionized (DI) water (H_2O) to 25 g of oven-dried soil and measuring with a bench-top electronic pH meter. Efforts were focused on the top 30 cm of soil, as this is the depth where roots are most concentrated and therefore the most likely source of Mo from the soil (Day et al. 2013).

Because of the low concentration of Mo in spodosols and plants growing on those soils, several steps were taken to minimize contamination. Centrifuge tubes and caps used in the extraction protocols were soaked for >48 h in 0.5 M hydrochloric acid (HCl). All glassware was acid washed with the same protocol and rinsed with 18 MΩ Milli-q water. Digestions and extractions were prepared in trace-metal-clean laboratories at the Keck Biogeochemsitry Laboratory at Arizona State University (Phoenix, Az.) and SUNY-Stonybrook (Stonybrook, N.Y.).

Total Mo was determined using the Environmental Protection Agency (EPA) protocol 3052 digestion. Samples were homogenized by sieving oven-dried mineral soil to pass a 2-mm screen, and then 100 mg of soil was dry ashed at 600 °C. This soil was then microwave digested in a MARS 5 digester (CEM Corp., Matthews, N.C.), with 7.0 ml HCl, 2.5 ml hydrofluoric acid (HF), and 12.0 ml nitric acid (HNO_3^-). Trace-metal-clean, concentrated acids were used for digestion and all subsequent extractions. Soils typically took several runs (up to 5) on the digestor to completely go into solution. The digestor was set at 200 °C and ran for 30 min, and samples were allowed to cool for 1 h. Samples were then diluted 10 times prior to introduction on the ICP-MS.

One plant-available Mo pool was determined with a modified ammonium oxalate extraction protocol (Liu et al. 1996). One g of sieved soil was extracted in 10 ml of 0.3 M ammonium oxalate (pH 6.0) and shook for 18 h at 500 rpm on a reciprocal shaker. The samples were centrifuged at 2000 rpm for 5 min. To keep Mo in solution prior to analysis, 1 ml HNO_3^- and 25 μl HF were added to the extract. One ml of the soil/ammonium oxalate/acid supernatant was transferred to a sample tube and diluted 15 times with deionized water. This extraction protocol was used to determine Al, Mn, and Fe oxides in addition to Mo (Carter 1993).

An ethylenediamenetetraacetic acid (EDTA) extraction was employed, as this is a strong chelating agent and is often used in agronomic settings to estimate total metal content of soils (Carter 1993). However, metals extracted using EDTA likely represent a more labile pool of metals than the total soil digest described previously, and it is a potentially useful extraction for determining plant-available Mo (Labanowski et al. 2008). For this extraction, samples were suspended in 0.05 M EDTA for 24 h, filtered, and then diluted (10 times) for analysis. All samples mentioned were analyzed at Arizona State University's Keck Biogeochemistry Laboratory (Arizona State University, Phoenix, Az., USA).

EPA protocol 3050B was also used as a comparison of plant-available Mo (U.S. EPA 1996). For this method, soils were dried for 72 h at 60 °C in a Fisher-Isotemp oven (Thermo Fisher Scientific Inc., Waltham, Mass., USA) and sieved to 2 mm. Samples were digested using repeated additions of nitric acid and hydrogen peroxide (H_2O_2). The Mo concentrations were quantified using a Thermo-Finnigan Element2 inductively coupled plasma mass spectrometer (Thermo Fisher Scientific, Waltham, Mass.). The EPA extraction was carried out on 12 samples (six ambient and six elevated plots), and these samples were analyzed at SUNY-Stony Brook.

Pressurized hot water has been used as a means for determining plant-available boron (Webb, Hanks, and Jolley 2002), and this was explored as a means for extracting plant-available Mo. The same sieved soil samples were used, and 20 g of soil was put into an

acid-cleaned single-cup espresso maker, fitted with an acid-washed Whatman filter paper (GE Health Sciences, Little Chalfont, UK). Eighteen MΩ Milli-q water was used as the extractant, and the soil extract was collected from the espresso maker into acid-washed specimen cups. The espresso maker parts that came in contact with soil were thoroughly washed with 0.5 M HCl between sample extracts.

To determine plant Mo, foliar tissues were dried at 60 °C prior to preparation for element analysis. To create chamber-level foliar samples, 500–600 mg of dried leaf sample were ashed for *Galactia elliottii* and *Quercus myrtifolia* at 600 °C from five different branches from each chamber prior to acid digestion. After acid digestion, plant samples were diluted 10 times in 18 MΩ Milli-q water.

Digestion and extraction blanks were prepared for all samples to evaluate possible contamination introduced by reagents and sample preparation. Blanks were prepared in the same manner as soil samples and in the same acid-washed sample tubes but soil was left out. Cody Shale, SCo-1 (Flanagan, 1976) were used as standard references for Al, Fe, Mn, and Mo in soils. There were very high rates of Mo recovery from the standards, with percent error ranging from 0.11% at the greatest concentration to 4.29% error at the lowest Mo concentration of 0.08 ng g^{-1} (Table 1). Apple leaf (NIST SRM 1515) was used as a digestion standard for plant tissue (100 ± 10% recovery). Rhodium (^{103}Rh) was used as an internal instrument standard.

There was not a significant CO_2 effect on plant or soil Mo concentrations at the Florida experiment (Duval et al. 2013), and therefore researchers lumped treatment and control plots in the comparison of methods to increase sample size ($n = 16$ compared to $n = 8$). Pearson's correlation analysis was employed to determine the strength of association between different analytical techniques for assaying soil Mo and to determine how those related to plant foliar Mo concentration for a N_2-fixing legume (*Galactia elliottii*), which likely has relatively high Mo requirements, and a nonfixing oak species (*Quercus myrtifolia*), which has presumably lower Mo needs (*sensu* Anderson and Spencer 1950). Correlation analysis was used to make inferences about how the oxides of Al, Mn, and Fe relate to different extractable Mo fractions. Statistical tests were performed in R (R Core Development Team 2013).

Table 1
Mean recovery values (ppb) of molybdenum from the NIST standard SCo-1, analyzed via ICP-MS

Standard (dilution ratio)	Defined [Mo]	Measured [Mo]	Error	Error (%)
Blank	0.00	0.00	0.00	0.00
SCo-1 (1:25,000)	0.08	0.07	0.003	4.29
SCo-1 (1:7,000)	0.28	0.26	0.01	3.85
SCo-1 (1:500)	3.86	4.03	0.17	4.22
SCo-1 (1:75)	26.36	26.07	0.71	2.72
SCo-1 (1:15)	129.5	129.56	0.14	0.11

Notes. Dilution ratios refer to quantity of standard in sample matrix introduced into the instrument. Defined molybdenum equals NIST published values for Mo in the SCo-1 standard, corrected for our dilution; measured Mo concentration is our measurement of the standards.

Results

The five extraction techniques yielded different amounts of Mo from the study soils, in the order of total digest > EPA protocol 3050 > ammonium oxalate ≥ EDTA > pressurized hot H_2O (Table 2). Four of the methods resulted in greater Mo concentrations in the A horizon soils (0–10 cm), with the exception of EDTA Mo, which showed a greater Mo concentration in the E horizon, 10–30 cm (Table 2). The assays yielded a different order of concentrations for Al, Fe, and Mn compared to Mo (Table 2). The total acid digest yielded the greatest concentration for all metals, but a divergence from Mo was that the EDTA extraction resulted in greater concentrations of Al, Fe, and Mn compared to the ammonium oxalate extraction, and the hot water extract resulted in greater Mn concentrations than ammonium oxalate (Table 2).

In relation to plant uptake, only the ammonium oxalate extraction yielded a positive relationship with *Galactia elliottii* foliar Mo (Table 3). No significant relationships were found between any of the soil extraction assays and *Quercus myrtifolia* foliar tissue Mo (Table 3). However, the Mo concentration of bulk fine roots sampled between 10 and 30 cm (E horizon) was significantly correlated with ammonium oxalate soil Mo (Table 3; $r = 0.50$, $P < 0.05$).

As the oxides of Al, Fe, and Mn are important controls on free Mo (Carter 1993), we also compared concentrations of those metals with the ammonium oxalate–extractable Mo pool. In the upper 10 cm of the soil column, weak, nonsignificant correlations were observed between Al and Fe oxides ($r = 0.29$ and $r = 0.26$, respectively) and plant-available Mo (Table 4). However, plant-available Mo in the E horizon (10–30 cm) significantly correlated ($P \leq 0.001$) with Al, Fe, and Mn oxides (Table 4). This is perhaps surprising as concentrations were lower for all four metals in the E horizon, but in these sandy soils, organic matter that can also bind free Mo is concentrated in the upper soil layers (Kabata-Pendias 2001).

Discussion

Parent material determines overall Mo content in soils (Manheim and Landergren 1978; Brady and Wiel 2002). Highly weathered sands derived from sandstone sediments with stable minerals are generally low in Mo due to drainage losses (Gupta 1997), so low total Mo concentrations in these coastal spodosols were expected. However, total Mo proved to be a poor predictor of plant Mo, and the ammonium oxalate extraction was the only assay to significantly correlate with legume and fine root Mo, even though this constituted 0.32–0.42% of total Mo (Tables 2 and 3).

The Mo deficiency would likely be most important in ecosystems reliant on N import from N_2-fixing symbionts (Hungate et al. 2004). An isotope-tracing technique might more accurately assess plant Mo uptake, whereby plants are grown in a medium of known ^{97}Mo:^{95}Mo, or in a substrate fertilized with Mo of a pure (99.9%) stable isotope. However, that could be cost prohibitive, and results could be confounded by biological fractionation or isotope discrimination (Barling, Arnold, and Anbar 2001). Therefore, correlations between ammonium oxalate Mo and *Galactia* Mo are relevant for researchers trying to establish relationships between soil Mo availability and plant Mo nutrition. Results show that ammonium oxalate–extractable Mo provides reasonable predictive power for plant Mo in a legume and is inexpensive and simple to perform in the laboratory.

Table 2
Aluminum, manganese, iron, and molybdenum concentrations in coastal Florida spodosols, as determined by five digestion and extraction methods

Extraction method	Soil depth	Al ($\mu g \cdot g^{-1}$)	Mn ($\mu g \cdot g^{-1}$)	Fe ($\mu g \cdot g^{-1}$)	Mo ($ng \cdot g^{-1}$)
Total digest	A horizon	32571 (3715)	436.1 (23.8)	12529 (1190)	2375 (193.2)
	E horizon	33636 (5576)	561.6 (38.8)	15655 (2919)	2055 (254.0)
EDTA	A	194.2 (30.8)	2.82 (0.30)	289.0 (59.6)	7.58 (0.57)
	E	141.4 (57.6)	0.25 (0.03)	135.9 (53.6)	8.48 (0.43)
Ammonium oxalate	AAa A	108.51 (26.3)	1.12 (0.21)	95.1 (32.4)	9.96 (2.47)
	E	87.5 (24.1)	0.70 (0.10)	65.7 (23.2)	6.66 (0.43)
EPA 3050B	A	187.2 (48.9)	2.35 (0.12)	151.0 (30.4)	28.2 (1.95)
	E	317.9 (101.3)	3.02 (0.20)	244.7 (65.8)	20.7 (1.70)
Hot H_2O	A	0.18 (0.03)	3.68 (0.72)[a]	57.9 (16.8)[a]	0.15 (0.01)
	E	0.06 (0.01)	0.67 (0.13)[a]	24.5 (8.50)[a]	0.08 (0.01)

Note. Values are mean concentrations with SEM in parentheses.
[a]Units are $ng \cdot g^{-1}$.

Table 3
Correlation analysis results comparing the relationships between five different methods of determining molybdenum from soil and plant tissues of the dominant oak species (*Quercus myrtifolia*) and a symbiotic N_2-fixing legume (*Galactia elliottii*)

Extract	*Quercus myrtifolia* [*r* (*P* value)]	*Galactia elliottii* [*r* (*P* value)]
Foliar tissue		
Total digest	0.01 (0.97)	0.28 (0.32)
Ammonium oxalate	−0.03 (0.90)	0.44 (0.10)*
EDTA	0.13 (0.64)	−0.01 (0.98)
EPA	0.22 (0.52)	−0.02 (0.98)
Hot H_2O	0.10 (0.72)	0.33 (0.23)
Coarse roots (>2 mm) 0–10 cm		
Total digest	−0.06 (0.82)	
Ammonium oxalate	0.04 (0.88)	
EDTA	−0.04 (0.88)	
EPA	−0.26 (0.33)	
Hot H_2O	0.29 (0.28)	
Fine roots (<2 mm) 0–10 cm		
Total digest	0.03 (0.91)	
Ammonium oxalate	0.02 (0.92)	
EDTA	−0.13 (0.63)	
EPA	0.09 (0.74)	
Hot H_2O	0.22 (0.41)	
Fine roots (<2 mm) 10–30 cm		
Total digest	−0.19 (0.48)	
Ammonium oxalate	0.50 (<0.05)	
EDTA	0.28 (0.29)	
EPA	−0.29 (0.28)	
Hot H_2O	−0.18 (0.50)	

Notes. Results are from Pearson's correlation test, and *P* values are in parentheses.

Table 4
Correlations between ammonium oxalate molybdenum and the oxides of Al, Mn, and Fe in sandy soils

Horizon	Metal oxide	Correlation coefficient	*P* value
A (0–10 cm)	Al	0.29	0.28
	Mn	0.08	0.77
	Fe	0.26	0.33
E (10–30 cm)	Al	0.84	<0.001
	Mn	0.72	0.001
	Fe	0.80	<0.001

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