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Correcting Micronutrient Deficiency Using Metal Hyperaccumulators: *Alyssum* Biomass as a Natural Product for Nickel Deficiency Correction

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***Abstract*. The existence of nickel (Ni) deficiency in certain horticultural crops merits development of fertilizer products suitable for specific niche uses and for correcting or preventing deficiency problems before marketability and yields are affected. The efficacy of satisfying plant nutritional needs for Ni using biomass of Ni hyperaccumulator species was assessed. Aqueous extraction of *Alyssum murale* (Waldst. & Kit.) biomass yielded a Ni-enriched extract that, upon spray application, corrects and prevents Ni deficiency in pecan [*Carya illinoinensis* (Wangenh.) K. Koch]. The Ni-*Alyssum* biomass extract was as effective at correcting or preventing Ni deficiency as was a commercial Ni-sulfate salt. Foliar treatment of pecan with either source at ≥10 mg·L^{-1} Ni, regardless of source, prevented deficiency symptoms whereas treatment at less than 10 mg·L^{-1} Ni was only partially effective. Autumn application of Ni to foliage at 100 mg·L^{-1} Ni during leaf senescence resulted in enough remobilized Ni to prevent expression of morphologically based Ni deficiency symptoms the following spring. The study demonstrates that micronutrient deficiencies are potentially correctable using extracts of metal-accumulating plants.**

Many plant species have specialized physiological processes enabling metal accumulation throughout the growing season. Species accumulating trace elements (e.g., Ni, Cu, Co, Mn, Se, Zn) at more than 1000 µg·g^{-1} shoot dry weight when they grow on soils where they evolved are considered hyperaccumulators (Reeves and Baker, 2000). A review of phytoremediation research by Salt and colleagues (1998) reports that hyperaccumulation occurs in ≈45 plant families and ≈400 taxa, with biotypes accumulating trace metals at concentrations as high as 3% dry weight in whole shoots and 4% in leaves. It was recognized by Chaney (1983) that hyperaccumulation offered a means for soil remediation. He subsequently introduced the concept of phytoextraction as a technique for phytoremediation of metal-contaminated or geogenic high-metal soils.

A research consortium has developed commercially feasible phytoremediation and phytomining technologies for reclaiming Ni-contaminated soils (Chaney et al., 2005; Li et al., 2003a) via usage of *Alyssum murale* and *A. corsicum* as Ni hyperaccumulators. The genus *Alyssum* (Brassicaceae) contains a large number of Ni hyperaccumulating species (Broadhurst et al., 2004a), many of which accumulate Ni at more than 2% dry foliage (Baker and Brooks, 1989). These species are typically native to serpentine soils throughout Mediterranean Europe, yet also grow well on a wide variety of soil types (Broadhurst et al., 2004a; Li et al., 2003a, b). *Alyssum* hyperaccumulator species primarily store Ni in epidermal cell vacuoles or in the basal portions of stellate trichomes (Ni at ≈15% to 20% dry weight) (Broadhurst et al., 2004b). The cultivation of perennial *Alyssum* on high-Ni soils not only provides a means of soil remediation, but also a means of mining the soil for the valuable Ni metal. Additionally, the biomass is a potential organic fertilizer source for Ni and certain other trace nutrient elements. In addition, shoots of *Alyssum* Ni hyperaccumulator species contain high levels of malate, citrate, and malonate, which contribute to metal tolerance and storage in tissues (Brooks et al., 1981) and might improve the absorption of Ni by leaves compared with inorganic Ni solutions.

Spray-applied Ni is increasingly recognized as an essential mineral nutrient element for higher plants (Bloom, 2002; Epstein and Bloom, 2005; Gerendas et al., 1999; Marschner, 2002;), especially with the discovery of field-level Ni deficiencies severe enough to kill trees (Wood et al., 2004c). The discovery that Ni deficiency was the cause of both a mysterious malady of pecan, termed "mouse-ear" (Demaree, 1926; Wood et al., 2004a) and of an increasingly common replant malady in old, or second generation, pecan orchards (Wood et al., 2004a, c) has established the need for commercial Ni fertilizers. Additionally, Ni deficiency has been documented as the cause of the "little-leaf" malady in containerized river birch (*Betula nigra*) (Ruter, 2005), and anecdotal evidence indicates that Ni deficiency occurs in many woody perennials and tropical legumes (Wood et al., 2006). Deficiency can also have major impact on primary and secondary metabolism (Bai et al., 2006), can potentially influence plant resistance to certain diseases (Wood and Reilly, 2006), and is especially significant to nitrogen catabolism (Bai et al., 2006).

It is becoming apparent that Ni is likely a far more limiting factor in agriculture than previously supposed (Bai et al., 2006, Nyczepir et al., 2006; Wood and Reilly, 2006; Wood et al., 2006). Thus, potential sources of Ni fertilizers are likely to be increasingly needed depending on usage situations. The discovery of field-level nickel deficiency in agriculture provides, for the first time, the novel approach and opportunity to correct micronutrient deficiencies using biomass of Ni hyperaccumulating plants such as *A. murale*. Organic sources of microelements are of special potential in cropping niches where "organic" certification is important. We hypothesized that aqueous extracts of *Alyssum*, a Ni hyperaccumulator also high in organic acids, could correct Ni deficiency. Our objectives were 1) to determine whether extracts of *A. murale* biomass could correct Ni deficiency of pecan and 2) to evaluate the efficacy of *A. murale* extracts against an easily absorbed, commercially available Ni salt for correction of Ni deficiency.

Materials and Methods

Efficacy of Ni Sources as Foliar Sprays

The ability of *A. murale* extracts to prevent the expression of morphological symptoms of Ni deficiency was assessed for greenhouse-grown pecan trees. Seedling trees were grown in 18 × 18 × 18-cm plastic pots with a loamy sand soil (Tifton loamy

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sand) from a commercial pecan orchard that exhibited Ni deficiency. Two-year-old seedlings of Desirable' were rated for severity of Ni deficiency based on morphological symptoms (referred to as mouse-ear) expressed the growing season previous to treatment (Wood et al., 2004a). Seedlings exhibiting symptoms with severity classes of "7–9" (Nyczepir et al., 2006) were selected for Ni treatments the following spring and were subsequently blocked according to severity class. Assessment of degree of Ni deficiency exhibited by shoots and foliage of seedlings was based on the following scale developed for estimation of severity of Ni deficiency symptoms by Wood and colleagues (2004a): 1 = no Ni-associated morphological distortions of leaflets, leaves, or shoots (i.e., normal); 2 = 1% to 25% of leaflets on the seedling exhibiting Ni-deficient morphological distortions (i.e., slightly blunted); 3 = 26% to 50% of leaflets exhibiting some degree of Ni-associated morphological distortion; 4 = more than 50% of leaflets exhibiting morphological distortion; 5 = more than 50% morphological distortion of leaflets, plus leaflet cupping; 6 = rating 5 plus necrosis of leaflet tips; 7 = rating 6 plus necrosis of leaflet margins, crinkled leaflets, and dwarfed leaflets; 8 = rating 7 plus dwarfed shoots; 9 = rating 8 plus rosetting, or multiple shoots; and 10 = rating 9 plus tree death.

The experiment consisted of three Ni treatments (including untreated control) and eight replicates blocked by severity class. Ni sources (*A. murale* extract and $NiSO_4{\cdot}6H_2O$) were applied at a Ni concentration of 100 $mg{\cdot}L^{-1}$ formulated with 0.05% surfactant (Bio-surf) and deionized water. For the Ni-*Alyssum* treatment, the Ni-*Alyssum* stock solution was prepared via aqueous extraction of ground *Alyssum* biomass (i.e., ground *Alyssum* hay grown on Brockman variant gravelly loam near Cave Junction, Ore.). The powdered biomass was mixed with deionized water (1 g dry weight to 33 mL water), heated to 100 °C, and stirred for 24 hours as the solution cooled to ambient temperature. The mixture was then filtered to remove particulate matter, and the Ni extract was stored refrigerated until needed. Atomic absorption spectroscopy indicated that the extract contained Ni at 623 $mg{\cdot}L^{-1}$. Lower Ni-*Alyssum* concentrations used for Ni treatments were attained by appropriate dilution of the 634 $mg{\cdot}L^{-1}$ stock solution. The $NiSO_4{\cdot}6H_2O$ was an analytical grade salt (Sigma-Aldrich, St. Louis, Mo.). Treatments were applied via foliar spraying, being cautious to prevent soil contamination of potted trees by using a physical spray barrier. Sprays were timed to occur at the "parachute" stage (i.e., when the immature compound leaves are unfolding from the emerging shoot to present an appearance resembling an open parachute) of budbreak of the third leaf during the spring, and again 1 week later. Treatments were rated 5 weeks after budbreak. Ratings were severity classes based on morphological symptoms of Ni deficiency (Nyczepir et al., 2006; Wood et al., 2004a). Spray application was by a wand-type hand-pump sprayer, with foliage being sprayed until leaflet drip occurred. Treatment means with standard errors of the mean were calculated (Systat Software, Richmond, Calif.).

Efficacy of Ni-*Alyssum* as Foliar Spray Treatments in Spring or Autumn

The efficacy of Ni-*Alyssum* for correcting morphological Ni deficiency symptoms of orchard trees was evaluated using 5-year-old 'Desirable' trees exhibiting Ni deficiency symptoms that were growing in a commercial pecan orchard. Efficacy was evaluated within the context of two distinct studies assessing application in both autumn and spring application periods. For the first study, application occurred in mid Oct., about 1 month before natural leaf drop. Tree canopies received a single foliar Ni spray application of 100 $mg{\cdot}L^{-1}$ Ni with nonionic surfactant (0.05% Bio-surf). Nickel sources were the Ni-*Alyssum* and $NiSO_4{\cdot}6H_2O$ described previously. Treatments were two Ni sources, plus the nontreated control, replicated nine times in a randomized complete block design. Blocks were based on the severity of the morphological Ni deficiency symptoms (i.e., severity of mouse-ear-associated disorders). Experimental units were comprised of single trees. Treatments were evaluated about 4 weeks after budbreak the following spring for expression of morphological Ni deficiency symptoms using the scale described earlier. Spray application was until leaflet drip using a motorized 30-gal. power sprayer.

The second study was identical to the first study (i.e., autumn), except that Ni treatments were applied the following spring, when tree canopies were in the "parachute" stage of budbreak. Severity of Ni deficiency symptoms were assessed 4 weeks after budbreak. In both studies treatment means along with the standard error of the mean were calculated.

Concentration of Ni Needed to Correct Deficiency When Applied as a Foliar Spray

Nickel-deficient Desirable' seedlings (2-year-old) growing in pots (as described earlier) were selected about 2 d after budbreak (i.e., preparachute stage) and segregated according to the severity of morphological symptoms of Ni deficiency. Several concentrations of either Ni-*Alyssum* or $NiSO_4{\cdot}6H_2O$ were evaluated regarding their ability to correct Ni deficiency of pecan seedlings. Nickel sources were nickel sulfate hexahydrate and an *Alyssum* extract formulated as described earlier. The two Ni sources were applied at 0.001, 0.01, 0.1, 1, 10, 50, and 100 $mg{\cdot}L^{-1}$ Ni. The experiment contained two Ni sources at seven Ni concentrations arranged in a randomized complete block design with five replications. Blocking was by severity of Ni deficiency. Experimental units were single seedlings. Treatments were applied every 7 d for five applications, beginning at the "parachute" stage of spring budbreak and extending throughout Apr. Application was via foliar spray with a wand-type hand-pump sprayer, with foliage being treated until leaflet drip occurred. Soil surfaces were protected from exposure to drift or drip using a physical barrier. Treatments were visually assessed for symptoms of Ni deficiency on growth occurring after treatment application and were rated regarding severity of deficiency using a severity class scale ranging from 1 to 10 as described earlier.

Results and Discussion

Efficacy of Ni-*Alyssum* on Greenhouse-Grown Trees

Ni deficiency symptoms were largely prevented from occurring in 2-year-old greenhouse-grown Desirable' pecan seedlings receiving a single foliar postbudbreak spray of equimolar Ni treatments with Ni derived from either $NiSO_4{\cdot}6H_2O$ or Ni-*Alyssum* extracts (Fig. 1). Seedlings treated with an aqueous spray of the Ni-sulfate salt did not exhibit Ni morphological symptoms of Ni deficiency, although the nontreated control treatment exhibited symptoms of severe deficiency. By comparison, the Ni-*Alyssum* treatment also greatly reduced Ni deficiency symptoms, with results being only slightly more variable compared with Ni-sulfate. These results indicate that Ni-*Alyssum* extracts are efficacious for Ni deficiency correction.

Efficacy of Ni-*Alyssum* as Autumn or Spring Foliar Spray Treatments to Orchard Trees

Ni-*Alyssum* extracts applied during autumn were as efficacious as $NiSO_4{\cdot}6H_2O$ for preventing Ni deficiency symptoms (Fig. 2A). A single autumn foliar spray treatment (at 100 $mg{\cdot}L^{-1}$) of Ni nearly eliminated the expression of morphological Ni deficiency symptoms in shoot and leaf growth the subsequent spring. Treated trees only occasionally exhibited slight and sporadic Ni deficiency symptoms. These were occasional blunted leaflets within the tree canopy, whereas trees of the nontreated control exhibited Ni deficiency so severe that canopies were comprised of severely dwarfed shoots with foliage that was also severely dwarfed and blunted, and with necrotic leaflet tips and cupped leaflet margins. Mean late-Apr. leaflet Ni concentration of Ni-treated (in autumn) foliage was $\approx$0.98 $\mu g{\cdot}g^{-1}$ dry weight for either Ni-*Alyssum* or Ni-sulfate treatments and about $\approx$0.06 $\mu g{\cdot}g^{-1}$ dry weight for nontreated trees (data not included). These results indicate that autumn application of Ni to foliage of Ni-deficient trees was an effective means of preventing or minimizing disruptions in tree growth the following spring. Thus, Ni appears to be partially remobilized from senescing foliage and transported to dormant season reserve pools that are again mobilized in the spring to meet the growth demand for Ni by buds and shoots the following spring.

A single foliar application of either a Ni-*Alyssum* extract or a $NiSO_4{\cdot}6H_2O$ salt during

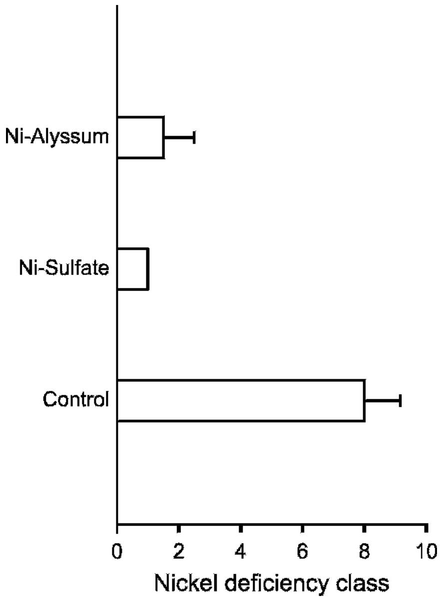

Fig. 1. Efficacy of Ni from *Alyssum* biomass and $NiSO_4 \cdot 6H_2O$ for correction of Ni deficiency of greenhouse-grown third-leaf Desirable' seedlings treated with a single postbudbreak foliar spray of Ni at 100 $mg \cdot L^{-1}$ with 0.05% surfactant (Bio-surf) and deionized water. Values are treatment means and standard errors. Nickel deficiency class is as follows: 1 = no Ni-associated morphological distortions of leaflets, leaves, or shoots (i.e., normal); 2 = 1% to 25% of leaflets on the seedling exhibiting Ni-deficient morphological distortions (i.e., slightly blunted); 3 = 26% to 50% of leaflets exhibiting some degree of Ni-associated morphological distortion; 4 = more than 50% of leaflets exhibiting morphological distortion; 5 = more than 50% morphological distortion of leaflets plus leaflet cupping; 6 = rating 5 plus necrosis of leaflet tips; 7 = rating 6 plus necrosis of leaflet margins, crinkled leaflets, and dwarfed leaflets; 8 = rating 7 plus dwarfed shoots; 9 = rating 8 plus rosetting, or multiple shoots; and 10 = rating 9 plus tree death.

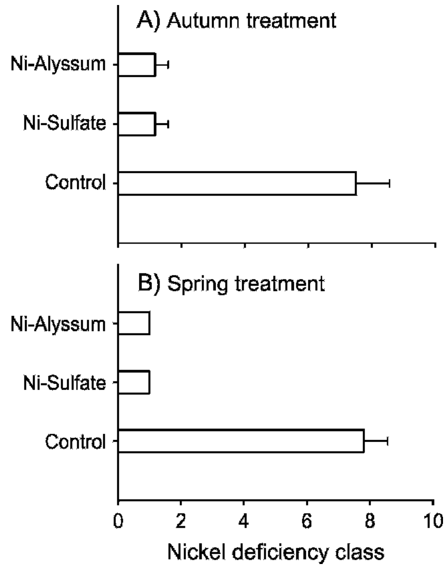

Fig. 2. Efficacy of Ni-*Alyssum* and $NiSO_4 \cdot 6H_2O$ for correcting or preventing morphological symptoms of Ni deficiency by 5-year-old 'Desirable' trees growing in a commercial orchard is compared for a single foliar spray at 100 $mg \cdot L^{-1}$ Ni. Treatments were applied in the autumn (Oct., A) or the spring (Apr., B). Values are treatment means and standard errors. See Fig. 1 for an explanation of Ni deficiency class.

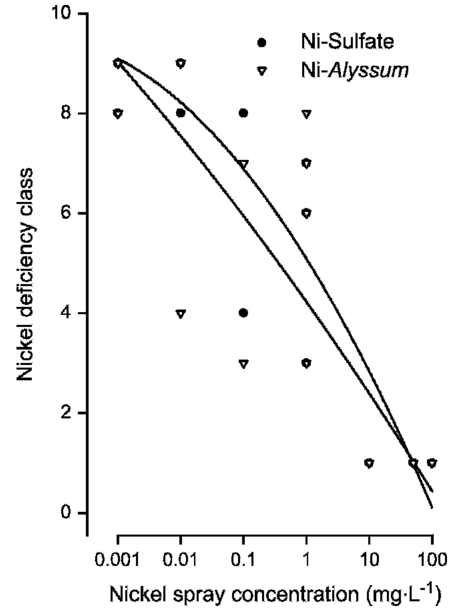

Fig. 3. Efficacy of foliar sprays of Ni-*Alyssum* compared with $NiSO_4 \cdot 6H_2O$, for correction of Ni deficiency of third-leaf greenhouse-grown Desirable' seedlings treated with five weekly applications of various Ni concentrations beginning at the "parachute" stage of spring budbreak. The two Ni sources were applied at 0.001, 0.01, 0.1, 1, 10, 50, and 100 $mg \cdot L^{-1}$. Curves denote quadratic regressions of each Ni source. See Fig. 1 for an explanation of Ni deficiency classes.

early budbreak at 100 $mg \cdot L^{-1}$ Ni prevented expression of morphological symptoms of Ni deficiency (Fig. 2B). By comparison, the control seedlings exhibited severe shoot dwarfing (i.e., short internodes) with foliage that was also severely dwarfed and blunted, and with necrotic leaflet tips and cupped leaflet margins.

These data indicate that pecan tree Ni deficiency symptoms can be prevented by either autumn or spring foliar applications of aqueous solutions with Ni being at least 100 $mg \cdot L^{-1}$. The correction or prevention of Ni deficiency in pecan using autumn Ni applications means that farmers can largely correct the Ni-associated nutritional problem before the initiation of growth the following spring. The autumn application approach is especially appealing in that many commercial orchard operations find it difficult or impossible to apply a ground spray timely just after budbreak, because of either excessively wet orchard floors or because there is insufficient spraying capacity for ensuring that trees get Ni in time to avoid either morphological or physiological harm to the tree. Thus, autumn application of Ni has the advantage of ensuring that orchard trees will experience little or no physiological or growth harm the following spring before a second application can occur. These results also indicate that Ni from *Alyssum* biomass is highly efficacious for correction of morphological deficiency symptoms of Ni deficiency of orchard trees, even when applied by spraying the previous growing season to correct problems in the subsequent growing season.

Concentration of Ni Needed to Correct Deficiency When Applied as a Foliar Spray

Nickel deficiency symptoms were corrected by foliar sprays of Ni from either *Alyssum* or $NiSO_4 \cdot 6H_2O$ (Fig. 3). Response curves for both sources were curvilinear with the quadratic nature of the curves differing only slightly, with the slope of the Ni-sulfate curve being slightly greater than that of the Ni-*Alyssum* curve. It is possible that the Ni-sulfate source was slightly more efficacious at low Ni concentrations than that of the Ni-*Alyssum* source, but any difference is small at best.

Both Ni sources had little or no influence on Ni deficiency symptoms when spray Ni concentration was less than 1 $mg \cdot L^{-1}$. Symptom severity showed distinct signs of being reduced with 1 $mg \cdot L^{-1}$ Ni sprays. Foliar spray concentrations of $\geq$10 mg $Ni \cdot L^{-1}$ were highly effective at preventing morphological symptoms of Ni deficiency in subsequent spring shoot and leaf growth. At Ni concentrations of 10, 50, and 100 $mg \cdot L^{-1}$, foliar sprays were equally efficacious for correction of Ni deficiency of seedling trees. This response of severely deficient seedlings to spray solutions containing as little as 10 $mg \cdot L^{-1}$ Ni indicates that the relatively small amount of Ni needed to satisfy metabolic and physiological needs can easily enter shoot and foliar tissues in sufficient quantity to satisfy plant needs. It also indicates that Ni from *Alyssum* biomass is as efficacious as that of Ni from $NiSO_4 \cdot 6H_2O$.

Conclusions

These results indicate that foliar treatment of Ni-deficient pecan trees with aqueous sprays of Ni at $\geq$10 $mg \cdot L^{-1}$ derived from either a Ni-containing *Alyssum* biomass or from an $NiSO_4 \cdot 6H_2O$ salt are efficacious for correcting Ni deficiency of pecan trees in both orchard or nursery operations. Autumn treatment of foliage with aqueous sprays of Ni offers a practical means of correcting deficiencies before the following spring's budbreak, when orchard situations can limit the orchard manager's ability to apply Ni sprays timely for fully satisfying metabolic and growth needs. The success of autumn treatments is evidence that small amounts of Ni are remobilized in senescing foliage and translocated to dormant season storage tissues and again mobilized for spring growth. The efficacy of Ni-*Alyssum* as a foliar spray indicates that incorporation of the Ni-enriched biomass into soils would be expected to provide for plant Ni needs via root uptake.

The efficacy of Ni-*Alyssum* biomass extracts for correction of Ni deficiency identifies for the first time the use of a plant extract for correction of a specific micronutrient deficiency. Therefore, *Alyssum* biomass provides a means for correction of Ni deficiency that appear to be fully compliant with criteria pertaining to organic agriculture certifications and that is likely to be cost competitive with mineral sources of Ni. The successful correction of Ni deficiency in pecan with aqueous extracts of a metal-hyperaccumulating species is evidence that biomass of selected metal hyperaccumulators used in soil remediation offer potential as cost-effective fertilizer sources for correcting micronutrient needs of crops.

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