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Highlights

A preliminary study of the role of nickel in enhancing flowering of the nickel hyperaccumulating plant *Alyssum inflatum* Nyár. (Brassicaceae)

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- We investigated if reproductive fitness in *A. inflatum* is enhanced by Ni.
- We documented survival and the proportion of individuals that flowered.
- We also quantified flower production as an indicator of plant fitness.
- Survival was not affected by the presence of Ni.
- Ni, however, stimulated flowering and may contribute to greater fitness.

1 Short communication

A preliminary study of the role of nickel in enhancing flowering of the nickel hyperaccumulating plant *Alyssum inflatum* Nyár. (Brassicaceae)

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ABSTRACT

Alyssum inflatum is a native of serpentine soils and is able to hyperaccumulate nickel (Ni), but the importance of Ni to reproduction in the species is unknown. We investigated if reproductive fitness is enhanced by Ni in the growth medium, and included a treatment involving a relatively high level of Mg to provide a comparison with elevated levels of another metal. Seedlings were grown in a modified Hoagland solution culture in an inert medium of Perlite and were treated with Ni (100 μM), a high concentration of Mg (5 mM), or under control conditions (solution culture without Ni or the addition of high Mg) for 14 months. We documented survival, as well as the proportion of individuals that flowered. We also quantified flower production as an indicator of plant fitness. Survival was not affected by treatment (87–90% for all treatments), but significantly more Ni-treated plants (63%) flowered compared with Mg-treated (19%) or control plants (12%). In addition, inflorescences per plant, inflorescence length, and number of open flowers per inflorescence were all significantly greater for Ni-treated plants relative to plants from the other treatments. Although high levels of Ni are not essential for growth and reproduction of the species, we suggest that Ni stimulates flowering in *A. inflatum* and may result in greater fitness for the species on serpentine soils.

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1. Introduction

The study of plant adaptations to serpentine soils is an active area of research (O'Dell and Rajakaruna, 2011). Serpentine soils differ from normal soils due to their unique physico-chemical and biological features that make them unfavorable for seed germination, seedling establishment, and subsequent growth (Brady et al., 2005; Kazakou et al., 2008). The unfavorable features include a Ca:Mg ratio <1, usually low concentrations of essential nutrients such as N, P and K, and generally low moisture holding capacity due to unstable, rocky, and shallow soil often found on usually open, steep landscapes upon which serpentine outcrops are frequently found (Kruckeberg, 1984; Rajakaruna et al., 2009). The soils are also rich in heavy metals such as Cr, Ni and Cd (Brady et al., 2005). Plants living in such harsh environments have evolved adaptations to overcome the stressors, including developing tolerance to low Ca:Mg ratios (Bradshaw, 2005; Grace et al., 2007; Palm et al., 2012), low essential nutrients (Brady et al., 2005), or elevated levels of heavy metals (O'Dell and Rajakaruna, 2011; Pollard et al., 2002), enabling them to even thrive on metalliferous soils.

Heavy metals become toxic to organisms if their concentrations in tissues exceed particular thresholds (Kabata-Pendias, 2001). For most plants these thresholds are at very low concentrations (Ahmad and Ashraf, 2011). Based on the tolerance of different species of plants to metals and their ecological breadth, Pollard et al. (2002) divided plants into four groups: those that solely grow on non-metalliferous soils and have no populations or ecotypes on metal rich soils (obligate non-metallophytes); plants endemic to metalliferous soils (obligate metallophytes); and two types of facultative metallophytes, those with a few tolerant populations and those with a few non-tolerant populations. The role of heavy metals in adaptation to metalliferous soils is unclear. Some heavy metals (e.g. Fe and Zn) have important roles in plants and are considered essential elements (Marschner, 1995). Others, such as Ni (Brown et al., 1987), are essential but plants usually need them in very low amounts (Epstein and Bloom, 2004; Polacco et al., 2013). High concentrations of these micronutrients can cause severe toxicities (Kabata-Pendias, 2001; Marschner, 1995) and, in some cases, even reduce both flower and ramet production (i.e. reproductive output) in metal-intolerant plants (Saikkonen et al., 1998).

Metallophytes may be restricted to metalliferous soils because they are poor competitors in non-metalliferous soils (Going et al., 2009; Kay et al., 2011; O'Dell and Rajakaruna, 2011), because they are

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relatively unprotected against herbivores and pathogens on non-metalliferous soils (Martens and Boyd, 1994; Rascio and Navari-Izzo, 2011; Strauss and Boyd, 2011), or because they require relatively high levels of metals for optimal growth and reproduction. Reports regarding the need of Ni hyperaccumulators for Ni are inconsistent. Some authors, e.g. Reeves and Baker (1984) and de Varennes et al. (1996), reported no difference in growth between hyperaccumulator species when grown in greenhouse or laboratory cultures on high- versus low-Ni media. Conversely, others (e.g., Boyd et al., 1994; Burrell et al., 2012; Krämer et al., 1996; Palomino et al., 2007; Saison et al., 2004) have reported a growth-stimulating effect of Ni. Few authors have proposed a mechanism for this effect: however, a recent review (Polacco et al., 2013) suggests that the growth stimulating effect may stem either from direct beneficial effects of Ni on N metabolism (because Ni is essential for the enzyme urease) or from indirect effects stemming from a potential role of Ni-containing urease in supporting plant pathogen defense.

Alyssum inflatum Nyár. is considered a metallophyte since most populations occur on serpentine soil, although Ghasemi and Ghaderian (2009) report at least one population found on non-serpentine soil. Like many other members of the genus (Cecchi et al., 2010), A. inflatum is able to hyperaccumulate Ni (Ghaderian et al., 2007; Ghasemi et al., 2009a), accumulating more than 1000 μg Ni g^{-1} dry weight in leaves of at least one population from its natural habitat (Van der Ent et al., 2013). In this study, we test the influence of Ni on survival and reproduction of A. inflatum to determine whether long-term treatment with Ni enhances the fitness of this Ni-hyperaccumulating serpentine plant.

2. Material and methods

2.1. Plant material

A. inflatum, a native of Anatolia (Baker and Brooks, 1989), is an outcrossing perennial from the section Odontarrhena (Brassicaceae). It flowers from June–July with fruits ripening in September. The population from which seeds were collected for this research occurs at Marivan, on serpentine soils in western Iran (N 35°, 13.625′ and E 46°, 27.184′). As reported by Ghasemi and Ghaderian (2009), these serpentine soils are high in Ni (total Ni 1600 μg g^{-1} and ammonium nitrate extractable Ni 1.6 μg g^{-1}), have high Mg levels (total Mg 90,000 μg g^{-1} and ammonium nitrate extractable Mg 1300 μg g^{-1}) and have low Ca:Mg ratios (0.04 for total and 1.4 for ammonium nitrate extractable concentrations). The elevation of this area is about 1600 m above sea level. Average yearly precipitation is more than 700 mm, while the daily maximum temperature in summer reaches 42 °C and the minimum temperature in winter reaches −20 °C (Ghasemi and Ghaderian, 2009). Seeds of A. inflatum were harvested in September 2009. Approximately 50,000 seeds were collected as a bulk sample from ca. 70 individual plants found on ca. 10 ha of serpentine outcrops in a region of ca. 100 ha. The bulk sample was mixed thoroughly prior to using the seeds for the study.

2.2. Experimental design

Seeds were sown on Perlite under greenhouse conditions and watered with distilled water during germination and seedlings remained on the Perlite medium for the duration of the experiment. After 10 days (when germination was complete), seedlings were irrigated with one-fourth strength Hoagland nutrient solution (Hoagland and Arnon, 1950) for 2 months until the seedlings were established. The modified Hoagland solution (pH 7) was composed of 0.5 mM Ca(NO$_3$)$_2$, 0.1 mM KH$_2$PO$_4$, 0.5 mM MgSO$_4$, 0.5 mM KNO$_3$, 0.2 μM CuSO$_4$, 0.2 μM ZnSO$_4$, 2 μM MnSO$_4$, 10 μM H$_3$BO$_3$, 0.1 μM Na$_2$MoO$_4$, 2 μM NaCl and 5 μM FeEDDHA (ferric ethylenediamine-di-2-hydroxyphenylacetate). After seedlings were established, plants were treated with Ni using NiSO$_4$ (100 μM). Prior work (Ghasemi and Ghaderian, 2009) using a

concentration of 100 μM resulted in shoot Ni concentrations similar to those of field-collected plants (3000 vs. 3700 μg Ni g^{-1}). Since the concentration of Mg in serpentine soils is high, for comparison the effect of high concentration of Mg (5 mM) was also studied. This level was selected because preliminary experiments showed that greater levels decreased plant growth. The concentration of Mg in the Hoagland solution (prior to the addition of high Mg) was 0.5 mM. The Ca:Mg ratios (mol:mol) were 1 and 0.1 at low and high Mg concentrations, respectively (if expressed in ppm:ppm, these were 1.67 and 0.167 at low and high Mg concentrations, respectively). Thirty plants in 10 pots (three plants per pot) were each treated with Ni and Mg, alongside a control consisting of the same sample size. The pots were arranged in a completely randomized design: each pot was placed into a separate plastic tray to which the appropriate nutrient solution was added to sub-irrigate the plants in that pot. The fluid level in each tray was kept constant by adding water as needed: daily during warm weather and less frequently (every few days) when evaporative demand was low. Nutrient solutions in trays were replaced every 10 days to maintain treatment effectiveness during the experiment.

The experiment was completely performed in partially climatically controlled conditions in a greenhouse. Temperature conditions ranged from about 36 °C/19 °C max./min. during summer (July/August) and 24 °C/13 °C max./min. in winter (January and February). Maximum sunlight into the greenhouse was almost 12 h in June and almost 8 h in January: no additional light was supplied. Seeds were sown in May and flowering occurred in June of the following year. At the end of the blooming period (July), all flowering stalks had senesced yet most plants remained alive. The number of plants that flowered was recorded, along with the number of inflorescences, length of each inflorescence to the nearest mm, and the number of open flowers produced by each inflorescence per plant.

2.3. Data analysis

Two types of statistical analyses were used. Data on survival and on plants flowering were analyzed using 3 × 2 contingency table analyses in the program StatView 5.0 (SAS Institute, 2005). If the full table showed significance (as it did for flowering), it was further subdivided into pairwise comparisons to determine which treatments differed from each other. Data on inflorescence number per flowering plant, mean inflorescence length per flowering plant, and mean number of flowers/inflorescence per flowering plant were analyzed using One-way Analysis of Variance (ANOVA) in StatView 5.0, followed by pairwise mean comparisons using Fisher's Protected Least Significant Difference (PLSD) Test if the ANOVA showed significance (SAS Institute, 2005). Count variables (inflorescence number and flower number) were log-transformed before analysis to minimize violation of ANOVA assumptions (Zar, 1996).

3. Results

3.1. Plant survival

Few plants died during the experiment: 90% of both Ni-treated and Mg-treated plants survived to the experiment's end, compared to 87% of control plants. Contingency table analysis showed no effect of treatment on survival (chi-square = 0.23, df = 2, P = 0.89).

3.2. Flowering response

Flowering occurred in spring of the year following germination. Treatment significantly affected flowering (contingency table analysis: chi-square = 19.4, df = 2, P < 0.0001). Sixty-three percent of Ni treated plants flowered, compared to only 19% of Mg-treated plants and 12% of control plants. Subdividing the contingency table showed that Ni-treated plants flowered significantly more than both Mg-treated plants

Fig. 2. Mean inflorescence length of flowering *A. inflatum* plants for the experimental treatments. Means represent 10 replicates each containing 3 plants (error bars = SD). Different letters show significantly different means (*P* < 0.05) based on Fisher's PLSD Test (SAS Institute, 2005).

(chi-square = 11, df = 2, *P* = 0.0009) and control plants (chi-square = 15, df = 2, *P* < 0.0001), whereas Mg-treated and control plants did not differ from each other (chi-square = 0.51, df = 2, *P* = 0.48).

Besides being more likely to flower, Ni-treated plants produced more inflorescences per flowering plant compared to flowering plants from other treatments. ANOVA showed a significant effect of treatment on inflorescence number per flowering plant ($F_{2,22}$ = 18, *P* < 0.0001): Ni-treated plants had significantly more inflorescences per flowering plant than either Mg-treated or control plants (Fig. 1: Fisher's PLSD Test: *P* < 0.004 in both cases). Similarly, inflorescence length and number of open flowers per inflorescence both were affected by treatment ($F_{2,22}$ = 20, *P* < 0.0001 and $F_{2,22}$ = 15, *P* < 0.0001, respectively). Ni-treated plants had significantly longer inflorescences (Fig. 2) and more open flowers per inflorescence (Fig. 3) compared to both other treatments (Fisher's PLSD Test: *P* < 0.0004 for comparison of Ni-treated plants compared to other treatments for both variables). Fig. 4 shows representatives of Ni-treated, Mg-treated, and control plants at the end of the experiment.

4. Discussion

The effects of Ni on Ni hyperaccumulating plants and other serpentine-tolerant plants (Lee, 1974) have not received much attention beyond the well-studied phenomenon of the role of Ni in plant defense against pathogens and herbivores (Boyd, 2007; Strauss and Boyd, 2011). Enhanced growth of some metal hyperaccumulator plants in the presence of higher concentration of metal has previously been reported (e.g., Burrell et al., 2012; Ingle et al., 2005; Krämer et al., 1996; Whiting et al., 2000) but no physiological mechanism for the enhanced growth has been suggested. Hanikenne et al. (2008) reported constitutive increased expression of genes responsible for metal transport in a Zn hyperaccumulator plant *Arabidopsis halleri* (L.) O'Kane & Al-Shebaz subsp. *halleri*. Similarly, Ingle et al. (2005) report constitutively high expression of the histidine biosynthetic pathway in the Ni-hyperaccumulating *Alyssum lesbiacum* (Candargy) Rech.f. These studies suggest that plants that hyperaccumulate metal are equipped with physiological mechanisms for both increased uptake and tolerance of those metals. The growth stimulating effect may also stem from direct beneficial effects of Ni on N metabolism or from indirect effects resulting from a potential role of Ni-containing urease in supporting plant pathogen defense (Polacco et al., 2013).

It has been proposed that hyperaccumulation (Ni or other metals) has varied advantages for a plant: Boyd and Martens (1992) summarized five potential explanations for metal hyperaccumulation. First, it could be a me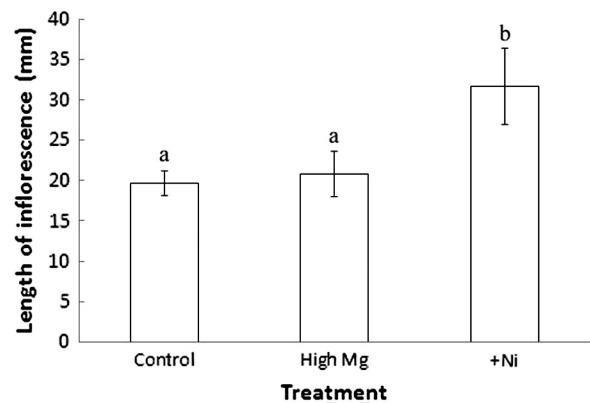chanism for metal tolerance so that the absorbed metal translocates into the shoot and is detoxified there or removed from the plant by shedding the leaves (Baker, 1981). Second, metal hyperaccumulation could be a mechanism to improve competitive ability against other plants (elemental allelopathy: Boyd and Jaffré, 2001). Concentration of the metal-rich plant materials around the hyperaccumulator may decrease growth of other less metal-tolerant plants. Third, a high concentration of stored metal could increase drought resistance (Baker and Walker, 1989). Fourth, metal hyperaccumulation could be the result of other properties of these plants, such as higher transpiration or higher activity of transporters of essential elements, leading to 'inadvertent' metal uptake (Cole, 1973). The last explanation (defense hypothesis) stresses the role of hyperaccumulated metal to reduce herbivory and pathogen attack (Reeves et al., 1981).

The above hypotheses show that elevated concentrations of metals may affect hyperaccumulator plant fitness through multiple pathways. The defense hypothesis has been relatively extensively investigated, and defense has been demonstrated in a number of studies (see review by Boyd, 2007), but evidence for other hypotheses is more equivocal. For example, the elemental allelopathy hypothesis has not been demonstrated for metals (Morris et al., 2009). However, recent investigation of elemental allelopathy as a benefit of hyperaccumulation of Se (a metalloid) has found multiple effects. Soil near Se hyperaccumulators had greater Se concentration, supported 10% less vegetative cover and decreased germination and growth of a Se-sensitive plant (El Mehdawi
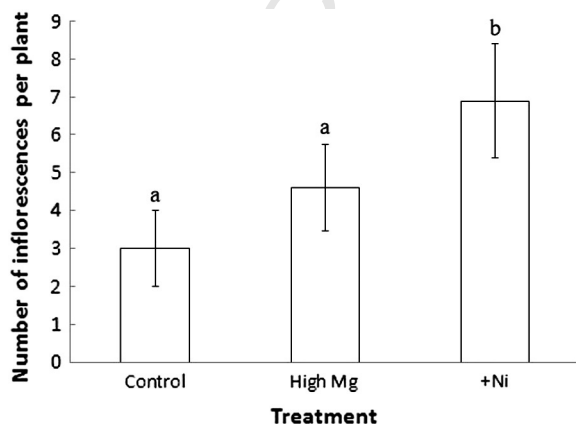

Fig. 1. Mean number of inflorescences per flowering *A. inflatum* plant for the experimental treatments. Means represent 10 replicates each containing 3 plants (error bars = SD). Different letters show significantly different means (*P* < 0.05) based on Fisher's PLSD Test (SAS Institute, 2005).
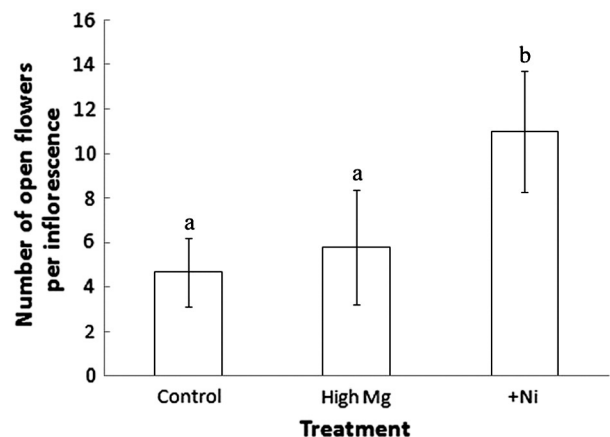

Fig. 3. Mean number of open flowers per inflorescence of *A. inflatum* for each treatment. Means represent 10 replicates each containing 3 plants (error bars = SD). Different letters show significantly different means (*P* < 0.05) based on Fisher's PLSD Test (SAS Institute, 2005).
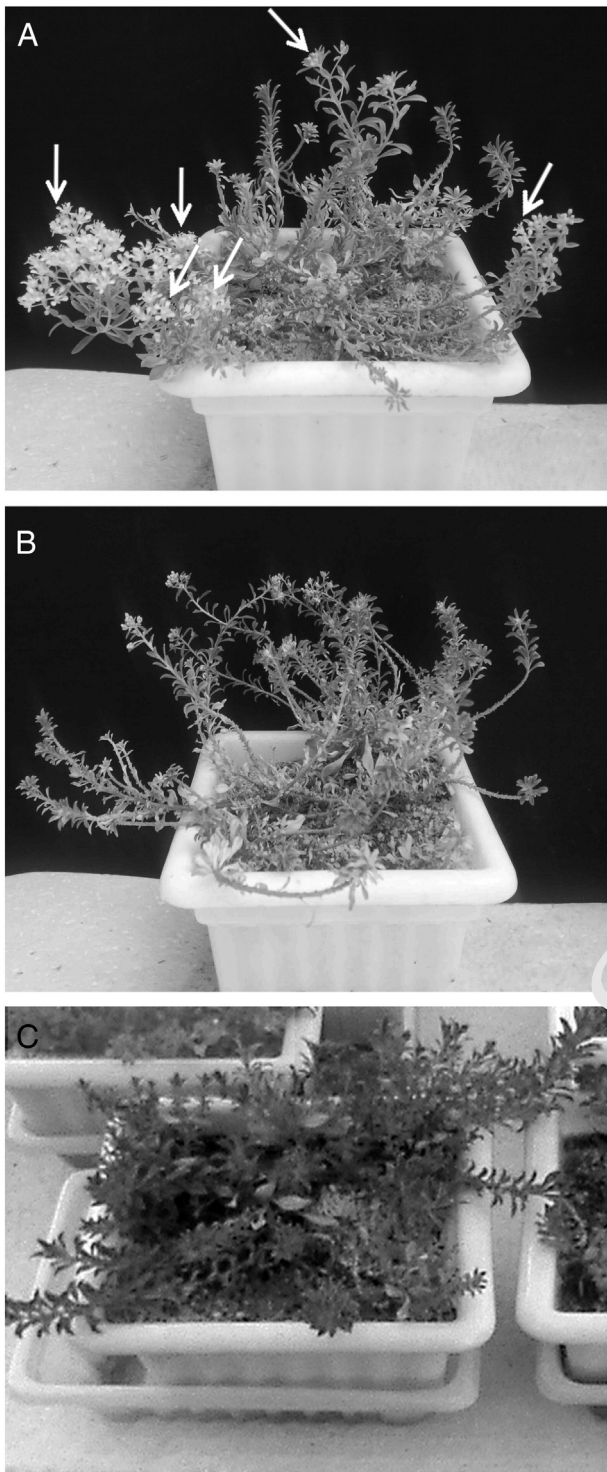
Fig. 4. Photographs of representatives of Ni-treated (A), Mg-treated (B), and control (C) plants at the end of the experiment. Inflorescences with at least one open flower are indicated by arrows.

et al., 2011a). However, further investigation showed that Se-tolerant plant species benefitted from growing near Se hyperaccumulators because the higher Se concentrations of their tissues protected them from herbivory (El Mehdawi et al., 2011b). It is likely that similar combinations of effects operate in the field situation for our Ni hyperaccumulator species and these should be explored in future experiments.

Because the experiment described here was performed on seeds collected from the Ni-enriched serpentine soils, some of the differences we observed may result, in part, from maternal effects (Mousseau and Fox, 1998; Roach and Wulff, 1987; Wright and Stanton, 2011). Maternal effects are most likely to influence seedling characteristics and to diminish over time (Donohue et al., 2005; Roach and Wulff, 1987; Wright et al., 2006) and our variables were all measured on adult plants. Hence, we assume in our discussion that maternal influence is either minimal or uniform across treatments in its potential effects.

Our finding of a stimulatory effect of Ni on flowering suggests a new hypothesis for the role of Ni in hyperaccumulator plants: that Ni can stimulate flowering and enhance reproductive fitness. We note that, because these are long-lived plants and our experiment only documented first-year's reproductive effort, we are documenting an initial fitness advantage of Ni-treated plants (as measured by greater flower production) rather than a lifetime fitness difference. Furthermore, few seeds were produced by plants studied by us (probably due to lack of pollinators in the greenhouse setting) so that we necessarily used flower number as an indicator of seed production under more natural conditions. Our data clearly show, however, that the Ni-treated plants had an initial reproductive advantage and, since plant size was not observed to be different, we expect that this initial advantage would continue into future flowering seasons.

We are unable to determine definitively if the increased flowering documented in our experiment resulted from enhanced growth in the presence of Ni, or if Ni had a direct effect on stimulating flowering, since we do not have total biomass data for the plants at the flowering stage. However, there were no obvious visible differences in plant size associated with treatments during the experiment. Furthermore, Ghasemi and Ghaderian (2009) reported similar relative growth of this species (at a short timeframe: 4 wk after transplanting into treatment solutions) at Ni concentrations of 0, 10, 100 and 250 μM across a range of Ca/Mg ratios. Finally, data from another experiment (Ghasemi and Zare, unpublished results) show that shoot biomass of *A. inflatum* is unaffected by Ni concentrations of up to 250 μM (compared to an unamended control). It is therefore possible that Ni had a direct effect on flowering. Additionally, we are not able to absolutely verify if the increased flowering resulted from reduced infection/herbivory caused by a defensive effect of Ni. Herbivore and pathogen damage are not always easily detected (especially some pathogens) and so we cannot absolutely eliminate that as a potential explanation, but we do not think it is a likely explanation for our results. Ultimately, the increase in flowering suggests that Ni is directly or indirectly responsible for increased fitness in this Ni-hyperaccumulating taxon. Additional studies, such as cultivating these plants in aseptic culture, are required to better elucidate the mechanism of Ni in enhancing fitness.

The mechanism underlying the flowering response by the Ni-treated plants is unknown: flowering is a complicated phenomenon and, apart from its genetic basis (Bernier and Perilleux, 2005), environmental conditions (Mouradov et al., 2002) such as soil nutrient and moisture status could affect it. No specific Ni transporter, Ni metallochaperone or Ni response element has yet been found in plants (Eitinger et al., 2005; Krämer and Clemens, 2006; but see Nishida et al., 2011). Urease (E.C. 3.5.1.5) is perhaps the most important of the several known Ni-requiring enzymes in higher plants (Liu, 2001). Nickel works as a cofactor to enable urease to catalyze the conversion of urea into the ammonium ion, which plants can use as a source of N. Without the presence of Ni, urea conversion is impossible. Nickel deficient plants develop leaf chlorosis and leaf tip necrosis (Malavolta and Moraes, 2007), symptoms that can be prevented with the application of Ni which increases leaf urease activity and prevents urea accumulation (Eskew et al., 1984). Thus, in generally N-poor soils such as serpentine (Kruckeberg, 1984), nickel may be particularly important for N acquisition and metabolism. Thus the stimulatory effect we have observed in flowering could be in response to better N metabolism in those plants exposed to Ni. In fact, Roach and Barclay (1946) showed that Ni significantly increased the yield of potato, barley, and wheat. Additionally, Ni deficiency can also lead to oxalic and lactic acid accumulation and toxicity, greatly

disrupting carbon metabolism (Bai et al., 2006). In addition to influencing photosynthesis, these toxicities can disrupt the conversion of organic acids to other metabolites necessary for optimal growth and development, including reproduction. Nickel is also critical for N-fixation in the Fabaceae, as deficiency contributes to delayed nodulation and reduced efficiency of N-fixation (Brown, 2006). Hence, the increased reproductive effort we have observed in *A. inflatum* in response to the addition of Ni could simply be a byproduct of increased N and C metabolism in the presence of Ni. It is tempting to speculate that the requirement for Ni may be greater in Ni-hyperaccumulating plants and increased Ni contributes to both more efficient N and C metabolism, thereby promoting growth, including reproduction.

It is also possible that Ni acts by changing the balance of other elements in the tissues, and such ion balances directly or indirectly contributed to enhanced flowering. Nickel may show strong interference with other essential elements such as Ca, Mg, Fe, or Cu, and Zn in uptake, transport and translocation in plants (Brown, 2006; Chaney et al., 2008; Ghasemi et al., 2009b; Nishida et al., 2011). It may be that Ni directly affects flowering gene expression or indirectly affects it by changing the status of available elements (including N), enzymes, and hormones, contributing to reproductive effort in plants. Because our modified Hoagland solution (control and Mg treatments) did not contain deliberately added Ni (small amounts of Ni were probably present as a contaminant of other ingredients), it is difficult to determine if high concentrations of Ni contribute to enhanced reproduction. Additional studies, exposing plants to a range of Ni concentrations, including those found in non-serpentine and serpentine soils, can better elucidate the role of Ni in enhancing reproductive effort in serpentine-tolerant plants. Further investigation of this stimulatory effect can also lead to a better understanding of the adaptive significance of hyperaccumulated metals in plants.

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