

Nickel Increases Susceptibility of a Nickel Hyperaccumulator to *Turnip mosaic virus*

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ABSTRACT

Hyperaccumulated Ni can defend plant tissues against herbivores and pathogens. The effectiveness of this defense, however, has not been tested with a viral pathogen. *Turnip mosaic virus* (TuMV) accumulation was studied in two serpentine species of *Streptanthus* with different Ni uptake abilities. Plants of a Ni hyperaccumulator, milkwort jewelflower (*S. polygaloides* Gray), and a non-hyperaccumulator, plumed jewelflower (*S. insignis* Jepson), were grown on Ni-amended and unamended soils. Plants were inoculated with TuMV at three different phenological stages: basal rosette, bolting, and flowering. Susceptibility of experimental plants to TuMV was determined either by the magnitude of TuMV accumulation (measured by indirect enzyme-linked immunosorbent assay [ELISA]) or by plant survival. *Streptanthus polygaloides* plants grown on high-Ni soil were more susceptible to TuMV than low-Ni *S. polygaloides* at all three phenological stages. All rosette and pre-bolt *S. insignis* plants were infected by TuMV, but survival and TuMV accumulation were not significantly affected by soil Ni. At flowering, only high-Ni *S. polygaloides* plants became infected. For *S. polygaloides*, elevated tissue Ni concentrations enhanced TuMV infection instead of defending plants from the virus. To reduce risks to nearby agricultural crops, future phytoremediation and phytomining operations using this species should incorporate management plans to prevent the creation of artificial reservoirs of TuMV inoculum.

CERTAIN plants incorporate remarkably high levels of metals into their tissues (Brooks et al., 1977). As defined by Baker and Brooks (1989), a plant containing $>1000 \mu\text{g g}^{-1}$ of Co, Cu, Cr, Pb, or Ni or $>10\,000 \mu\text{g g}^{-1}$ of Mn or Zn in its tissues is considered to be a metal hyperaccumulator. Metal hyperaccumulators occur naturally on soils arising from ultramafic substrates (Brooks, 1987). Ultramafic soils, often called serpentine soils, are high in ferromagnesian minerals and have high concentrations of other metals such as Ni, Co, and Cr (Brooks, 1987). These unique plants are potentially important tools for the recolonization and phytoremediation of soils contaminated with metals from anthropogenic sources (Brooks et al., 1998; McGrath, 1998; Rugh et al., 1998; Robinson et al., 1999). Economically, hyperaccumulators have potential value as a means for phytomining metals from either contaminated soils or soils that have naturally high metal contents, but are not suitable for traditional mining techniques (Nicks and Chambers, 1995; Robinson et al., 1997; Anderson et al., 1998). Most studies involving metal hyperaccumulators have centered on their use for phytoremediation or recolonization of anthropogenically contaminated soils

(e.g., mine tailings, industrial sites). Although these contaminated sites occur globally, many phytoremediation and phytomining efforts are located in North America and Europe (Nicks and Chambers, 1995; Robinson et al., 1997, 1999). In the USA, Superfund legislation has made it financially attractive for land owners to remediate polluted soils (USEPA, 1993). With the emergence of new technologies, the phytoremediation market is projected to grow to \$370 million by the year 2005 (Watanabe, 1997; Anonymous, 1998).

A survey of the literature has shown that metal hyperaccumulators occur in at least 50 plant families distributed worldwide. Of these, Ni hyperaccumulators comprise the most taxa; to date, at least 320 species have been reported from 43 families (Reeves et al., 1999), with centers of diversity occurring in subtropical (Cuba) and tropical (New Caledonia) regions. However, most species investigated previously for metal extraction belong to the same plant family, Brassicaceae, a family distributed mainly in temperate and cold regions of the Northern Hemisphere (Brooks, 1987). Many proposed phytoremediation sites in North America and Central Europe (USEPA, 1993) co-occur with the geographical distribution of natural and agricultural populations of many members of Brassicaceae (Zomlefer, 1994). In 1997, production of vegetable crops belonging to Brassicaceae [e.g., broccoli (*Brassica oleracea* var. *italica* Plenck), rutabagas (*Brassica napus* L. var. *napobrassica* (L.) Reichb.), cabbage (*Brassica oleracea* L.)] within the USA totaled more than \$1.1 billion (USDA National Agricultural Statistics Board, 1999). Since these agricultural crops and many hyperaccumulators may have common pathogens and insect pests, and the economic values of these crops are high, investigation of the dynamics of diseases and other pests within phytoremediation crops is necessary before widespread implementation of phytoremediation technologies may begin.

Hyperaccumulators sequester potentially toxic metals in their tissues. Therefore, it is vital to understand the natural interactions (both mutualistic and antagonistic) between hyperaccumulators and other biotic components of serpentine communities. Herbivores that have adapted to feed upon high-metal tissues may provide a gateway for the movement of heavy metals into the food web (Boyd, 1998). Altered or sustained susceptibility of hyperaccumulators to herbivory or diseases could create new reservoirs of pathogens or herbivores on phytoremediation sites (Bañuelos et al., 1992). Also, no studies to date have examined pollination and seed dispersal mutualisms of metal hyperaccumulators (Boyd and Martens, 1998a). Potential alterations in local agroecosystems caused by biotic interactions with hyperaccumu-

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Abbreviations: ANOVA, analysis of variance; DPI, days post inoculation; ELISA, enzyme-linked immunosorbent assay; PLSD, protected least significant difference; TuMV, *Turnip mosaic virus*.

lators may place nearby natural and agricultural areas at risk.

Only a small fraction (1–2%; Reeves, 1992) of serpentine taxa hyperaccumulate metals. The rarity of this characteristic may indicate that it is a novel adaptation to some component of the serpentine community (Boyd and Martens, 1998a). Several hypotheses regarding the evolutionary “raison d’être” of metal hyperaccumulation were reviewed by Boyd and Martens (1992) and, of these, the metal defense hypothesis has garnered the most supporting evidence (see Boyd and Martens, 1998b; Boyd, 1998). Metals in plant tissues have been shown to deter feeding (Pollard and Baker, 1997), delay larval development (Martens and Boyd, 1994; Boyd and Moar, 1999) and cause acute toxicity in certain herbivores (Boyd and Martens, 1994; Martens and Boyd, 1994; Boyd and Moar, 1999). Accumulated Ni has also been demonstrated to negatively affect both fungal and bacterial plant pathogens (Boyd et al., 1994; Ghaderian et al., 2000). However, not all organisms using hyperaccumulators as hosts are negatively affected by metal-based plant defenses. Several types of pests have been observed attacking natural populations of the Ni hyperaccumulator, *S. polygaloides*, including aphids (Boyd and Davis, personal observation, 1996; Boyd and Martens, 1999), mirids (Wall, 1999), and a parasitic plant (Boyd et al., 1999). Boyd and Martens (1999) experimentally demonstrated that aphids are unaffected by the metal defense of *S. polygaloides*. Since aphids are common vectors of plant viruses both within and among plant populations, the possibility exists that new reservoirs of aphids or virus inoculum may be created on phytoremediation sites. To address this concern, we studied the effects of elevated Ni in plant tissues on the susceptibility of two congeneric serpentine plant species in the family Brassicaceae to inoculation with TuMV, a common pathogen of natural and agricultural members of the family Brassicaceae (Shattuck, 1992). A Ni hyperaccumulator and a non-hyperaccumulator were compared to address the specific effects of hyperaccumulation on susceptibility to infection by TuMV. Plants were inoculated during three phenological stages to examine the effects of age on response of plants to TuMV infection.

MATERIALS AND METHODS

Study Species

The Ni hyperaccumulator, *S. polygaloides*, is endemic to serpentine chaparral in the western foothills of the Sierra Nevada of California (Reeves et al., 1981; Kruckeberg, 1984). It is one of many species being evaluated for use in phytoremediation and phytomining (Nicks and Chambers, 1995). Nickel concentrations measured in tissues from natural populations range from 1100 to 16 400 $\mu\text{g g}^{-1}$ dry wt. (Reeves et al., 1981; Kruckeberg and Reeves, 1995). Plants were raised in a glasshouse in Auburn, AL from seeds collected in the Red Hills Management Area, near Chinese Camp, Tuolumne County, CA (Favre, 1987). Seeds were sown on both high-Ni soil (ProMix [Premiere Horticulture, Red Hill, PA] amended with NiCl_2 to obtain a soil Ni concentration of $\sim 1000 \mu\text{g g}^{-1}$ dry wt) and low-Ni soil (unamended ProMix). For comparison,

a congeneric non-hyperaccumulator, *S. insignis*, was also grown on high- and low-Ni soils. *Streptanthus insignis* is an annual that is usually limited to serpentine soils of the Southern Coast Ranges of California (Hickman, 1993). Seeds were collected from a population growing on serpentine soil west of Panoche Pass in San Benito County, CA. A small subset of plants were harvested and analyzed at the beginning of the experiment to determine mean plant Ni contents resulting from these soil treatments.

Turnip mosaic virus is a cosmopolitan potyvirus and important pathogen of both agricultural and natural populations of plants in the family Brassicaceae (Shattuck, 1992). In North America, the virus has caused important crop losses for cabbage, cauliflower (*Brassica oleracea* var. *botrytis* L.), horseradish (*Armoracia rusticana* P. Gaertn. et al.), endive (*Cichorium endivia* L.), escarole (*Cichorium endivia* L.), turnip (*Brassica rapa* L. var. *rapa*), collards (*Brassica oleracea* L. var. *viridis* L.), rutabaga, and statice [*Goniolimon tataricum* (L.) Boiss.] (Shattuck, 1992). Although TuMV is most prevalent in hosts from the family Brassicaceae, it is known to infect members of at least 13 other plant families (Shattuck, 1992; Stobbs and Stirling, 1990). The virus often overwinters in populations of both native and introduced weeds within and near crop fields (Stobbs and Stirling, 1990).

Virus Inoculum and Inoculation Procedures

Turnip mosaic virus was obtained from Dr. T.P. Pirone, University of Kentucky, Lexington. It was propagated in smooth-leaf mustard (*Brassica campestris* L.) plants by mechanical passage and maintained in a temperature-controlled (average temperatures of 21°C during the day and 16°C at night), aphid-free glasshouse. Inoculum consisted of TuMV-infected leaves of mustard ground in 50 mM potassium phosphate buffer (pH 8.0) at a ratio of 1:10 (w/v). Inoculum was applied by rub-inoculation to selected leaves (see below) that were dusted with carborundum just prior to inoculation. Plants were evaluated for response to TuMV inoculation at three phenological stages of growth: rosette, bolting, and flowering. Aboveground portions of rosette plants consisted of only basal leaves. Bolting plants had elongated stems (<2.0 dm) and both cauline and basal leaves. Stems of flowering plants were fully elongated (>5.0 dm) and retained only cauline leaves. Basal leaves of plants in the rosette stage were inoculated, whereas for bolting plants, TuMV was inoculated onto both cauline and basal leaves. Only cauline leaves were inoculated on plants at the flowering stage. For plants in the flowering stage, only those grown in high-Ni soil plants were inoculated since low-Ni *S. polygaloides* plants of that age are unlikely to occur in either natural or phytoremediation populations. Each treatment consisted of 4 to 12 plants, depending on availability of plant material.

Response of Jewelflower Plants to TuMV Infection

Plants were monitored daily for the timing of symptom appearance, and for the type and severity of symptoms. Indirect ELISA (Voller et al., 1979) was used to confirm TuMV infection and compare the relative amount of virus accumulation in leaves of plants subjected to the different treatments. For analysis, leaf samples (collected from uninoculated portions of plants near the apical meristem) were ground in 50 mM carbonate buffer (pH 9.6) and 100 μL of the extract was added to microtiter plates at a final dilution of 1:50 (grams of tissue to milliliters of buffer). Microtiter plates were then kept at 4°C for at least 12 h. Following three rinses with phosphate buffered saline containing 0.05% Tween 20 (PBS-T;

Sigma–Aldrich, St. Louis, MO), anti-TuMV immunoglobulin (ATCC PVAS-134), diluted in PBS-T to $1.0 \mu\text{g mL}^{-1}$, was added to microtiter plates ($100 \mu\text{L}$ per well) and incubated at 4°C for at least 12 h. Microtiter plates were rinsed three times with PBS-T. Goat anti-rabbit immunoglobulin conjugated to alkaline phosphatase (Sigma Chemical Co., St. Louis, MO) was added to microtiter plates at a dilution of 1:6000 in PBS-T and incubated at 37°C for 3 h. After three rinses with PBS-T, substrate (1 mg mL^{-1} of *p*-nitrophenyl phosphate in 10% diethanolamine, pH 9.8, $100 \mu\text{L}$ per well) was added to microtiter plates and reactions were allowed to develop for 30 min at room temperature. Reactions were read using a Dynatech MR 700 microplate reader (Dynatech Laboratories, Chantilly, VA) at $\lambda = 405 \text{ nm}$. Samples were considered positive for the presence of TuMV when the ELISA absorbance value was greater than the mean plus three standard deviations of comparable healthy control samples.

Enzyme-linked immunosorbent assay values were analyzed by one-way analysis of variance (ANOVA), and post hoc means separations were performed using Fisher's protected least significant difference (PLSD) test (Abacus Concepts, 1991). Kaplan–Meier analysis was used to model survival functions of rosette stage plants and the Peto–Peto–Wilcoxon rank test was used to detect differences in survival (SAS Institute, 1998). This analysis is desirable since it does not make the assumption that survival probabilities are constant with respect to time (SAS Institute, 1998). All statistical comparisons were considered significant at $\alpha \leq 0.05$.

RESULTS

Symptom Development

Plants inoculated as rosettes experienced high mortality; 100% of high-Ni plants of both *S. polygaloides* and *S. insignis* died by 55 days post inoculation (DPI). Prior to death, shoot apical meristems became necrotic followed by a rapid wilting of basal leaves (28 DPI for *S. polygaloides* and 38 DPI for *S. insignis*). No other symptoms (e.g., mosaic, vein-clearing) were apparent.

Plots of percent survival over time indicated that *S. polygaloides* was more severely affected by TuMV sooner after inoculation (~ 35 DPI) than *S. insignis* (Fig. 1), but by 55 DPI there was no difference in survival between species ($P = 0.511$, Peto–Peto–Wilcoxon rank test). At 45 DPI, survival of high-Ni plants of both species was 30% below that of low-Ni plants, although this difference was not statistically significant ($P = 0.079$, Peto–Peto–Wilcoxon rank test). There was no difference in survival between high- and low-Ni *S. polygaloides* ($P = 0.3908$, Peto–Peto–Wilcoxon rank test) by 55 DPI, but comparison of high- and low-Ni *S. insignis* at that same date indicated a trend for decreased survival of high-Ni plants ($P = 0.071$, Peto–Peto–Wilcoxon rank test).

In contrast to plants in the rosette stage, no mortality occurred in TuMV-infected plants inoculated at bolting or flowering stage. Although differences occurred in levels of virus accumulation among treatments for bolting plants (see below), no visible differences were detected in symptom development. At 21 DPI, plants of both species inoculated at the bolting stage had shortened internodes and dwarfed, curled leaves, resulting in severe stunting.

Whereas all inoculated plants in the bolting stage expressed disease symptoms, the only flowering stage plants that expressed symptoms were the *S. polygaloides* plants grown on high-Ni soil. Stunting did not occur with infected high-Ni *S. polygaloides* plants that were inoculated at the flowering stage, although some young leaves were dwarfed and curled by 21 DPI.

Turnip Mosaic Virus Accumulation

Excessive mortality prohibited analysis of plants for TuMV accumulation by ELISA on plants inoculated at the rosette stage. For plants inoculated at the bolting

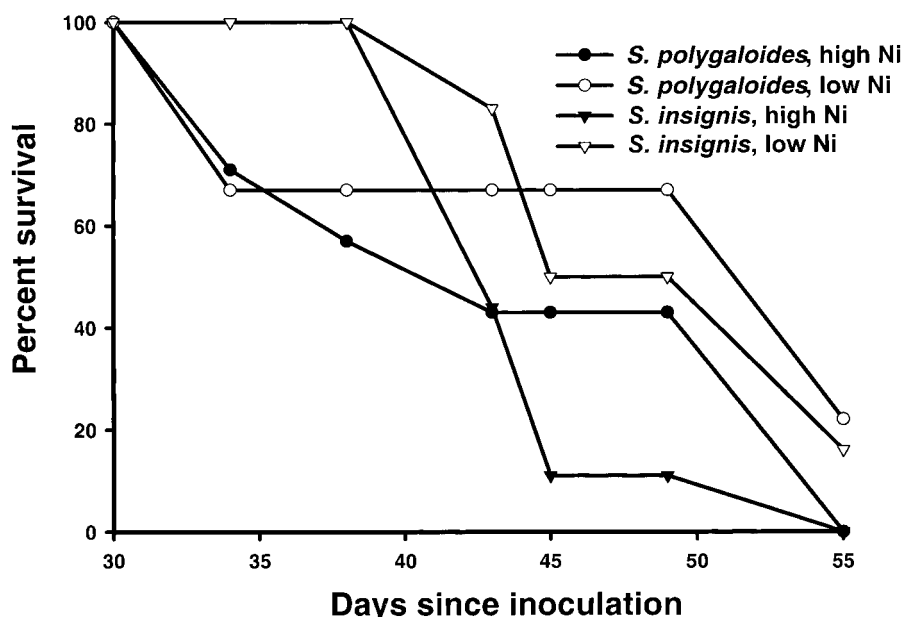


Fig. 1. Percent survival of high- and low-Ni *Streptanthus polygaloides* and *S. insignis* plants inoculated with Turnip mosaic virus (TuMV) during rosette stage.

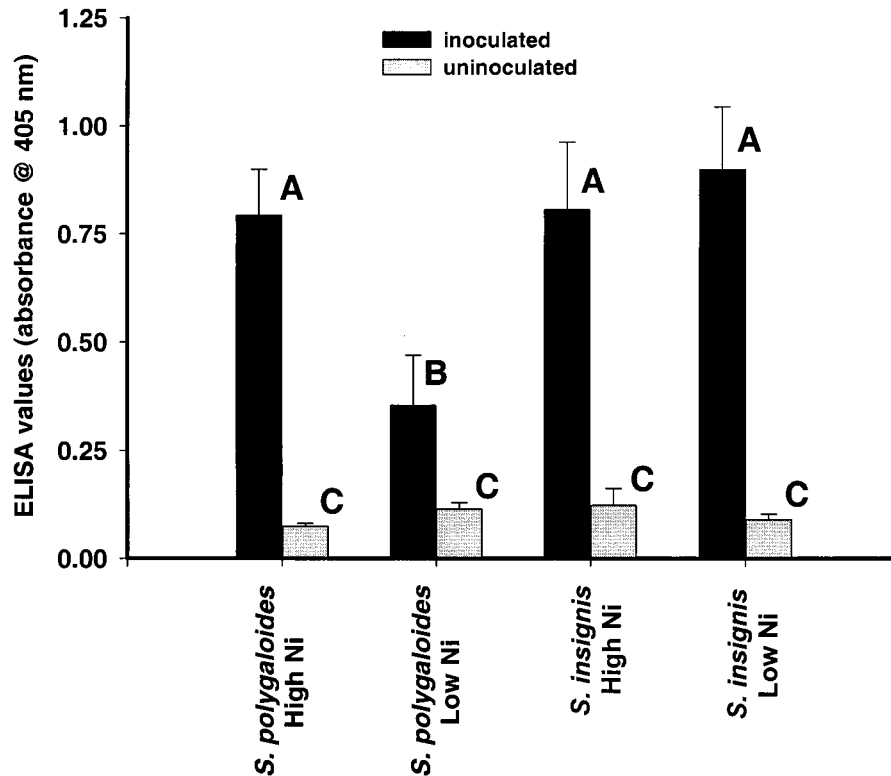


Fig. 2. Turnip mosaic virus (TuMV) accumulation (enzyme-linked immunosorbent assay [ELISA] values) in high- and low-Ni *Streptanthus polygaloides* and *S. insignis* plants inoculated during the bolting stage. Uninoculated controls (light gray bars) are included to indicate background ELISA values. Columns with the same letters do not differ significantly ($\alpha \leq 0.05$). Error bars denote 95% confidence intervals.

stage, soil Ni affected virus accumulation (ANOVA: MS = 0.97, $F_{7,48} = 8.55$, $P < 0.0001$). Bolting *S. polygaloides* plants grown on high-Ni soil had significantly

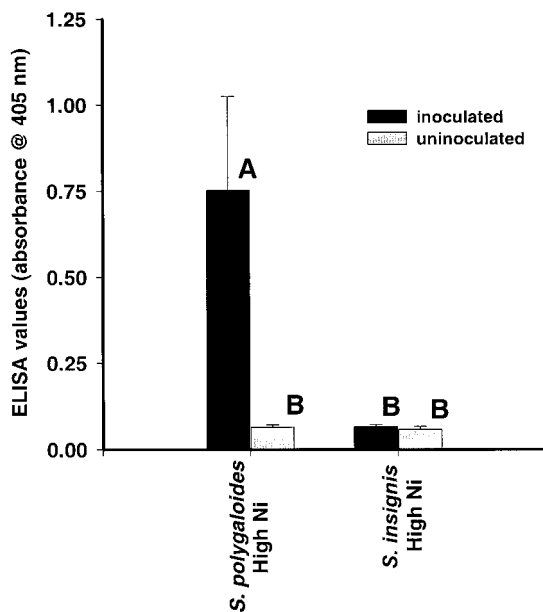


Fig. 3. Turnip mosaic virus (TuMV) accumulation (enzyme-linked immunosorbent assay [ELISA] values) in high-Ni *Streptanthus polygaloides* and *S. insignis* plants inoculated during the flowering stage. Uninoculated controls (light gray bars) are included to indicate background ELISA values. Columns with the same letters do not differ significantly ($\alpha \leq 0.05$). Error bars denote 95% confidence intervals.

higher levels of TuMV accumulation than those grown on low-Ni soil ($P = 0.011$, Fisher's PLSD) (Fig. 2). In contrast, TuMV accumulation did not differ significantly between high- and low-Ni *S. insignis* plants in the bolting stage ($P = 0.567$, Fisher's PLSD), nor did TuMV accumulation in high- and low-Ni *S. insignis* plants differ from levels in high-Ni *S. polygaloides* plants ($P = 0.9374$ and $P = 0.4673$ for high- and low-Ni *S. insignis*, respectively, Fisher's PLSD).

Significant differences in TuMV accumulation among plants in the flowering stage were found (ANOVA: MS = 0.57, $F_{3,14} = 5.39$, $P = 0.011$) (Fig. 3). High-Ni *S. polygaloides* had significantly greater levels of TuMV accumulation than high-Ni *S. insignis* ($P < 0.005$, Fisher's PLSD) (Fig. 3). In fact, ELISA detected no TuMV accumulation in high-Ni *S. insignis* plants in the flowering stage.

DISCUSSION

The Ni hyperaccumulator, *S. polygaloides*, was highly susceptible to TuMV infection during vegetative growth based on high levels of TuMV accumulation and mortality. Clearly, hyperaccumulated Ni did not defend *S. polygaloides* from infection by TuMV. Conversely, virus accumulation and plant mortality data indicated that *S. polygaloides* plants with elevated tissue Ni concentrations were more susceptible to TuMV than low-Ni *S. polygaloides*. This pattern was apparent with the bolting plants, as high-Ni *S. polygaloides* plants accumulated 44% more TuMV than low-Ni plants of the same spe-

cies. For plants in flower, only the high-Ni *S. polygaloides* exhibited any evidence of TuMV infection. Based on the poor initial survival of high-Ni *S. polygaloides* plants, this same pattern was noticeable, although not statistically supported, with the infected rosette plants (Fig. 1).

Few investigations have examined the effects of soil metal(s) on plant viral diseases and this is the first study to investigate host-virus interactions within a hyperaccumulator. Here, TuMV accumulation was higher in high-Ni *S. polygaloides* than in low-Ni *S. polygaloides*, but did not differ between high- and low-Ni plants of the congeneric non-hyperaccumulator, *S. insignis*. To our knowledge, only one published study exists that has examined the effects of Ni on plant viruses. Singh and Singh (1974) showed that elevated Ni in growth solutions increased the titre of *Potato virus X*, a potyvirus, in tomato (*Lycopersicon esculentum* var. *esculentum*) plants grown in nutrient solutions. Studies of effects of other metals on plant viruses have yielded varied results. Analogous to our results, Mn caused increased infection (increased number of local lesions) of *Potato spindle tuber viroid* (PSTVd) in *Scopolia sinensis* (Jacq.) plants (Singh et al., 1974) and increased foliar Cd resulted in an increase in number of local lesions in *Tobacco mosaic virus* (TMV)-infected bean (*Phaseolus vulgaris* L.) and tobacco (*Nicotiana tabacum* L.) plants (Harkov and Brenna, 1981). Conversely, Cd inhibited systemic infection of tobacco by *Turnip vein-clearing virus* (TVCV) (Citovsky et al., 1998; Ghoshroy et al., 1998) and Mg decreased the concentration of TMV in eggplant (*Solanum melongena* L.) leaves (Seaker et al., 1982). The paucity of (and sometimes contradictory) data makes it difficult to make comprehensive statements regarding the overall effects of metals on plant response to viral infections.

Certainly, our experiment showed that TuMV accumulation within two congeneric species responded differently to soil Ni treatments. Generally, mineral nutrition conditions that favor optimal plant growth are also those that foster increased susceptibility to most plant viruses (Matthews, 1991). Hyperaccumulators often grow in soils that are not favorable for normal plant growth. However, the high concentrations of Mg, Ni, Zn, and Cd present in serpentine soils (Brooks, 1987) that would be toxic to many unadapted plants, do not limit metal hyperaccumulators. In fact, many hyperaccumulators grow best in the presence of heavy metals. Brown et al. (1995a,b) showed that the Zn and Cd hyperaccumulator *Thlaspi caerulescens* J. and C. Presl produced the greatest shoot and root biomass when grown in nutrient solutions containing high concentrations of Zn (1000 μ M) and Cd (20 μ M). The Ni hyperaccumulator, yellow-tuft (*Alyssum murale* Waldstein and Kitabel), also yielded more shoot and root biomass when grown in solution cultures with elevated Ni, Cd, and Zn concentrations (Bernal and McGrath, 1994). Elevated soil Ni levels increased biomass accumulation of *Alyssum pintodasilvae* Dudley, another Ni hyperaccumulator (de Varennes et al., 1996). For our study, increased TuMV titre in high-Ni *S. polygaloides* may be due to

the increased robustness of *S. polygaloides* grown on high-Ni (versus low-Ni) soil.

The developmental stage of the host during virus infection could greatly affect the productivity of phytoremediation crops. Here, young jewelflower plants (of both species) infected with TuMV died quickly. The rapid death of these plants would diminish the likelihood of a phytoremediation site becoming a source of inoculum for nearby crops, but the value of the plants as phytoremediators would also be reduced. Older TuMV-infected *S. polygaloides* plants, however, would probably persist and could represent important inoculum sources of TuMV.

Aphids are common vectors of plant viruses. Turnip mosaic virus is transmitted in a nonpersistent (stylet-borne) manner by at least 89 species of aphids (Edwards and Christie, 1986). The green peach aphid (*Myzus persicae*) and the grey cabbage aphid (*Brevicoryne brassicae*) are two of the most common vectors (Tomlinson, 1970). Aphids have not been shown to be harmed by the Ni defense of *S. polygaloides* (Boyd and Martens, 1999) and also use these plants as hosts in California serpentine communities (Boyd and Davis, personal observation, 1997; Boyd and Martens, 1999). In addition to climatic conditions, natural TuMV epidemiology is dependent on vector (aphid) performance and behavior (Shattuck, 1992). Given that high-Ni *S. polygaloides* is highly susceptible to TuMV and apparently is an attractive host for aphids, phytoremediation management plans should include disease and pest control strategies.

There is concern that artificial populations of hyperaccumulators on phytoremediation sites may become reservoirs of agricultural pests or pathogens (Bañuelos et al., 1992). Many potential phytoremediation sites in the Northern Hemisphere share common distributions with the agricultural production of cruciferous crops (e.g., rapeseeds, turnips, broccoli) and weeds (e.g., members of the genera *Thlaspi*, *Lepidium*, *Brassica*, and *Arabis*). Since many phytoremediation plants (e.g., *S. polygaloides*, *T. caerulescens*, Indian mustard [*Brassica juncea* (L.) Czern.]) are also in the family Brassicaceae, the potential exists for the creation of pest reservoirs on phytoremediation sites. Future considerations of the costs and benefits of phytoremediation versus conventional remediation should include risk assessments of potential dangers to nearby agricultural production.

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