

## THE RELATION BETWEEN NICKEL AND CITRIC ACID IN SOME NICKEL-ACCUMULATING PLANTS

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**Key Word Index**—*Sebertia*; Sapotaceae; *Hybanthus*; Violaceae; *Homalium*; Flacourtiaceae; nickel accumulation; citric acid levels; nickel-citrate complexes.

**Abstract**—A strong correlation has been found between the levels of nickel and citric acid in the leaves of 17 New Caledonian plant species which show a range of nickel-accumulating ability: *Sebertia acuminata*, *Psychotria douarrei*, *Geissois pruinosa*, *Hybanthus austrocaledonicus*, *Hybanthus caledonicus*, and 12 species of *Homalium*. Purified extracts of these plants contain nickel as a citrate complex. Three nickel-accumulating plants from beyond New Caledonia (*Alyssum bertolonii*, *Alyssum serpyllifolium* s.sp. *lusitanicum* and *Pearsonia metallifera*) do not contain unusually high levels of citrate, nor is citrate present in purified nickel-containing extracts of these species.

### INTRODUCTION

Phytochemical studies on several plants from New Caledonia [1] have recently led to the isolation of nickel-containing extracts in which much of the nickel exists as an anionic citratonickelate(II) complex. This complex was found in *Sebertia acuminata* Pierre ex Baill., *Homalium francii* Guillaumin, *H. guillainii* (Vieill.) Briq., *H. kanaliense* (Vieill.) Briq., *Hybanthus austrocaledonicus* Schinz et Guillaumin, and *H. caledonicus* (Turcz.) Cretz. These plants are of special interest because they contain remarkably high concentrations of nickel (> 1000 µg/g on a dry weight basis), and have been classified as hyperaccumulators [2]. Six other New Caledonian plants are known to be nickel hyperaccumulators (*Geissois pruinosa* Brongn. et Gris., *Psychotria douarrei* (G. Beauvis.) Däniker, *Homalium austrocaledonicum* Seeman, *H. deplanchei* (Vieill.) Warb., *H. mathieuanum* (Vieill.) Briq., and *H. rubrocostatum* Sleumer), and several other species of *Homalium* may be classified as strong accumulators of nickel (100–1000 µg/g dry weight).

Several workers [3–8] have shown that increased concentrations of major nutrient cations (e.g. K, Ca) are associated with elevated levels of total ether-extractable organic acids, or of specific acids such as malic and citric acids. Torii and Laties [9] concluded that increased organic acid synthesis is a response to excess cation uptake, and that transport of the cation into the vacuole is facilitated by the formation of metal–organic complexes of moderate stability.

Correlations have also been found between malate or citrate and some elements normally present in low concentrations in plants. Brown and Tiffin [10] noted a strong correlation between iron and citrate in soybean stem exudate. Vergnano Gambi *et al.* [11] measured an increase of citric acid in *Avena* and *Phaseolus* plants grown in nickel-treated sand cultures and in serpentine soil cultures. In the investigation of zinc-resistance in herbage plants Mathys [12] found large differences in malic acid

content between zinc-resistant and zinc-sensitive plants, the zinc-resistant ecotypes containing much higher concentrations of the acid in their leaves than the sensitive ones.

Since citrate has been shown to be complexed with nickel in six New Caledonian species, there is reason to suppose that a more widespread correlation between nickel and citric acid levels may be found. The present work was carried out to ascertain whether such a correlation existed in a variety of nickel-accumulating and non-accumulating species of New Caledonia. If a strong relationship could be demonstrated, this would lend support to the argument for *in vivo* involvement of citrate in nickel accumulation and nickel tolerance.

### RESULTS AND DISCUSSION

The following New Caledonian species were studied: *Sebertia acuminata* [13], *Hybanthus austrocaledonicus*, *H. caledonicus* [2], *Psychotria douarrei*, *Geissois pruinosa* [14], and 12 of the 16 New Caledonian species of *Homalium* [2, 15]. Three nickel-accumulators from outside New Caledonia were also investigated: *Pearsonia metallifera* Wild [16, 17], a legume from Rhodesia, *Alyssum serpyllifolium* Desf. s.sp. *lusitanicum* Dudley & P. Silva [18] and *A. bertolonii* Desv. [19], perennials from Portugal and Italy, respectively.

Table 1 shows the levels of nickel and citric acid (on a dry weight basis) in the leaves of all the hyperaccumulator plants studied, and in some of the New Caledonian *Homalium* species that accumulate lesser amounts of nickel from serpentinitic soils. Data for all specimens examined (except the *Sebertia* latex) are plotted in Fig. 1, which shows the correlation observed between the nickel and citric acid concentrations in the leaves of the New Caledonian species. Only mature leaves were sampled, but because of the disjunctive geographical distribution of the different species, collection dates varied considerably. Nevertheless, the data plotted on

Table 1. Nickel and citric acid concentrations in plant species showing a wide range of nickel-accumulating ability

Species	Nickel ( $\mu\text{g/g}$ )	Citric acid ( $\mu\text{g/g}$ )	Mole ratio nickel: citric acid
<i>Homalium mathieuanum</i>	14.7	3170	0.015
<i>H. polystachyum</i>	46.4	3000	0.051
<i>H. intermedium</i>	74.6	1190	0.205
<i>H. le-ratiorium</i>	446	7170	0.204
<i>H. decurrens</i>	505	5590	0.296
<i>H. austrocaledonicum</i>	622	5630	0.362
<i>H. kanaliense</i>	3730	14800	0.825
<i>H. francii</i>	7210	12600	1.88
<i>H. guillainii</i>	9580	13200	2.38
<i>Hybanthus caledonicus</i>	6820	10900	2.04
<i>H. austrocaledonicus</i>	14900	18600	2.62
<i>Geissois pruinosa</i>	6720	9760	2.24
<i>Psychotria douarrei</i>	13400	9740	4.52
<i>Sebertia acuminata</i> (leaves)	10200	11100	3.00
<i>S. acuminata</i> (latex)	167000	250000	2.19
* <i>Alyssum bertolonii</i>	2590	2500	3.39
* <i>A. serpyllifolium</i> s.sp. <i>lusitanicum</i>	9330	2650	11.5
* <i>Pearsonia metallifera</i>	10600	2500	13.9

\* Hyperaccumulators from beyond New Caledonia.

logarithmic scales as shown, a very highly significant correlation is found ( $r = 0.855$ ,  $P < 0.001$ ).

Citric acid levels of 1000–4000  $\mu\text{g/g}$  are characteristic of those New Caledonian species which generally have less than 100  $\mu\text{g/g}$  nickel. Where nickel is present at 100–1000  $\mu\text{g/g}$ , elevated citric acid concentrations (4000–8000  $\mu\text{g/g}$ ) are found, and the nickel hyperaccumulators show citric acid levels of 8000–20000  $\mu\text{g/g}$  (0.8–2.0%). In the extreme case of the *Sebertia acuminata* latex, one of the most remarkable of all biological fluids, removal of

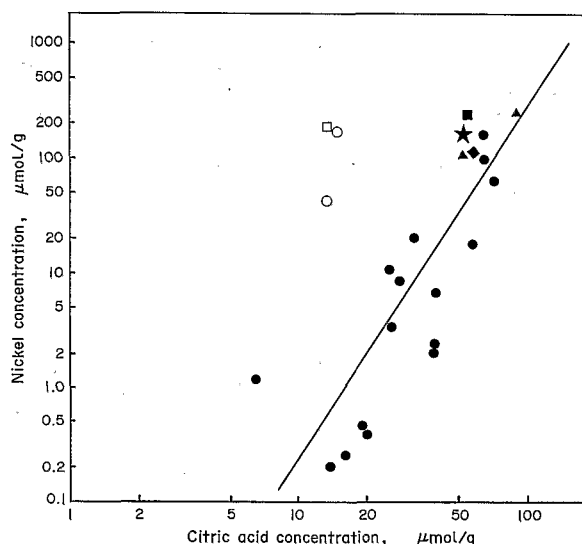


Fig. 1. Relation between citric acid and nickel concentrations in the leaves of *Homalium* species (●), *Hybanthus* species (▲), *Sebertia acuminata* (★), *Geissois pruinosa* (◆) and *Psychotria douarrei* (■), all from New Caledonia. Values for *Alyssum* species (○) and *Pearsonia metallifera* (□) are also shown.

water gives a solid typically containing 16.7% nickel and 25.0% citrate. In the latex, nickel is present both as the aquo-cation and as an anionic citratonickelate complex [1].

The citratonickelate complex previously isolated from six New Caledonian hyperaccumulators [1] has now also been found in aqueous extracts of leaves of *Psychotria douarrei* [20] and *Geissois pruinosa*, using GLC of trimethylsilyl (TMS) derivatives of extracts purified by gel chromatography on Sephadex G-10 and G-25. Electrophoretic evidence indicates the presence of anionic citratonickelate in extracts of these species and of several other nickel accumulators (*Homalium austrocaledonicum*, *H. decurrens*, *H. deplanchei*) which typically contain 100–1200  $\mu\text{g/g}$  nickel). In view of this association of citrate with nickel in the aqueous extracts of all New Caledonian accumulator plants studied so far, the correlation observed in the leaf material is not surprising.

The three hyperaccumulators from beyond New Caledonia are noteworthy in that they contain only 'normal' levels of citrate, insufficient to complex a significant proportion of the total nickel present. The nature of the nickel chelation in these plants is discussed in more detail below.

GC-MS studies on silylated organic acid extracts from leaf material showed some significant differences among the various species. Although the GLC traces show many minor peaks which were not identified, the TMS derivatives of the major acids were well resolved and most were readily identified from their retention times and from comparison of their mass spectra with those of authentic silyl derivatives.

In the leaves of the New Caledonian nickel accumulators, typified by *Homalium kanaliense*, citric, malic, quinic and an unknown (B) were present in the largest amounts. Citric and malic were the major acids of the TCA cycle present, although small quantities of malonic and aconitic acid were also observed. Among the *Homalium* species there were no important differences in the identity of the organic acids present, but the hyperaccumulators had much higher levels of citric acid, in particular, and of malic acid. Direct silylation of the *Sebertia acuminata* latex confirms the predominance of citric acid, and shows the presence of traces of malic and malonic acids. GLC traces of TMS derivatives of purified nickel-containing extracts of all the New Caledonian hyperaccumulators show the presence of citric acid alone.

In the Rhodesian hyperaccumulator *Pearsonia metallifera*, malic and malonic acids predominate in the organic acid extract. Moderate amounts of quinic acid and an unknown (C) were detected, and only a relatively small concentration of citric acid was present. The purified nickel-containing extract from the leaves of this species was shown by GC-MS to contain only the unknown acid C. Although its retention time is identical with that of mesotartaric acid, accurate mass measurements and a study of the fragmentation pattern suggest that C is a trihydroxycarboxylic acid  $\text{C}_5\text{H}_{10}\text{O}_5$ .

Pelosi *et al.* [21] have provided evidence that nickel in extracts of *Alyssum bertolonii* is associated with malic and malonic acids. Our work with *A. serpyllifolium* s.sp. *lusitanicum* by GLC shows the predominance of these acids in this species also and follows a similar discovery by the Italian workers (pers. comm. O. Vergnano Gambi to R. R. Brooks). These two acids were also the only important acids in purified nickel-containing extracts

of the leaves of *A. serpyllifolium* s.sp. *lusitanicum*. Since the stability constants for nickel malate ( $pK = 3.17$ ) and nickel malonate ( $pK = 3.30$ ) are considerably smaller than that for the citrate ( $pK = 5.40$ ) [22], it appears that the rather small amounts of citrate present in the leaves of *P. metallifera* and the *Alyssum* species are not available for complexing with nickel.

Our results indicate that elevated levels of citrate occur in the leaves of all New Caledonian nickel accumulators studied so far, and that citratocomplexes of nickel are present in aqueous extracts of their leaves and in the *Sebertia acuminata* latex. However, malic and malonic acids are associated with nickel in two *Alyssum* species, and the nickel in *Pearsonia metallifera* is complexed with another carboxylic acid, incompletely identified. To account for these differences, and to gain a better understanding of the mechanisms of nickel tolerance and nickel accumulation, further work is needed on the behaviour of nickel and organic acids in all parts of these plants and in the surrounding soils.

#### EXPERIMENTAL

**Extraction of organic acids.** Freeze-dried material (2 g) was homogenized with 50 ml 80% EtOH (acidified with 0.1%  $\text{HCO}_2\text{H}$ ). After filtration, the residue was combined with 5 g of cation exchange resin (Amberlite IR-120,  $\text{H}^+$ ) and extracted with 50 ml  $\text{H}_2\text{O}$  by shaking for 2 hr. The suspension was filtered and the filtrate combined with the EtOH extract. Cations remaining in soln were removed on a  $10 \times 50$  mm cation exchange column. Organic acids were eluted from a  $10 \times 100$  mm anion exchange column (Dowex 1-X8, formate) with 25 ml of 50%  $\text{HCO}_2\text{H}$ . The eluate was dried under red. press. at  $40^\circ$ , redissolved in 10 ml  $\text{H}_2\text{O}$  and dried again to ensure complete removal of formic acid.

**Determination of citric acid.** 1 ml aliquots of the organic acid extract were assayed for citric acid colorimetrically by the pentabromoacetone method [23, 24].

**Determination of nickel.** Samples of freeze-dried leaf material (1 g) were ashed at  $450^\circ$  in a muffle furnace and the residue dissolved in 2 M HCl for analysis by atomic absorption.

**Gas chromatography.** In the presence of trifluoroacetic acid, aliquots containing 100–400  $\mu\text{mol}$  of organic acids were reacted with *N*-(trimethylsilyl)imidazole in dimethyl sulphoxide, giving trimethylsilyl (TMS) derivatives of the organic acids in the upper clear layer of the reaction mixture. GLC was carried out with a Packard 802 chromatograph (200 cm  $\times$  2.5 mm glass column, 3% OV-101 on Chromosorb W, AW-DMCS, 100–200 mesh,

flame ionisation detector), using 1  $\mu\text{l}$  samples injected directly on to the column head with injection port temp.  $250^\circ$ . Gas flow rates were 45 ml/min  $\text{N}_2$ , 30 ml/min  $\text{H}_2$ , 450 ml/min air. The oven temp. was programmed from 90 to  $220^\circ$  at  $8^\circ/\text{min}$ .

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