Effects of Three Nickel Salts on Germinating Seeds of Grevillea exul var. rubiginosa, an Endemic Serpentine Proteaceae

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Received: 12 August 2004 Returned for revision: 28 October 2004 Accepted: 23 November 2004 Published electronically: 10 January 2005

**Background and Aims** Serpentine soils are usually quite infertile, arid and toxic, mainly because they contain high levels of heavy metals such as Ni. The aim of the present work was to assess the effects of Ni on the germinating seeds of Grevillea exul var. rubiginosa, an endemic serpentine Proteaceae of New Caledonia. In addition, the distribution of macronutrients and the Ni levels in germinating seeds were examined.

**Methods** Seeds were sown in glass Petri dishes and exposed to increasing concentrations of Ni (5 to 500 mg Ni L\textsuperscript{-1}) using Ni chloride, Ni sulphate and Ni acetate. The germination percentage and root length were measured after 40 d. Longitudinal frozen sections of germinating seeds growing in the presence of Ni (500 mg Ni L\textsuperscript{-1} for all three salts) were used for X-ray microanalysis and X-ray elemental mapping using scanning electron microscopy (SEM).

**Key Results** Ni chloride resulted in the greatest reductions in germination and root growth, particularly at 500 mg L\textsuperscript{-1}, followed by Ni sulphate and Ni acetate. SEM images revealed Ca crystalline structures in the seed coat for all the samples. S/Ca and Mg/P/K/Mn were found to be distributed differently in Ni-treated samples, whereas they all followed the same pattern in the controls. For all three salts, the Ni added to the medium had accumulated in the seed coat, whereas the endosperm seemed to be devoid of Ni.

**Conclusions** It is assumed that the seed coat is able to reduce the amount of Ni entering the seed, and that a high level of Ni induced the mobilization of macronutrients.

**Key words:** Ni, SEM, energy dispersive spectrometry (EDS) microanalysis, macronutrient mapping, seeds, Ca crystals, serpentine, Proteaceae.

**INTRODUCTION**

One third of the largest island of New Caledonia is covered with outcrops of ultramafic soils. The mineral composition of these soils is not favourable for plant growth, because they contain low levels of essential macronutrients (N, P, K, Ca), and high levels of heavy metals such as Ni (Enright \textit{et al}., 2001), the range of exchangeable Ni concentrations being 50–1000 μgNi g\textsuperscript{-1} (Walker, 1954; Jaffré, 1980). Even though Ni is considered to be an essential nutrient for higher plants (Brown \textit{et al}., 1987), it can also be toxic in these soils. The phytotoxic threshold in soil depends on environmental and species-specific factors (Gabrielli \textit{et al}., 1991).

Eighty percent of New Caledonian ultramafics are covered by scrubby vegetation known as ‘maquis minier’ (Proctor, 2003). This is xerophyllous shrub vegetation, 2–8 m tall (Proctor, 2003), and consists of 92.7 % endemic species (Jaffré \textit{et al}., 2001). The dominant families in the ultramafic maquis include Proteaceae (Jaffré \textit{et al}., 1987). One common species of this family, Grevillea exul var. rubiginosa, is used as a pioneer to restore degraded areas around New Caledonian Ni mines (Jaffré and Pelletier, 1992). Species growing on soils containing high levels of Ni are usually assumed to be Ni-resistant (Farago and Cole, 1988). However, no Ni hyperaccumulating Proteaceae has ever been identified (Brooks, 1987), although some species accumulate Mn in their leaves (Jaffré, 1980). The physiological susceptibility of Grevillea exul var. rubiginosa towards Ni has not been studied before, and so the present study was intended to find out whether this species is Ni-resistant or not.

Seed germination is sensitive to the Ni status of the environment, because minerals enter the seed during imbibition. Cations are preferred to anions during the uptake process, and high concentrations of some cations accumulate (Larcher, 1995). Previous investigations have demonstrated the beneficial effects of Ni on seed germination in Ni-resistant species or serpentine species (Homer \textit{et al}., 1991; Welch, 1995; Rout \textit{et al}., 2000; Peralta \textit{et al}., 2001). The effects of Ni on the uptake of other nutrients have already been studied in adult plants and seedlings (Cataldo \textit{et al}., 1978; Gabrielli \textit{et al}., 1991; Kovačević \textit{et al}., 1999), however there have been few studies of germinating seeds. The assimilation, concentration and distribution of heavy metals in seeds have been explored in species used by the food industry (Lange-Hesse \textit{et al}., 1994; He \textit{et al}., 2000; Mengchang \textit{et al}., 2000), but there have been few studies of the seeds of serpentine species (Psaras and Manetas, 2001).
The sulphate (Brown et al., 1987; Kovacević et al., 1999; Rout et al., 2000) and chloride (Cataldo et al., 1978; Boyd and Martens, 1994; Issa et al., 1995; Psaras and Manetas, 2001) salts of Ni are those usually used to investigate Ni stress. In the present study, three different salts (Ni sulphate, Ni chloride, Ni acetate) were used in a germination bioassay to determine the effect of the chemical form of Ni on its assimilation by distribution in seeds. Indeed the toxicity of the chloride ion, the catabolism of acetate and the fundamental structural role of sulphates in metallothioneins might induce specific effects that could mask the effects of the Ni ions. The phyto-available chemical form of Ni in New Caledonian ultramafics is not known.

The objective of this study was to investigate the distribution of macronutrients and Ni levels in germinating Grevillea exul var. rubiginosa seeds exposed to three different Ni salts using scanning electron microscopy (SEM).

MATERIALS AND METHODS

Harvesting the seeds

Fruits of Grevillea exul (Lindley) var. rubiginosa (Brongniart and Gris) were collected in March 2003 from ultramafic soils near the Pirogues River in the south of New Caledonia. The adult trees from which the fruits were gathered were well-represented in the area and no visual signs of damage were observed. The fruits were dried for 1 week under light (38 °C) and then the seeds were sorted and placed in water-tight flasks. They were then kept at 4 °C to limit the development of fungi and other pests. A seed of Grevillea exul var. rubiginosa is shown in Fig. 1. Fruit is produced annually. The fruit is dehiscent, woody, aggregated in bunches and generally contains two seeds (Virot, 1968). The seeds are oval and flat, with a thin seed coat. Previous experiments in our laboratory have shown that seed dormancy is partly controlled by seed coat inhibition (unpublished data).

The viability of the harvested seeds was tested by incubating the seeds for 24 h in 2,3,5-triphenyl tetrazolium (C₁₅H₁₅N₄Cl) solution (1 %) (modified from Bennett and Loomis, 1949). The seeds were then cut in half longitudinally and deposited on a sterile Whatman No. 1 filter paper impregnated with 1 % tetrazolium chloride. The viability of seeds was observed 2 d later.

Seed sowing

Three months after being harvested, the seeds were surface-disinfected for 30 min in a 4 % solution of sodium hypochlorite, and then rinsed four times in sterilized distilled water. The seeds were sown in 90-mm sterilized glass Petri dishes (15 seeds per dish, four dishes per treatment) containing three pieces of sterilized Whatman No. 1 filter paper, and 10 mL of sterilized Ni chloride (NiCl₂·6H₂O), Ni acetate (C₂H₄NiO₂·H₂O) or Ni sulphate (NiSO₄·7H₂O) solution were then added (5, 10, 50, 100 and 500 mg Ni L⁻¹). All chemicals were analytical reagent grade. Sterilized distilled water was used for the control. Each treatment was replicated four times, and the whole experiment was repeated twice. The Petri dishes were incubated at 30 °C in darkness.

After 40 d, the root length of the seedlings and the number of germinated seeds were recorded. The percentage of germinated seeds was calculated for each Petri dish. Averaged percentages for the four replicates were used for statistical analysis in Excel, and the results were expressed as a percentage of the control.

Cryosectioning and scanning electron microscopy

Only the 500 mg Ni L⁻¹ treated seeds were used for the mineral analysis, because the Ni concentrations used for the lower dose could not be detected in the seeds by EDS (energy dispersive spectrometry) microanalysis. Germinated seeds with 1 mm of root were selected and rinsed four times in sterilized distilled water. They were embedded in Cryomount (Tissue-Tek® O.C.T. compound) and then frozen in liquid nitrogen. Longitudinal sections 40 μm thick were cut at −25 °C using a cryomicrotome (Cryo-cut II microtome Reichert-Jung). They were immediately placed on SEM specimen holders, and then stored at −32 °C until carbon metallization occurred (10–15 mm) and observed under an ESEM Philips XL 30 microscope.

The EDS spectra for the compositional analyses were performed for Ca and Ni by point analysis with a collection time of 5 min and 1500–2000 cps (excluding C and O from the normalized percentages; three replicates per analysis). The findings of the elemental analysis are expressed as percentages of the dry matter. X-ray mapping was performed for 2 h to give the elemental distribution for each selected element (Ca, K, Mg, Cl, Mn, S, P, Ni). In all cases the voltage was 20 kV. SEM images were obtained with back-scattered electron imaging.

![Fig. 1. Seed of Grevillea exul var. rubiginosa; a: seed coat; b: position of the embryo; c: endosperm area. Scale bar = 1 mm.](https://academic.oup.com/aob/article-abstract/95/4/609/199540)
RESULTS

Germination percentage and root length

Results for the germination percentage and the root length of the germinating seeds grown in different Ni salts at varying concentrations are presented in Fig. 2 (A and B, respectively).

Ni chloride treatments. All the concentrations of Ni chloride resulted in germination percentages that were significantly lower than the control, but there was no difference between 5, 10 and 50 mg Ni L\(^{-1}\) treatments. 100 and 500 mg Ni L\(^{-1}\) both produced marked and significant inhibition of germination. Significant inhibition of root elongation was seen from 50 mg Ni L\(^{-1}\). The 100 and 500 mg Ni L\(^{-1}\) concentrations depressed root elongation considerably more than the 50 mg Ni L\(^{-1}\) concentration.

Ni sulphate treatments. Significant inhibitory effects of Ni sulphate were seen on germination at concentrations from 100 mg Ni L\(^{-1}\) and on root elongation from 50 mg Ni L\(^{-1}\). The 100 and 500 mg Ni L\(^{-1}\) concentrations induced similar effects for both germination and root length.

Ni acetate treatments. No effect of Ni acetate was detectable up to 100 mg Ni L\(^{-1}\) for the seed germination, but it appeared to be very toxic at 500 mg Ni L\(^{-1}\). Ni acetate inhibited root length at concentrations from 50 mg Ni L\(^{-1}\), and this effect was greater at 500 mg Ni L\(^{-1}\).

Ni chloride appeared to be the salt with the greatest inhibitory effect on seed germination. There was no significant difference from the control in the root length at 5 or 10 mg Ni L\(^{-1}\) for any of the salts tested. For all three salts, the relative effects of the 500 mg Ni L\(^{-1}\) concentration on germination percentage and on root length were similar: the least inhibition was produced by Ni sulphate, whereas Ni chloride and Ni acetate showed similar, highly inhibitory effects.

Some microbial growth was observed on the seeds and on paper exposed to the control, 5 and 10 mg Ni L\(^{-1}\) solutions.

![Figure 2. Germination (A) and root length (B) according to the salt and the Ni concentration in treatment. Different letters indicate significant differences between means (P < 0.05; Student’s t-test).](https://academic.oup.com/aob/article-abstract/95/4/609/199540)
SEM analysis

X-ray mapping. Figures 3 (control), 4 (Ni chloride), 5 (Ni sulphate) and 6 (Ni acetate) show the X-ray maps of longitudinal sections of germinating *Grevillea exul* var. *rubiginosa* seeds. The first electron micrograph in each figure shows exactly which part was examined. It always included part of the seed coat and the endosperm.

Maps of the distribution of Ca in the control and treated samples (for all three salts) were similar, with a line of Ca slabs inside the seed coat, and a wide band of Ca aggregates corresponding to the external zone of endosperm. The inner part of the endosperm contained little Ca. For all three Ni treatments (Ni acetate, Ni sulphate, Ni chloride), the distributions of S/Ca and of Mg/P/K/Mn differed sharply: K, Mg, Mn and P were located in the places devoid of Ca and S, corresponding to the middle of the seeds where the embryo axis was developing. This feature was not observed in the control seeds, where the distribution of the various elements seemed to be homogeneous. No Ni was detected in the control, whereas Ni mapping of the treated seed demonstrated that for all three salts, most of the Ni stopped at the seed coat. However, little Ni was found inside the endosperm after exposure to Ni chloride (map of Ni in Fig. 4, in comparison with those in Figs 3, 5 and 6). Cl entered inside the seed with the Ni chloride treatment.

Semi-quantitative mineral analysis. The Ni/Ca ratio in the endosperm was very low for all three salts (Fig. 7). However, the Ni_{seed coat}/Ni_{endosperm} ratio was three times higher for sulphate than for chloride or acetate. The values of the Ni/Ca ratio in the seed coat were greater than 1 for all three salts (3.5 to 12), and slightly higher for sulphate and acetate.

SEM image. The *Grevillea exul* var. *rubiginosa* seed coat was made up of a thin outer layer and a hypodermal sclereid layer (Fig. 8B, C). Some of the sclereid cells in the seed coat were incrusted with crystal formations. Point analysis of these crystals revealed that they consisted essentially of Ca (Fig. 8D, E; 9D), which was associated with Ni (Fig. 8D, E). Figure 9C presents the findings of point analysis of a Ni particle (920 nm) located in the vicinity of a Ca crystal (analysed in Fig. 9D). The Ca peak in Fig. 9C was probably due to the proximity of Ca crystals.

**DISCUSSION**

These experiments use a new protocol for locating minerals in seeds. This approach was attempted because the high level of metal tolerance of this species meant that the Ni content reached levels that could be detected. However, there are few related studies available for the purposes of comparison because the level reached in most species is usually below the threshold of detection. This experiment shows that exposure to Ni alters the partial redistribution of minerals in seeds that occurs during germination, and thus affects germination and root growth.
**Fig. 4.** Elemental maps of longitudinal section from *Grevillea exul* var. *rubiginosa* germinating seed, treated with Ni chloride at 500 mg Ni L$^{-1}$. The first image shows the analysed zone: part of the seed coat (a) and underlying cell layers of the endosperm. Scale bar = 100 μm.

**Fig. 5.** Elemental maps of longitudinal section from *Grevillea exul* var. *rubiginosa* germinating seed, treated with Ni sulphate at 500 mg Ni L$^{-1}$. The first image shows the analysed zone: part of the seed coat (a) and underlying cell layers of the endosperm. Scale bar = 100 μm.
Microbial infections around *Grevillea exul* var. *rubiginosa* seeds are regularly observed, regardless of where they have been collected or of the year they were collected. These microorganisms are probably cellulotic fungi, and live inside the seed coat. Previous work in our laboratory (unpublished data) has shown that they do not affect the germination rate. No contamination has been found following exposure to high levels of Ni, probably because of Ni toxicity.

**Seed development**

Seed germination and root elongation were partly inhibited by the Ni treatments, but the critical phytotoxic concentration and the severity of the effects depended on the Ni salt used.

In the case of root elongation, similar results were obtained for all the salts up to 50 mg Ni L\(^{-1}\). Differences between the salts appeared at the 100 and 500 mg Ni L\(^{-1}\) concentrations: Ni chloride resulted in the poorest root elongation, and Ni sulphate gave the best growth at 500 mg Ni L\(^{-1}\). However, it is not possible to conclude which salt is most favourable for root growth, because differences were too small. For germination, the increasing phytotoxicity gradient is: Ni acetate < Ni sulphate < Ni chloride. The effects of Ni on seed germination thus depend to some extent on the co-transported ion. Ni salts probably have specific potential binding sites and properties. The low phytotoxicity of Ni acetate can be explained by the bulkiness of the acetate molecule, resulting in steric hindrance.
that limits its ability to penetrate the seeds. Moreover, the uptake of organic compounds, such as acetate, during the primary metabolism may stimulate germination, and thus mask the effects of Ni. Ni chloride was the Ni treatment that resulted in the lowest germination. This can be explained by the toxicity of the chloride ion, which has been demonstrated in studies of NaCl toxicity in plants (Britto et al., 2004). At 500 mg Ni L$^{-1}$, Ni sulphate was the least toxic treatment with regard to both germination and root growth. This may be due to the fact that, unlike the chloride ion, which is a micronutrient, sulphate is a macronutrient and is involved in the synthesis of cell detoxification molecules, such as the metallothioneins. Curtin et al. (1993) found that sulphate salt induces a better growth than chloride salt in barley. However, the investigation of the chemical forms available to plants in natural serpentine soils could help clarify which salt is more significant in practice.

Grevillea exul var. rubiginosa can be classified as an Ni-resistant species on the basis of the fact that germination was similar to controls up at least to 50 mg Ni L$^{-1}$ (in the case of Ni chloride, this treatment produced only 30 % inhibition).
EDS microanalysis

A possible explanation for Ni-resistance has been provided by EDS microanalysis. SEM is the conventional method of studying seed structure and of locating and quantifying mineral elements (Przybylowicz et al., 1997; Gierth et al., 2000; Psaras and Manetas, 2001). The microscopic methods used to analyse biological samples usually require a cryochamber inside the scanning electron microscope to preserve the tissue structures (Gierth et al., 2000). However, the low water content of the seeds does not make this condition necessary. The method presented here consisted of gluing the frozen SEM specimen straight onto the holder, and then fixing the structures by carbon-coating. The SEM images of the carbon-coated sections displayed little tissue damage. Moreover, there is little risk of mineral leakage, unlike methods that use fixing baths. This made it easy to recognize cell walls and the various tissues, suggesting that this easy and quick method is suitable for mineral analysis of this particular biological material.

This study shows that Ca is well-represented in *Grevillea exul* var. *rubiginosa* seeds and located specifically in the cells of the seed coat and in the endosperm. Scanning electron micrographs revealed that Ca is present in crystalline form inside the cells of the seed coat. Barnabas and Arnott (1990) found a similar disposition of calcium in the form of Ca oxalate crystals in the hypodermal sclereid cells of the bean seed coat. Seeds of many angiosperms contain crystalline calcium oxalate, which is commonly deposited in the seed coat (Ilarslan et al., 2001). Two of the main known functions of calcium crystals are to provide a large reservoir of Ca and to protect plants against bacteria, fungi and herbivorous animals (Barnabas and Arnott, 1990; Jáuregui-Zúñiga et al., 2003). Ca crystal formation is under genetic control, and occurs in membrane-like compartments within the vacuoles (Nakata and McConn, 2000). The Ca present in *Grevillea exul* var. *rubiginosa* seeds must have been endogenous Ca accumulated during fruit formation, because no Ca was added in the medium. The endogenous Ca may therefore be correlated with the natural soil environment of this species. The ultramafic soils of

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**Fig. 9.** SEM images and point analyses of the seed coat of germinating seed of *G. rubiginosa* var *exul* treated with 500 mg Ni L⁻¹. (A) The seed coat has been split in two, and Ca crystals can be seen inside the seed coat: a, cotyledons; b, seed coat with a hypodermal layer incrusted with Ca crystals; c, external side of seed coat. (B) Detail of the area indicated in (A); the particle that is circled in the image has been analysed, and the micrograph obtained is presented in (C); crystals have been analysed, and the micrograph obtained is presented in (D). (C) Mineral composition of a Ni particle and its immediate environment. (D) Mineral composition of a Ca crystal.
New Caledonia are characterized by their low Ca/Mg ratio, which dramatically reduces the absorption of Ca ions (Walker et al., 1955; Jaffré, 1977). However, Ca crystal formation depends on the supply of calcium (Ilarslan et al., 2001; Zindler-Frank et al., 2001). The capacity of Grevillea exul var. rubiginosa to accumulate enough Ca in their seeds for the formation of calcium crystals suggests that the adult trees must have strategies for extracting large amounts of Ca from serpentine soils, or for recycling Ca already present in the plant.

Even though the Ni maps clearly show that Ni did enter the seed after Ni chloride treatment, most of it was found in the seed coat after Ni chloride treatment, which dramatically reduces the absorption of Ca ions (Witkowski et al., 1997; Issa et al., 1995). It can be surmised that Ca crystals could induce the crystallization of Ni by a heterogenic nucleation mechanism that forms a barrier to Ni entry. The heterogenic nucleation of calcium oxalate has been studied in a model used to elucidate kidney stone formation (Christmas et al., 2002). However, the implication of Ca crystals in protecting seeds from metal toxicities has never been studied previously, and our assumptions need to be confirmed by more detailed experiments.

The seed coat may be composed of structures that block Ni within the tissues of the seed coat. The disposition of Ca crystals in the seed coat may be correlated with the accumulation of Ni, because we have demonstrated that Ni is sometimes found with these crystals. Previous studies have shown that Ca can reduce the incidence of Ni toxicity in plants (Brooks, 1987; Issa et al., 1995). It can be surmised that Ca crystals could induce the crystallization of Ni by a heterogenic nucleation mechanism that forms a barrier to Ni entry. The heterogenic nucleation of calcium oxalate has been studied in a model used to elucidate kidney stone formation (Christmas et al., 2002). However, the implication of Ca crystals in protecting seeds from metal toxicities has never been studied previously, and our assumptions need to be confirmed by more detailed experiments.

Ca deposition was similar in the treated and control seeds. In the Ni-treated samples, S and Ca were located in the external part of the endosperm, whereas P, K, Mn and Mg were located where Ca and S were not found. The chemical form of Ni does not seem to influence this process, because similar results were obtained for all three salts. Because the mineral concentrations of minerals in distilled water are too low to influence the microanalysis, we can assume that the concentrations of the mineral elements detected resulted from the mobilization of endogenous seed reserves. These contrasting distributions did not appear in the control, suggesting that they were induced by the Ni treatments. Accumulation of Ni in the seed coat could be a stress signal that induces mineral mobilization processes. Previous studies have revealed that Ca in the seed coat may act as a secondary messenger (Witkowski et al., 1997; Gaspar et al., 2002). Contact between Ni ions and Ca elements in the seed coat may be implicated in the transduction of environmental stimuli to the underlying seed tissues. Why this should happen is not clear. Nevertheless, since these differences are correlated to the poor root development and low germination observed with the high dose treatment (500 mg Ni L⁻¹), it is possible that high concentrations of nutrients in the endosperm reveal metabolic processes in response to the Ni stress.

We were able to cast some light on the role of the chemical form of the Ni. Our data indicate that the translocation of Ni into the endosperm was lower, and the accumulation of Ni in the seed coat was higher for the sulphate and acetate salts than for the chloride salt. Cl ions had diffused inside the seed, which could have had toxic effects on the embryonic tissues. Indeed, the germination results suggested that Ni chloride was the most toxic salt. This would fit in with the general toxicity of chlorides towards plant cells.

A more precise technique, PIXE (Particle Inducted X-ray Emission), would be useful to detect affinity for Ni inside the endosperm. The pathways of the acetate and sulphate co-transported with Ni into the seed remain to be elucidated.

The main hypotheses to emerge from this study are the relative infertility of the Ni chloride salt for developing tissues in Grevillea exul var. rubiginosa seeds, and that the seed coat has a role in excluding Ni and is rich in calcium crystals. The exclusion of Ni and the calcium crystals border could be correlated, but this hypothesis needs to be confirmed by further experiments.

ACKNOWLEDGEMENTS

We thank the Southern Province of New Caledonia for its financial support. We thank Prof. Viano for providing facilities during experiments in Marseille, and R. Chandra for her contribution in this study. The skilful technical assistance provided by A. Tonetto and G. Girard is gratefully acknowledged.

LITERATURE CITED


