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Contact Address

Biology Dept. SPAS. USP

Chandra_ro@usp.ac.fj

Permanent Address

G.P.O. Box 12467, Suva

romi2chandra@yahoo.com

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Contact Address

Biology Dept.,
SPAS. USP
Chandra_ro@usp.ac.fj

Permanent Address

G.P.O. Box 12467
Suva
romi2Chandra@yahoo.com.

EFFECTS OF NICKEL ON SOME PHYSIOLOGICAL ASPECTS OF DEVELOPMENT OF *GREVILLEA EXUL* VAR. *RUBIGINOSA*, A NEW CALEDONIAN ENDEMIC SPECIES

By

ROMIKA ROMELESH CHANDRA

A thesis submitted in fulfillment of the requirements for the degree of Master of Science.

Department of Biology
The University of the South Pacific
In collaboration with:
Laboratory of Applied Plant Biology and Physiology
University of New Caledonia

July 2005

DECLARATION

I, Romika Romelesh Chandra declare that this thesis is my own work and that, to the best of my knowledge, it contains no material previously published or substantially overlapping with material submitted for the award of any other degree at any other university, except where due acknowledgement is made in the text.

Rhandra

20th July 2005

CERTIFICATE

This is to certify that this thesis entitled "Effects of Nickel on some Physiological Aspects of Development *of Grevillea exul* var. *rubiginosa*, a New Caledonian Endemic Species" submitted for the degree of Master of Science in the subject of Biology, to the University of the South Pacific, is a bonafide research work carried out by Ms. Romika Romelesh Chandra under our supervision and guidance. No part of this thesis has been submitted for any other degree in any University.

The assistance, help, guidance and supervision received during the course of this research work has been duly acknowledged.

Signature:…… Date:……

Dr. Anand P. Tyagi
Principal Supervisor (USP)
Associate Professor and the Head of Department of Biology
The University of the South Pacific
P. O. Box 1168, Suva
Fiji Islands.

Signature:…… Date:……

Dr Saliou Bouraïma Madjébi
Principal Supervisor (UNC)
Lecturer
Laboratory of Applied Plant Biology and Pathology
University of New Caledonia
BP 4477, F – 98847 Noumea Cedex
New Caledonia.

ACKNOWLEDGEMENTS

This is my chance to thank all those people who helped in this research and writing of this thesis; without their help this study would have not been completed.

Firstly Dr Anand Prakash Tyagi (USP) and Dr Saliou Bouraïma Madjébi (UNC) my two supervisors whose help, constant encouragement and support have made this study possible.

The entire Laboratory of Applied Plant Biology and Physiology (LBPVA) team at the University of New Caledonia for their invaluable and timely assistance throughout this research.

The French Embassy in Suva for funding this study and not forgetting Mr. Philippe Liege (Counsellor for Culture and Cooperation) for understanding the importance of time as a factor in plant studies.

Special thanks to my family for making my dreams come true.

Last but not the least to all those people who were there to see me through the stage of research and writing of this thesis.

……

Romika Romelesh Chandra

TABLE OF CONTENTS

ABSTRACT

Endemic plant species have been considered as one of the best means of ecological restoration of exploited mine sites in New Caledonia. These plants have the adaptability characteristics that allow them to thrive on serpentine soils. This study has investigated the physiological effects of nickel concentrations at different developmental stages on *Grevillea exul* var. rubiginosa an endemic New Caledonian species. The first two stages of a plant life cycle were studied: germination and post germination under controlled and semi-controlled conditions respectively.

The initial stage of development involved Petri dish germination and growth of seeds and seedlings respectively with different salts of nickel (nickel acetate, nickel chloride and nickel sulphate) with 0 – 500 ppm concentrations. Results obtained show nickel chloride as the most toxic compared to nickel acetate and sulphate. Decrease in all studied parameters: germination rates, root lengths, total nitrate, total soluble protein and total soluble glucid contents were observed with increasing concentrations of nickel chloride as the salt solution supplied to seeds. No significant differences were observed for nickel sulphate and nickel acetate except for increase in all studied parameters for 50 and 100 ppm nickel acetate. These results suggest that the form of nickel used in laboratory experiments are essential for experimental work, in order to take into account the form in which it is present in the natural environment. Seedlings germinated with nickel chloride were analyzed for glutamine synthetase activity (GSA) and nitrate reductase activity (NRA). Results obtained show that 5 ppm of nickel caused a slight increase in GSA compared to 0 ppm (control), 10 ppm, and 50 ppm, whereas 100 ppm of nickel had the highest GSA. Nitrate reductase activity increased with increasing concentrations of nickel chloride in the medium, with 100 ppm having the highest NRA.

Seedlings germinated and treated with nickel chloride solution (0 – 500 ppm) under greenhouse conditions were also analyzed morphologically and biochemically. Morphological analysis included growth parameters such as root length, shoot length

and number of leaves whereas biochemical analysis included total nitrate, total soluble protein and total soluble glucid contents as well as GSA and NRA.

Plantlets treated with 0 – 500 ppm nickel chloride over a period of 3 months indicated the importance of a minimum concentration of nickel in the substrate for the species. Nickel salt effect was quite obvious with fresh root : shoot ratio, enzyme activities as well as in biochemical parameters analyzed.

Minirhizotrons provided a clear demonstration of root colonization in the two major soil types used during revegetation. Saprolite soil amended with organic matter proved to improve plant growth and development as compared to red laterite soil. Proteoid roots were observed on principal roots in both soil types with branching of roots prominent only in the upper zones of the compartments.

This project has hence provided important and useful information for the revegetation of mine sites in New Caledonia using *Grevillea exul* var. rubiginosa as a major part of the mine vegetation.

Results obtained in the present investigation would be useful to revegetate mining sites not only in New Caledonia but also anywhere in the world where nickel mining is a major mining activity.

CHAPTER 1

1.0 INTRODUCTION

New Caledonia, a South Pacific archipelago has a land area of 19000 km², spread over an area of 500 km long and 50 km wide. Approximately one third of the total surface area of the island 5500 km² is covered with serpentine soils (International Nickel Study Group 2001; McCoy *et al.,* 2003; Sarrailh & Ayrault, 2003). These soils are derived from ultramafic rocks as seen in figure 1.1, which are very rich in metals (nickel, cobalt, manganese and chromium) but poor in plant nutrients (calcium, potassium, phosphorus and nitrogen) as well as organic matter.

Figure 1.1 Serpentine soil in the Southern province of New Caledonia

New Caledonia is the third largest producer of nickel in the world, mining of nickel began around 1876 and the main form of mining was carried out in open pit mines. Intensive mining activities became a concern to the people when pollution began to affect their normal way of life. Dumping of mine wastes into surrounding valleys

caused sedimentation in coastal rivers and destruction of fringing reefs (Bird *et al*., 1984). Large columns of red smoke hang in the area above Noumea, and open cut soils that used to be seen as signs of economic wealth are now seen as a major problem. These situations generated increasing environmental awareness for the need to revegetate mine sites and to develop new mining techniques in order to reduce the harmful environmental impact (Pelletier & Esterle, 1995; McCoy *et al*., 2003; Sarrailh & Ayrault, 2003). In New Caledonia, 90 – 98% of the plants are endemic (Jaffre, 2003) and recent mine restoration efforts have focused on the diverse endemic ultramafic flora as an important source of species. These species are adapted to extreme substrate and climatic conditions of mine sites (Jaffre *et al*., 1997).

This study focuses on an endemic plant species of New Caledonia, *Grevillea exul* var. rubiginosa figure 1.2. It belongs to family Proteaceae, which is dominated by the genus *Protea* from South Africa. *Grevillea exul* var. rubiginosa is a xenomorphic, woody species and one of the first to be used in mining site revegetation with few others hence becoming important economically. The distinctive features of this species are broad leaves with reddish undersides, growth to a maturity of 5 – 10 meters and its ability to retain unstable rocks. Due to the importance of *Grevillea exul* var. rubiginosa for revegetation, it became necessary to study the physiological aspects in order to determine effective means of germination, growth and revegetation of exploited mine sites.

Plant development is a continuous process and seed germination is the beginning of this process. Seeds are a convenient source to begin with since they are quiescent, resting entities that represent a normal hiatus in the life cycle. Seeds are severely dehydrated. Their water content is normally about 5% or less (Hopkins, 1995) and metabolic reactions are scarcely detectable because these reactions take place very slowly. Hence seeds can be stated to be in a state of suspended animation, and are capable of surviving adverse conditions for long periods of time without growing.

Figure 1.2 *Grevillea exul* var. rubiginosa plant and seeds in the southern province of New Caledonia

When conditions are appropriate, the embryo renews its growth and the seeds germinate. The initial step of germination of seeds is the uptake of water and rehydration of the seed tissues by the process of imbibition (Hopkins, 1995). General activation of seed metabolism occurs after imbibition of water. According to Hopkins (1995) the specific biochemical events that trigger germination are unknown, but increased respiration was one of the earliest detected in moist seeds. This was closely followed by the release of hydrolytic enzymes that digest and mobilize the stored food reserves, and renewed cell division and cell enlargement in the embryonic axis. Detailed biochemical events that occur during germination have been studied thoroughly in only a few species of seed plants (Hopkins, 1995). The protrusion of the radical allows it to make contact with water, soil and nutrient salts required to support further growth of the young seedling.

Post germination phase begins after germination with the emergence of the cotyledons. In case of *Grevillea*, epigeal germination is characteristic where the U – shaped elongating hypocotyl pulls the cotyledons and epicotyls up above the ground. Once the embryonic tissues have elongated to establish the seedling above the soil, new cells, tissues and organs are formed by regions of active cell division and enlargement. Hence a brief survey of the principal stages in the development of a plant, from seed through shoot and root elongation, flowering, fruit development and vegetative growth see the plant through an unfailing and irreversible orderly progression in its life cycle.

Mineral nutrients have specific and essential functions in plant metabolism; by definition depending on how great the requirement is for growth for a given nutrient, the nutrient is referred to as either a macronutrient (N, P, K, S, Mg, Ca, C, H, O) or a micronutrient (Fe, Mn, Zn, Cu, B, Cl, Ni) (Marschner, 1995).

The term essential mineral element (or mineral nutrient) was proposed by Arnon and Stout (1939). They concluded that, for an element to be considered essential, three criteria must be met:

1. A given plant must be unable to complete its life cycle in the absence of that mineral element.
2. The function of the element must not be replaceable by another element.
3. The element must be directly involved in plant metabolism – for example as a component of an essential plant constituent such as an enzyme – or it must be required for a distinct metabolic step such as an enzyme reaction (Arnon & Stout, 1939).

A large number of mineral forms (Ni^{2+}) and chemical compounds containing nickel (Ni) occur in soils (Nriagu, 1980). Examples include ($Ni^{2+}(H_2O)_6$, Ni OH^+, Ni^+L (where L represents large organic chelates). However it is the availability of these forms to plant roots that is biologically and ecologically important (McIlveen *et al*., 1994). The concentration and solubility of any form of a metal influences the amount of available

and this in turn is also determined by soil pH, texture, organic composition and moisture.

The content of nickel in soils average a few tens of mg/kg in most cultivated soils (McGarth, 1995), but in areas where serpentine or ultra basic bedrock is present, it can reach >10 000 mg/kg (Brooks, 1992). The availability of nickel in soils and transfer to plants depend on the geochemical origin of the metal, on soil characteristics and possibly the efficiency of plant-root uptake (Baker & Walker, 1989).

Nickel is always present in plant tissues at concentrations that range from 0.05 to 8.0 ppm (Vanselow, 1966 and Cottenie *et al*., 1979) whereas Marschner (1995) stated that the nickel content in the vegetative organs of most plants ranges from 1 – 10 ppm dry weight and this range mainly reflects the difference between plant species in uptake and root to shoot transport of nickel. There is some confusion in the literature concerning the levels of nickel in plants, since some have been reported on an ash weight and some on dry weight basis. The confusion is compounded by the conversion in literature, ash weight values to dry weight values by the use of different conversion factors (Farago & Cole, 1988). Nickel was discovered as an essential micronutrient for plant (Brown *et al*., 1987, Farago *et al*., 1988, Eskew *et al*., 1983, Welch, 1981), at low concentrations, but at higher concentrations it proves to be toxic. One of the major roles of nickel is as a component of the enzyme urease, hence suggesting that nickel might have a specific function in higher plants. According to Eskew *et al* (1983) nickel was essential for nitrogen metabolism in soybeans [*Glycine max*. (L.) Merr.], either when nitrogen was supplied as NO_3^- and NH_4^+ or when the plants were dependent on nitrogen fixation. Plant species differ extensively for mineral uptake and accumulation and these differences often help explain plant tolerances to mineral deficiencies and toxicities.

Various authors over the years have reported serpentine soils as extreme and hostile habitats for plant life. These soils contain high concentrations of heavy metals, in particular nickel, which generally has an adverse effect on plant growth (Westerbergh,

1994). Another factor that may reduce growth in serpentine soils is a low concentration of calcium in relation to magnesium (Proctor, 1971). Serpentine soils are also dry and exposed because of the granular texture and lack of organic material (Brooks, 1987). A combination of environmental factors is the reason for the infertility of serpentine soils and is called the ***serpentine syndrome*** (Brooks, 1987). A number of plant species have however, the genetic and ecological resources to overcome these adverse environmental factors thus distinct vegetation has evolved on serpentine soils (Brooks, 1987 and Rune & Westerbergh, 1992).

Work on serpentine soils began with the study of the presence of an unusual flora. Following this, the emphasis switched to the cause of the infertility of the soils derived from ultramafic. According to Brooks (1987) the serpentine factor may be defined as the causal factor or factors (chemical or physical) related to the infertility of serpentine soils and to the nature of the vegetation colonizing them. The development and adaptation of a large number of species and genera of nickel-tolerant plants in New Caledonia probably resulted from two main factors: (i) the relatively large area of ultra basic rocks on the island and (ii) the isolation of New Caledonia from continental masses. These two factors have provided unique opportunity for the flora with 90 – 98% endemism at the species level (Jaffre *et. al*., 1979 and Jaffre, 2003). Such a high endemicity level concentrated in so small a space is a source of great vulnerability, and some species have vanished even before being catalogued or studied. In all a total of 1,137 species are endemic both to the country and to the mining sites (Sarrailh & Ayrault, 2003).

Since it is difficult to regenerate the destroyed vegetation over nickel mined sites, however, research carried out by institutes such as Institute of Research and Development (IRD) and International Cooperation Center on Agrarian Research and Development (CIRAD) have facilitated the development of techniques necessary for revegetation. The natural process of recolonization of degraded soils by plant species is extremely slow and sometimes simply does not happen. Some reasons for slow or no colonization are infertile soil, high concentrations of toxic metals and erosion

vulnerability. In order to reduce the damage caused to slopes by mine runoff, revegetation has been carried out using exotic species such as *Acacias* or *Graminaceae* in order to produce a fast and inexpensive groundcover that check soil erosion. However it is hard to prevent introduced species from competing with endemic species, which ensure longer-term revegetation (Sarrailh & Ayrault, 2003). Fast growing native species such as *Acacia spirorbis* and *Casuarina collina* are nitrogen-fixing species and improve soil environmental conditions, hence they are widely used.

According to Sarrailh and Ayrault, (2003) the present trend is increasing towards revegetation by planting endemic New Caledonian scrub species suited to the substratum in order to recreate the original biodiversity. About 30 species are now being propagated, although their slow rate of growth sometimes necessitates a high planting density (10,000 plants per hectare) in order to give sufficient ground cover. Certain faster growing family shrubs such as Proteaceae (*Grevillea spp*.), Myrtaceae (*Carpoleois laurifolia*) and various other species of family Casuarinaceae (*Gymnostoma deplancheanum*) allow more reasonable densities (2,500 plants per hectare).

Red Laterite soils are the products of relatively more complete weathering of peridotites; where the original structures of the peridotites are no longer recognizable. Magnesia and silica contents are very low in such soils and the main components are crystallized ferric hydroxides (Pelletier, 1990).

Saprolite soils are partially weathered peridotites in which the original structures of the peridotites are preserved. Weathering also leads to the leaching of magnesia and a residual enrichment of iron (Pelletier, 1990). Figure 1.3 (below) shows the weathering profile of peridotites.

In the serpentine areas of New Caledonia the nickel has accumulated as a silicate by solution over very long periods of time in the lower layers of a laterite profile, especially in the underlying saprolite (Bradshaw, 1997) (Table 1.1)

Native species are being selected which can readily establish and contribute to soil stability and fertility. Native species have proved to be tolerant of local soil conditions, such as high Magnesium and of the mined areas. According to Jaffre *et al*., (1994) the endemic native nitrogen-fixing species of the genera *Acacia, Casuarina* and *Gymnostoma* are particularly promising since they nodulate well and must therefore accumulate nitrogen freely.

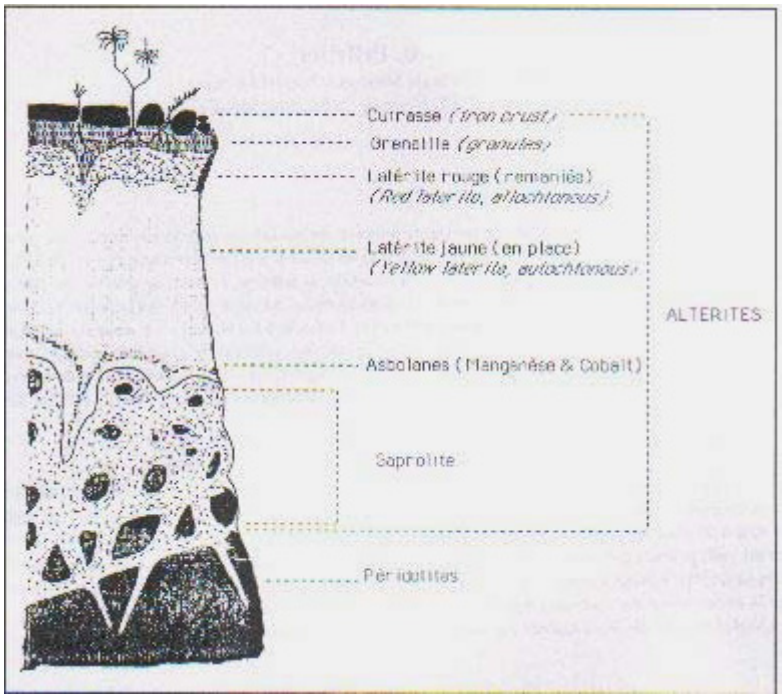

Figure 1.3 weathering profile on peridotites (nickel ores mined in New Caledonia are saprolites in which the nickel grade is high). (Pelletier, 2001)

Table 1.1 Average composition of mineral oxides (Selon Canterford, 1978) Distinction between lateritic and saprolitic oxide mineral compositions.

% Oxides	**saprolitic minerals**	**lateritic minerals**
Ni	1 - 3	0.8 - 1.8
Co	0.05 - 0.08	0.10 - 0.25
Fe_2O_3	10 - 30	50 - 75
MgO	25 - 38	0.5 - 5
SiO_2	40 - 55	1.5 - 6

Hence it is important to determine how native species can be restored. Trials with a variety of other endemic species show that these can be established best on saprolite substrate which suggests that it would be preferable that these should be left at the surface after mining (Bradshaw, 1997). Studies of the physiological aspects of growth and development of *Grevillea exul* var. rubiginosa are important in order to provide the information for proper revegetation procedures. In order to fully understand the biochemical processes in detail at the species level, a great deal of time and dedication is necessary.

Literature reviewed to date have not defined *Grevillea exul* as either a tolerant or hyper accumulator since it is a new endemic plant that is being used to restore mine sites. Hence the work conducted with this species is particularly important and data obtained significant. The roles of nickel in plant metabolism remain mostly unknown. The broad range of effects attribute to a nickel deficiency suggests that it maybe involved in several physiological processes (Brown *et al*., 1987). Most literature cited concludes that 5 ppm dose of nickel stimulate root and shoot elongation whereas a high concentration significantly reduce the ability of the seeds to germinate and grow.

Therefore the aims and objectives of the present investigation were:

1. To study the effects of high concentrations of nickel in the first two stages of plant life cycle that is germination and post germination of *Grevillea exul* var. rubiginosa
2. To determine the response of different salts of nickel on the species at different concentrations.
3. Determining suitable biochemical analysis methodology for total protein content in *Grevillea exul* var. rubiginosa.
4. To study the root colonization of *Grevillea exul* var. rubiginosa using minirhizotrons with the two main soil types, present in mining sites selected for revegetation.

CHAPTER 2

2.0 LITERATURE

Because of the confusing and often contradictory nature of studies on the serpentine factor, it is very difficult to assess the evidence and draw conclusions in a logical and straightforward manner (Brooks, 1987). This is a very common problem that is faced by many researchers and scientists who begin work with serpentine soils and its vegetation. Once a scientist claims to have solved a problem or even part of the problem, only to have their claims contradicted from another by fresh evidence. Most experiments conducted are designed with one or two variables while keeping all others constant, so when results are obtained there is a tendency to assume that these variables alone are the components of the serpentine factor. Hence it would be appropriate to consider several variables simultaneously, more closely approximating field conditions but interpretation of these data are virtually impossible. This has led to scientists tackling the serpentine factor and revegetation studies with one or two variables only. Brooks (1987) discussed the infertility of serpentine soils under five main headings:

1. the toxic effects of nickel, chromium and cobalt;
2. the toxicity of excess magnesium;
3. infertility due to the low calcium content,
4. problems arising from an adverse calcium/magnesium quotient in the substrate; and
5. infertility arising from low levels of plant nutrients.

This chapter focuses on the literature related to toxic effects of nickel and infertility arising from low levels of plant nutrients in serpentine soils. Concentrations of nickel in plants vary with plant species and soil types. Jaffre *et al*., (1979) reported that 18 hyperaccumulators were found only in New Caledonia. These include the following species: *Homalium, Hybanthus, Geissois, Psychotria* and *Sebertia.* In fact two types of plants occur on serpentine soils, generally the xerophytic shrub that are supported by serpentine soils are termed “maquis minier”. These include metal tolerant and hyperaccumulators. A plant is defined as a nickel hyperaccumulator when any part

above ground contains more than 1000 μg/g (0.1%) in a range of 1000 – 5000 μg/g (0.1 – 0.5%) nickel dry weight (Reeves 1992, Brooks *et al*., 1977_a, Kersten *et al*., 1979, Jaffre *et al*., 1979). Normally nickel content of plants is less than 1 μg/g (dry mass), significantly higher levels 10 – 100 μg/g are found in serpentine floras (Jaffre *et al*., 1979).

Most authors have studied the toxic effect of nickel and other heavy metals (Zn, Cu, Cd and Pb) in leafy vegetables, cereal crops and legumes due to the growth of these plants in contaminated sites and their essentiality as food sources. Brief reviews of some of the work research conducted are stated below.

Piccini and Malavolta (1992) worked with two common bean cultivars (*Phaseolus vulgaris L*.) to study the effects of nickel. They concluded that both cultivars developed nickel toxicity symptoms in roots, shoots and especially in leaves.

Investigations on the effects of nickel on two maize (*Zea mays*) cultivars related to growth, structure, nickel concentration and localization were conducted by L'Huillier *et al*., (1996) who found that nickel – induced reduction of growth was mainly the consequence of depressed mitotic cell division in the root meristem. Two non-exclusive mechanisms were proposed for this, firstly the direct toxic effect of nickel on the root meristem and secondly reduced carbohydrate supply to roots.

According to Parida *et al*., (2003) who conducted greenhouse experiments with fenugreek (*Trigonella corniculata L*.) found that growth and development of crops grown with 10 and 20 mg Ni/kg soil was normal and slightly better than those grown in pots without nickel. However growth of the crop declined above 20 mg Ni/kg soil and at levels $\geq$ 40 mg/kg soil. nickel toxicity was evident as interveinal yellowing followed by general chlorosis of younger leaves resembling Iron (Fe) deficiency. The affected plants showed highly stunted growth and reduced branching, at very high levels of nickel application even the germinated seedlings failed to grow. They also noted serious restricted root growth in plants suffering from nickel toxicity.

These effects on plant roots support the fact that high concentrations of nickel and other heavy metals have toxic effects on the roots before any other part of the plant is affected. Translocation to other plant parts determines how and where nickel is transported and the concentrations in which they are carried before toxicity symptoms would be observed. Usually nickel concentrations are highest in roots with stems and leaves following in that order depending on species.

Though it is quite obvious that different plant species show characteristic toxicity symptoms yet the general nickel phytotoxicity guidelines can be considered as general. These guidelines are:

- chlorosis,
- reduced plant growth,
- low yield depression and
- disorder in plant metabolism (Yang *et al*., 1996_a).

Crooke (1956) while working with oats (*Avena sativa*) demonstrated that increasing soil pH by applying lime decreased the amount of extractable nickel from soil, and the amount present in oat tissues. For example results showed that increasing soil pH from 5.2 to 7.1 reduced nickel in oat foliage from 144 ppm to 40 ppm. Fertilizer application also tended to reduce the amount of nickel uptake at lower pH. The study indicated that much of the nickel is attached to the soil organic matter, although it is freely available to plants. This observation was supported by Soon and Bates (1982) who also found that soil nickel was extractable by acids and that half was complexed with organic matter.

McCoy *et al*. (2003) in their report presented data on relative growth rate and maximum height of exotic and endemic plant species planted on different laterite substrates with NPK amendment at establishment phase. *Grevillea exul* var. rubiginosa showed a significant increase in growth rate and height in a mixture of red laterite and ironcap substrate. Whereas no significant differences were observed in height for ironcap,

yellow laterite and weathered gabbro substrates. Though it was noticed that ironcap reduced growth rate dramatically. The following confirm the importance of substrate and species in revegetation without eliminating the substrate amendment wherever applied. McCoy *et al.* (2003) also stated that endemic species were genetically predisposed to slow growth even when the species were grown on fertile soil. However endemic plant species are used for revegetation of mine sites since they have the adaptability characteristics to grow and survive in infertile soil conditions and this is mainly due to a long evolutionary process.

Eskew *et al*. (1983) demonstrated through their work on soyabean (*Glycine max* L. Merr.) plants deprived of nickel accumulated toxic concentrations of urea (2.5%) in necrotic lesions in their leaflet tips. This occurred regardless of whether the plants were supplied with inorganic nitrogen or were dependent on nitrogen fixation. Nickel deprivation resulted in delayed nodulation and reduction in early growth, whereas addition of nickel (1 µg/l) to the nutrient media prevented urea accumulation, necrosis and growth reductions. According to their results urea is produced under normal nitrogen metabolism in higher plants, and that nickel as a component of the enzyme urease is required to prevent the accumulation of toxic concentrations of urea.

Studies carried out by Sen and Bhattacharyya (1994) on the uptake and toxic effects of nickel ions on *Salvinia natans,* showed that nickel ions promoted senescence of *Salvinia* plants by general inhibition of biosynthesis of cellular metabolites, impairing the degradation of the biochemical processes and also interfering with photosynthesis and protein synthesizing machinery in the plant. Nickel ions were found to be toxic to *Salvinia* plants at most of the morphological, physiological and biochemical levels.

Investigations conducted by Chiarucci *et al*. (1998) on the effects of nutrient addition on species diversity and ground cover of “serpentine vegetation” demonstrated that many of the species already present in the experimental plots increased in ground cover after fertilization (N, P, K & Ca) but there was no significant colonization by other species. Interesting observations in their experiment were that when plots were supplemented

with N, P, K and Ca, there was a reduction in nickel and calcium availability. However, the response of different vegetation (plant species) was different.

Proctor and Nagy (1992) in a review on ultramafic rocks and their vegetation suggested that water and nutritional stress were probably more important than metal availability as factors contributing to the infertility of ultramafic soils. A similar conclusion that is metal toxicity cannot be regarded as the main limiting factor for vegetation on ultramafic soils was noted by Chiarucci *et al*. (1998). However the fact that species composition was not significantly altered by fertilizer application suggested that the species growing in the fertilized plots were probably adapted to a high metal content.

Kevresan *et al*. (2001) carried out a comparative study on the effect of Mo, Ni, Cd and Pb on the metabolism of nitrogen and proteins in young pea (*Pisum sativum*) plants. At low concentrations of nickel (0.01 ppm) a stimulative effect on the growth of shoots and roots was observed. As reported by Brown *et al*. (1987) and Petrovic & Kastori (1994) lower concentrations on Ni in the nutrient medium had a favorable effect on the growth of a number of plant species, whereas in the excess it could completely inhibit plant growth (Sheoran *et al*. 1990). Nitrate content, nitrate reductase activity, glutamine synthetase activity and total soluble protein decreased with increasing concentrations of nickel in the medium for pea plants.

Effects of Copper and nickel on nitrate reductase, urease and glutamine synthetase of two strains of *Bradyrhizobium* sp., *Cajanus cajan* (pigeon pea) and *Vigna radiata* (mungbean) were investigated by Singh (2002). Addition of 1 μg/l of Ni^{2+} to the nutrient medium enhanced the activity of glutamine synthetase (GS) and urease but strongly inhibited nitrate uptake and nitrate reductase. On the basis of the results obtained, by Singh (2002) it was concluded that incorporation of traces of Ni^{2+} will support the growth and production of legumes through the enhancement of the activity of enzymes directly or indirectly involved in symbiotic nitrogen fixation.

Kevresan *et al*. (1998) investigated changes in nitrate and protein metabolisms in sugar beet plants (*Beta vulgaris* L., hybrid NS Hy-11) caused by Ni, Cd and Mo present in 3 different concentrations. Nitrate and protein contents, glutamine synthetase and nitrate reductase activities decreased with increasing concentrations of nickel. Results suggest that the different effects of a particular heavy metal on plant growth are usually observed at high concentrations.

Changes in chloroplast ultra structure and total chlorophyll concentration in cabbage leaves caused by excess of organic nickel (II) complexes were examined by Molas (2002) who showed that phytotoxicity of nickel was apparent from reduction of leaf chlorophyll concentration and damage of chloroplasts depended on the form in which the metal occurred in the plant root environment. Differences in the toxic influence of the examined organic nickel (II) complexes on photosynthetic apparatus of cabbage plants resulted from different concentrations of nickel in leaves and its accumulation in chloroplasts. However, the differences in the bioaccumulation of nickel supplied from the examined nickel complexes depend on the nature of chelating agents. Nickel supplied as Ni (II)-EDTA was the least toxic and was accumulated in leaves and in chloroplasts in the smallest amounts. The toxicity of Ni (II)-Glu and Ni (II)-citrate was similar, but it was considerably higher than the toxicity of Ni (II)-EDTA.

Peralta *et al*. (2001) reported in their study on the uptake and effects of five heavy metals on seed germination and plant growth in Alfalfa (*Medicago sativa* L.) that high concentrations of nickel ions (40 ppm) had a detrimental effect on germination rate, root and shoot length. Claire *et al.* (1991) obtained similar results in a study with nickel, whereas concentrations of 5 ppm nickel ion increased root and shoot length.

Effects of nickel and copper ions on bilberry (*Vaccinium myrtillys* L.) seed germination and seedling development were investigated by Lyanguzova (1999), which showed that both the type and concentration of heavy metals affected seed germination and retardation of seedling development.

Rtout *et al*., (2000) investigated the effects of Cr and Ni on germination and growth in tolerant and non-tolerant populations of *Echinochloa colona* (L.) Link. Seed-based experiments indicated that the populations growing naturally on uncontaminated sites, germinated better in nutrient solutions without metal than those collected from mine waste dumps. Metal tolerance indices were greater in the plant populations derived from metal contaminated sites and better growth of these plants was noted on mine spoil-soil mix in the ratio of 1:1. The percentage of seed germination and the rate of seedling growth however declined in a soil compost containing 25% mine soil and 75% uncontaminated (control) soil. Populations of *Echinochloa colona* occurring naturally on chromite mine soils, therefore appear to have developed metal tolerance, which according to Rtout *et al*., (2000) is maintained by a balance and stable genetic system built up and adjusted by natural selection.

CHAPTER 3

3.0 MATERIALS AND METHOD

Grevillea exul var. rubiginosa is a woody species and belongs to the family Proteceae, common in New Caledonia. It produces fruits once a year during the summer months (October – March), fruits have a hard covering that protect the two seeds inside (figure 3.1). For the present investigation fruits were collected from the Southern province in February 2003 and left under artificial light to dry until the seeds inside were released (~1 week). Seeds were stored in airtight storage bottles at -4°C for later use.

3.1 Seed Viability Test

Under sterile conditions in the laminar flow cabinet, *Grevillea exul* var. rubiginosa seeds were washed with 4% sodium hypo chlorite solution for 30 minutes once, then four times with sterile water for 5 minutes each and left in 1% tetrazolium chloride for 48 hours. After 48 hours the seeds were halved and paced in 8 mm Petri dishes lined with 3 layers of Whatman filter paper containing 8 ml of 1% tetrazolium chloride as seen in figure 3.2. The seeds color change was noted after 48 hours. Viable seeds were noted with a color change to violet.

3.2 Controlled Germination

To determine seed germination percentage, *Grevillea exul* var. rubiginosa seeds were washed with 4% sodium hypochlorite solution for 30 minutes once, then four times for 5 minutes each with sterile water before being sowed in 8 mm Petri dishes lined with 3 layers of Whatman filter paper moistened with 8 ml of nickel solutions (0 – 500 ppm concentrations). All Petri dishes were sealed once they were set up. Three salts of nickel were used: nickel acetate, nickel chloride and nickel sulphate. Sterile conditions were maintained at all times to minimize the risks of contamination by working in the laminar flow.

Germination was allowed to occur in a dark incubator at 30°C as seen in figure 3.3, the following germination conditions were maintained: a temperature of 30°C, darkness, 1ml of nutrient solution per cm Petri dish, because they were the most appropriate for the species according to Léon (personal communication). Each concentration of nickel for the 3 salts (acetate, chloride and sulphate) had 4 replicates and the experiment was carried out twice to confirm the results obtained. Number of germinated seeds and root lengths were recorded twice a week until the germination rate became constant (~1 month). The seedlings were then washed with sterile water to remove excess nickel, frozen in liquid nitrogen and stored at -18°C; to be used for further biochemical analysis, which included nitrate, protein and glucid contents, and enzyme analysis which included glutamine synthetase and nitrate reductase activities.

3.3 Semi – Controlled Germination and Treatment

The *Grevillea exul* var. rubiginosa seeds were sowed in pots containing organic matter (potting mix from Dalton's gardening products) and vermiculate[1] in 1:1 ratio with water retention capacity of 52.48 ml/g. Seeds were sowed at 1 seed per square pot of 15 cm sides in the greenhouse and watered once a day. Each treatment (0, 5, 10, 50, 100 and 500 ppm concentrations) had 12 replicates and the experiment was conducted once due to time constraints. Two months later the plantlets were selected for treatment according to the number of leaves and length of stem. Each pot was treated manually with nickel chloride solutions 0, 5, 10, 50, 100 and 500 ppm concentrations of nickel once a week, in addition to automatic watering continued throughout the week for 3 min/day. After three months of treatment the morphological characteristics of the plants were recorded. These included number of leaves, stem and root lengths, root, leaves and stem fresh weights. The various parts of the plants were frozen in liquid nitrogen and preserved at -18°C for further biochemical analysis, which included nitrate, protein, glucid, glutamine synthetase, and nitrate reductase activity.

Figure 3.1 *Grevillea exul* var. rubiginosa fruit and seeds

Figure 3.2 Seed viability test setup for *Grevillea exul* var. rubiginosa

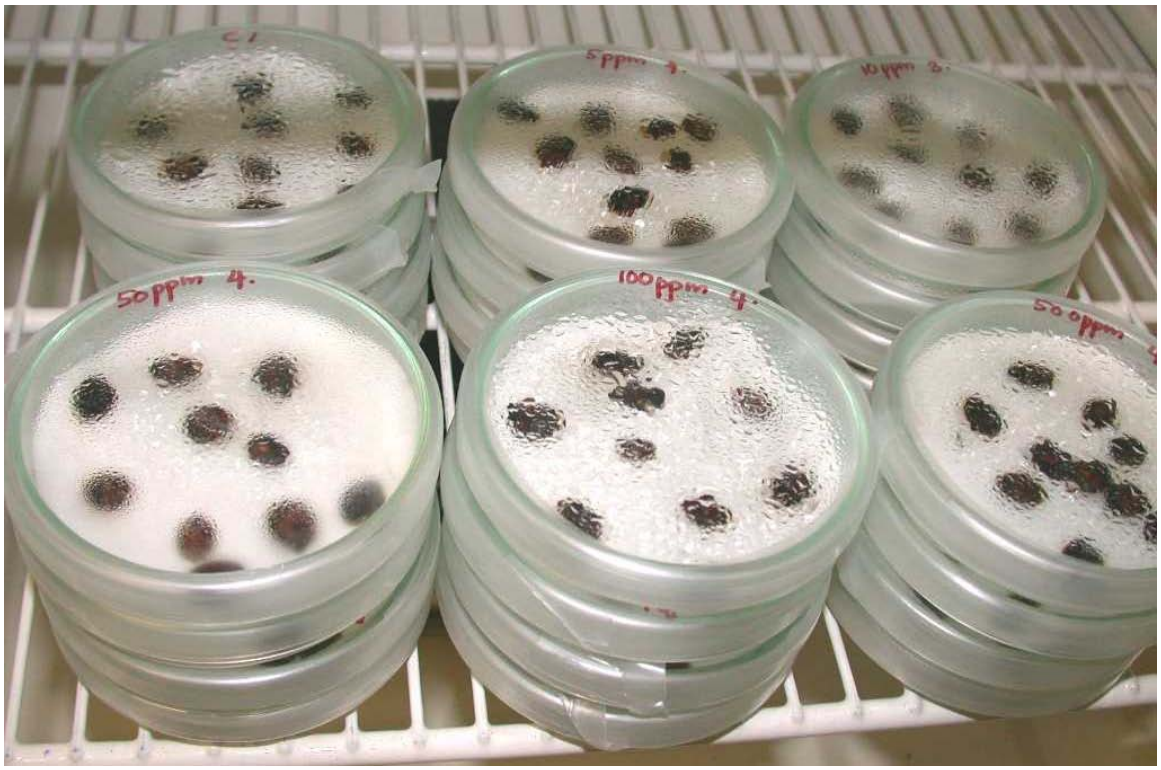

Figure 3.3 *Grevillea exul* var. rubiginosa seeds under controlled conditions.

Figure 3.4 Plantlets of *Grevillea exul* var. rubiginosa setup in the greenhouse for treatment with nickel chloride (semi – controlled conditions).

3.4 Aqueous Extraction

The seedlings were ground in pre-chilled motar and pestle with liquid nitrogen and transferred to falcon bottles. These ground samples were freeze dried in a lyophilisator for 48 hours below -55°C at 1 atmosphere. Cool and sterile conditions were maintained at all times.

To 200 mg of frozen dried sample, 10 ml of sterile water was added in centrifuge tubes and incubated for one hour at 45°C in a water bath. The samples were agitated in the agitator (Agitest® 86212) at a speed of 7/sec, after which they were centrifuged for 15 minutes at 5000 rev/min at 25°C. Supernatants were collected and stored at -18°C for nitrate, protein and glucid analysis.

Each treatment (nickel concentrations 0, 5, 10, 50, 100, 500 ppm) had three replicates for the following biochemical and enzyme analysis: nitrate, total soluble protein, total soluble glucid, glutamine synthetase and nitrate reductase activities. And each analysis was repeated twice.

3.4.1 Nitrate Analysis.

Nitrate analysis was carried our as per method described by Cataldo *et al*. (1975) using 800 µl of 5% salicylic acid (5 g salicylic acid in 100 ml 95% sulphuric acid) was carefully added to 200 µl of aqueous sample in Pyrex tubes. The tubes were allowed to cool to room temperature (~20 minutes), to which 19 ml, of 2 M sodium hydroxide was added. Once the temperatures of the tubes were similar to room temperature, absorbance was recorded at 410 nm. Potassium nitrate was used as reference (0.1 – 15 mM).

3.4.2 Total Soluble Protein Analysis (non purified extracts):

Total soluble protein was analyzed using the method described by Lowry *et al*. (1951). To 100 µl of aqueous sample, 5 ml of reagent was added. Reagent was prepared by mixing 50 ml of solution A (2% sodium carbonate in 0.01 M sodium hydroxide) and 1ml of solution B (0.5% sodium potassium tartrate). Solution A and B were mixed just before use. The tubes were agitated and left for a minimum of 10 minutes at room temperature. Diluted 0.5ml (X2) Folin Reagent was added and agitated immediately (within a couple of seconds). The tubes were allowed to stand for 30 minutes in darkness. Sample absorbances were read spectrophotometically at 750 nm. Bovine Serum Albumin (BSA) 0.35 mg/ml (0.1 – 1.0 ml) were used as references.

3.4.3 Total soluble Glucid Analysis:

The method used to analyze total soluble glucid was as follows. Sulphuric acid 70% was prepared at least 12 hours before utilization. Anthrone reagent was prepared on the day of experimentation with 0.092% thiourea and anthrone in 70% sulphuric acid. In Pyrex tubes 50 – 100 µl of aqueous samples were diluted with sterile water to give a total volume of 200 µl. Tubes were immediately agitated as soon as 2 ml of anthrone reagent was added. All tubes were capped with marbles and placed in 95°C water bath for exactly 10 minutes, after which the tubes were immediately immersed in ice water bath for 10 minutes. Absorbances were recorded at 635 nm. Dextrose ± glucose 1% was used as reference (0.5 – 12 mM).

3.5 Extraction of Proteins

Fresh plant materials were ground in pre-chilled motar and pestle with liquid nitrogen under cool conditions. The ground fresh material was transferred to centrifuge tube to which was added imidazole buffer[2] (0.2 M, pH 7.2) with 5 mM Na_2-EDTA and 1 mM

magnesium chloride supplemented with 1 mM DTT and 10 mM mercapto-2-ethanol (5 ml of buffer per gram of fresh material). With the aid of a polytron fine mixer, the solution was homogenized 3 times for 5 seconds each at high speed. The mixture was filtered using a gluter net (36 µm) and centrifuged at 47000 g for 30 minutes at 4°C. Supernatant was removed and stored at -18°C in hemolyse tubes for enzyme analysis.

3.6 Isolating Polyphenols From Protein Extracts

Polyvinyl poly pyrrolidone (0.1 g/g of fresh material) was used to purify the protein extracts obtained after centrifugation at 47000 g for 30 minutes. The supernatant was collected for glutamine synthetase and nitrate reductase analysis. In order to test the presence of large organic molecules such as poly-phenols, a few drops of $FeCl_{3(aq)}$ was added to the supernatant and precipitated. Colour change to violet indicated a positive change that is the presence of organic molecules.

3.7 Enzyme Assays: Total soluble protein analysis of extractions utilized for enzyme analysis was adapted and modified from Migg *et al*., (1997).

3.7.1 Glutamine Synthetase Assay

The reaction mixture contained 100 µl imidazole buffer (450 mM, pH 7.2), 100 µl magnesium chloride (450 mM), 100 µl hydroxylamine (60 mM), 100 µl Na-ATP[3] (80 mM) and 150 µl of sterile water. The reaction was initiated by adding 200 µl protein extract and terminated after incubation for 15 minutes at 30°C by adding 850 µl of stop solution containing 0.37 M Ferric Chloride[4], 0.2 M trichloroacetic acid and 0.67 N HCl. According to Wallsgrove *et al.,* (1979) hydroxylamine replaced the physiological substrate NH_4.

3.7.2 Nitrate Reductase Activity

Nitrate reductase activity was calculated from the increase of nitrite during the assay; the amount of nitrite was determined colorimetrically at 540 nm after azocoupling with sulphanilamide and napthyl ethylenediamine dihydrochloride. The reaction mixture contained 125 µl potassium phosphate (100 mM, pH 7.5), 500 µl potassium nitrate (100 mM), 125 µl α NADH (10 mg/ml), and 50 µl sterile water. The reaction was initiated by adding 200 µl protein extract and terminated by adding 250 µl sulphanilamide (1% w/v in 1.5 N HCl) and 250 µl naphthyl-ethylene diamine dihydrochloride (0.02% w/v) after 15-minute incubation at 30°C.

3.8 Mineral Analysis

To the Digesdahl Hach (model 23130-21) apparatus, 300 mg of freeze-dried plant material was deposited. Five milliliters of concentrated sulphuric acid (95 - 98%) was added and the temperature set to 440°C. Once the vacuum was set and refluxing of H_2SO_4 was visible, the sample was digested for 5 minutes, after which 17 ml of 30% hydrogen peroxide (H_2O_2) was added to the charred sample. Heating for another 5 minutes after addition of H_2O_2 completely boiled off excess H_2O_2. The sample was then cooled; 5ml of distilled water added and the system heated for 15 minutes at 204°C. The samples were diluted to 25 ml in volumetric flasks and analyzed by the Laboratory of Analytical Chemistry of the Institute of Research and Development in Noumea to determine concentrations of Ca, Mg, Na, K, P, Co, Fe, Mn, Ni, Al, Cu and Zn using the ICP plasma spectrophotometer (Perkin Elmer 3300 DV)

3.9 Ammonium Analysis from Mineralized Samples

To 1ml of mineralized samples in hemolyse tubes, 500 µl of Nessler reactive and 2 ml of sterile water was added to complete the reaction. Samples were homogenized and

absorbance recorded at 450 nm spectrophotometically after 15 minutes. Ammonium oxalate (0.0142% w/v in 30 mM H_2SO_4) was utilized as reference (0.1 –1 mM).

3.10 Minirhizotron Root Development

Minirhizotrons were used to study the characteristic distribution and colonization of one-year *Grevillea exul* plants in the two main types of soils found in mining sites in New Caledonia. Before considering the plant species it was important to study the physical characteristics of the soil types for revegetation of mining sites. Use of local pioneer plant species is one of the best strategies for revegetation of mine sites in New Caledonia. Mainly because these plants are naturally adapted to edaphic conditions of the mine sites, it is the long evolutionary process that has helped the plants to adapt to the phyto-toxicity of the soils and their low nutrient content.

The experiment was conducted in the greenhouse at the University of New Caledonia during the months of April to September 2003 in minirhizotrons 72 cm x 84 cm with individual compartments 72 cm x 28 cm each. Both soil types red laterite and saprolite were sieved through a 3 mm sieve including the organic matter it contains. Compartments I and II of both minirhizotrons were filled with red laterite and saprolite soils respectively whereas compartment III of both minirhizotrons were filled with a mixture of saprolite/red laterite plus organic matter in 2:1 ratio. Physical characteristics such as pH, conductivity and water retention capacity of both soil types and amendments were determined. Water was added to all compartments and the rate at which the soil types absorbed water was noted. Once the morphological characteristics of one-year-old plants were noted they were transferred into compartments II and III of both minirhizotrons where compartment I was kept as control. All compartments were watered twice a week and the root colonization followed for each compartment. Table 3.1 shows soil composition and plant characteristics before transplant

Table 3.1 Characteristics of substrate and plants before transplant

	Soil characteristics		Plant morphological characteristics	
Substrate	**pH**	**Conductivity**	**Number of leaves**	**Length of stem (cm)**
Laterite	6.62	0.15	14	19
laterite + organic	6.36	0.475	16	13.5
Saprolite	6.99	0.125	19	10.5
Saprolite + organic	6.61	0.71	19	14

3.11 Potential Hydrogen and Conductivity

3.11.1 Potential Hydrogen with Water

Twenty-five milliliters of distilled water was added to 10 g of soil sample (dried for a minimum of 48 hrs at 100°C) and agitated for 10 minutes, then filtered with a Buchnner funnel. The pH electrode (pH meter CG 840) was dipped into the solution and pH recorded once the solution environment stabilized (~10mins).

3.11.2.1 Potential Hydrogen with KCl

One molar KCl was used rather than distilled water and the method for pH determination with water was followed except solution environment stabilized after 1 minute. Estimated pH was determined using the following equation:

$$\Delta pH = pH_{water} - pH_{KCl}$$

3.11.2.2 Conductivity

Using the solutions prepared to determine pH with water, conductivity was measured by dipping the conductimeter (Consort K810, K=0.99) electrode in the solution until the solution environment stabilized.

Figure 3.3 Minirhizotron showing *Grevillea exul* var. rubiginosa plant in red laterite soil before treatment.

Figure 3.4 Minirhizotron showing *Grevillea exul* var. rubiginosa plant in saprolite soil before treatment.

3.12 Statistical Analysis

Germination rate (%) for each treatment was calculated as follows:

(no. of germinated seeds/total no. of seeds) x 100

Germination rate and root lengths were graphed to determine if a correlation exist between the two factors.

Seed viability test was conducted only once since the seeds utilized in the research were from the same season and site. Percentage viability was calculated as follows:

(no. of viable seeds/total seeds) X 100

Nitrate content per treatment was calculated as follows:

(Av. DO/m) x total sample vol. = KNO_3 in all the sample

where:

m = slope of reference graph

Av. DO = average optical density of samples

Once KNO_3 was calculated in terms of per gram of dry material, nitrate was then determined as 61.38% of KNO_3 . All results were expressed as mg/g DM.

Protein content was expressed as protein content equivalent to mg albumin bovine serum per gram of dry material, since protein content is determined from Bovine Serum Albumine.

Glucid content was expressed as content equivalent to mg D ± glucose/g DM.

In all three cases the volume of total analysis sample, mass of dry plant material and volume of water used to prepare aqueous extract were important factors in calculating and expressing total nitrate, protein and glucid contents.

Glutamine synthetase and nitrate reductase activities were expressed as DO/g of protein and were calculated as percent protein content.

Graphpad prism 4 statistical package and Microsoft excel programmes were used for data analysis.

[1] Vermiculite is a silicate material of the mica family. It expands on heating to produce a lightweight product that has high water retention.
[2] Imidazole buffer was decided since best activities were obtained, when compared to other commonly used buffers such as tris and phosphate that might be suitable for other plant species.
[3] Na-ATP stored at -4˚C and kept cool during experimentation.
[4] $FeCl_{(aq)}$ was used rather than powder since it was not practical to dissolve powdered $FeCl_3$ even with the aid of 100% methanol and ethanol.

CHAPTER 4

4.0 RESULTS

Results obtained on seed germination, root : shoot growth and development and various other responses by New Caledonian nickel tolerant endemic plant *Grevillea exul* var. rubiginosa are presented in this chapter. The results obtained are very interesting and informative.

4.1 SEED VIABILITY

Testing for seed viability is an important aspect of seed germination, root: shoot growth and development. To determine the seed viability of *Grevillea exul* var. rubiginosa 1% tetrazolium chloride was used in the experiment. Seed viability varied between 90 – 100% depending upon the season of seed collection. Seeds collected in the season had gone into a state of dormancy since they did not germinate under normal germination conditions. Ongoing season seeds were the ones that were collected from the plants.

Only two replicates were set with a total of 20 seeds per replicate to test the viability of *Grevillea exul* var. rubiginosa due to the loss of seeds during a cyclone that season, hence minimizing the number of seeds and replicates. Results obtained are presented in Table 4.1.

Table 4.1 *Grevillea exul* var. rubiginosa seed percentage viability

Set	No. of viable seeds	% Viability
1	18	90
2	20	100

Figure 4.1 Viable seeds of *Grevillea exul* var. rubiginosa

4.2 CONTROLLED GERMINATION

4.2.1 Germination Rate and Root Lengths

Germination rates (GR) and root lengths (RL) were the two major indicators of the environment and nutrient effect on seeds during germination. Nickel chloride showed the usual response of high concentrations of nickel to *Grevillea exul* var. rubiginosa. The response being positive at 0 ppm, 5 ppm and 10 ppm for GR as well as RL, whereas decrease and finally inhibitory effect sets in with increasing concentrations of nickel chloride 50 ppm, 100 ppm and 500 ppm. Germination rates for nickel acetate were highest at 50 ppm, 5 ppm than 10 ppm with 100 ppm having the lowest GR. Nickel chloride and sulphate followed a similar trend with the highest GR at 5 ppm and 10 ppm then decreased dramatically at 50, 100 and 500 ppm for nickel chloride. Whereas for nickel sulphate GR remains constant for 5, 10 and 50 ppm but decreased at 100 ppm and the lowest GR was recorded in the highest (500 ppm) concentration of

nickel as indicated in Figure 4.2. At 50 and 100 ppm nickel acetate concentrations a significant increase in root lengths was noted as seen in Figure 4.3 as compared to 0, 5 and 10 ppm concentrations.

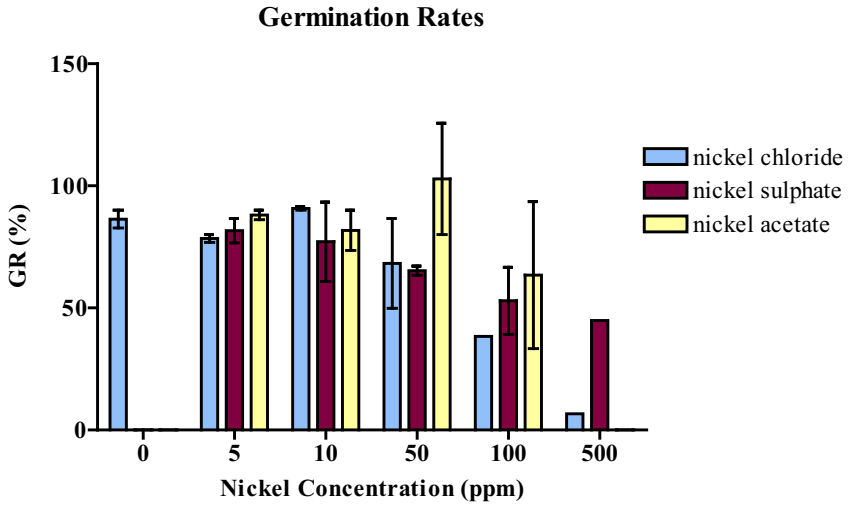

Figure 4.2 Seedling germination percentage of *Grevillea exul* var. rubiginosa in 3 nickel salt concentrations. Each treatment had 3 replicates and was repeated twice.

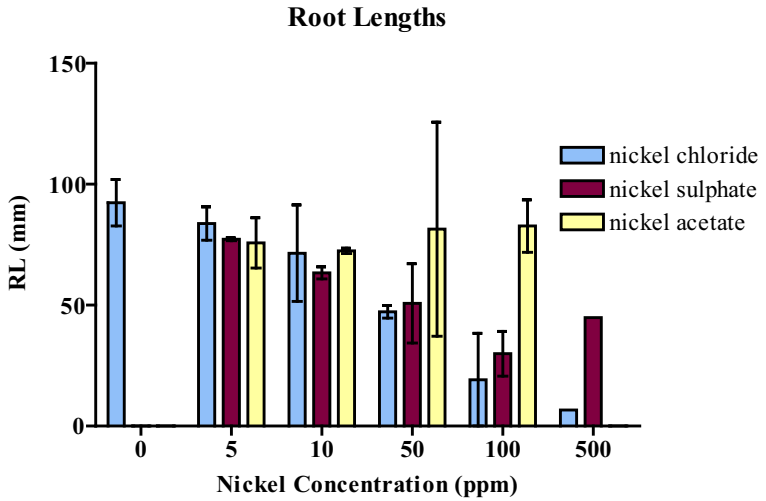

Figure 4.3 Seedling root lengths of 3 *Grevillea exul* var. rubiginosa in 3 nickel salts concentrations. Each treatment had 3 replicates and was repeated twice.

4.2.1.1 Nickel chloride

For nickel chloride the rate of germination over time (0 – 44 days) was highest in 5 ppm increasing gradually reaching a maximum of 80% on the 28th day and remained constant until treatment was terminated. A similar pattern was observed in 0 ppm and 10 ppm with 10 ppm having a higher GR which became constant at 66% on the 24th day and finally increased to 80% on the final (44th) day of observation as seen in Figure 4.3. For control (0 ppm) GR gradually increased reaching 73.3% on the final day (44th) of recording. At 50 ppm and higher concentrations the rate of germination decreased rapidly with 500 ppm concentration of nickel inhibiting germination almost completely. A similar pattern was observed for root lengths over time with 5 ppm of nickel, which had the longest roots followed by 10 ppm and control until the 32nd day. Treatment with concentration of 10ppm nickel had the longest roots of 92 mm (Figure 4.5), followed by control (83 mm) and 5 ppm treatment (78 mm) on the last day of recording. Root lengths begun to decrease at 50 ppm, the lowest observed at 100 ppm. An inhibitory effect at 500 ppm concentration was recorded.

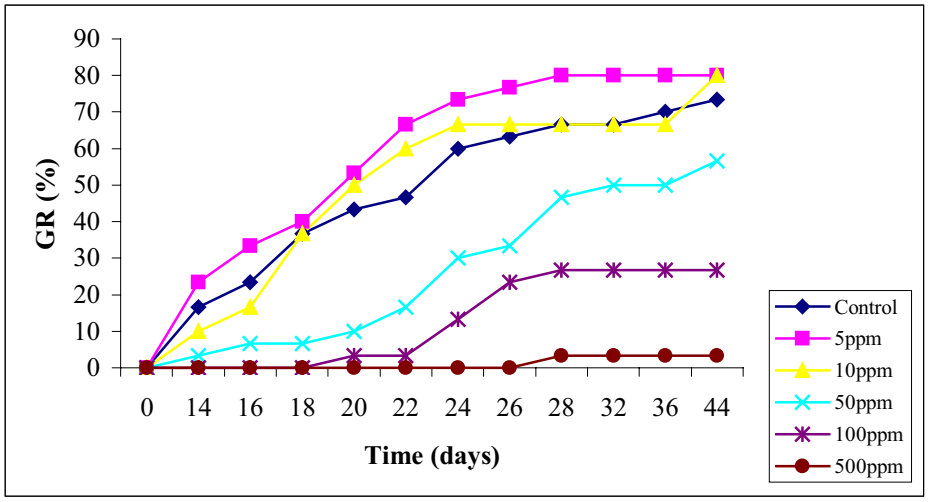

Figure 4.4 Seed Germination rate in nickel chloride germinated seedlings. Each treatment had 3 replicates and was repeated twice.

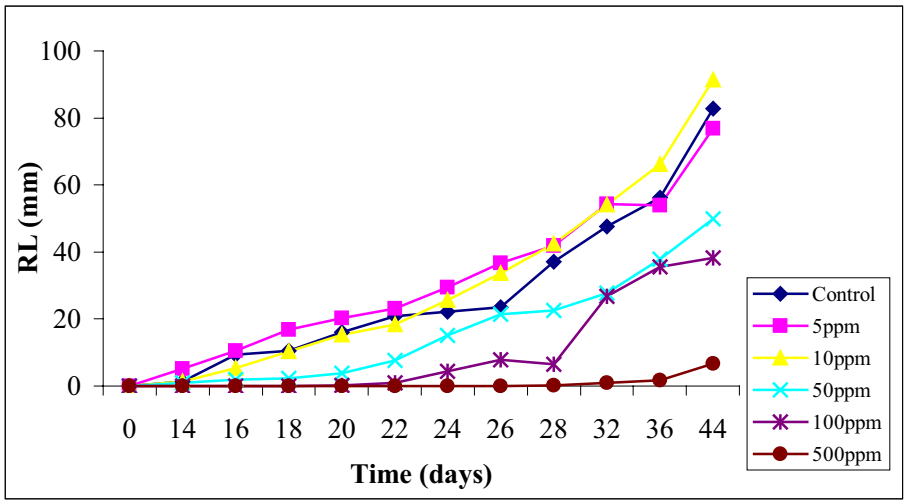

Figure 4.5 Root Lengths of germinated seedlings in nickel chloride. Each treatment had 3 replicates and was repeated twice.

4.2.1.2 Nickel sulphate

Nickel sulphate concentration on GR did not show differences between 0, 5, 10 and 50 ppm nickel concentrations but a decrease was noted for 100 and 500 ppm indicating the inhibitory effects of nickel at higher concentrations. As compared to control, GR was better in 5, 10, and 50 ppm after 28th day reaching a maximum 73.3% (Figure 4.6). The germination rate at 500 ppm in nickel sulphate showed lower inhibition rate as compared to nickel chloride. Germination in nickel sulphate begun after 16th day and continued to increase reaching a maximum of 23.3% on 44th (final) day. Germination in nickel chloride was delayed until 26th day, increased to 3.3% and remained constant until treatment was terminated.

Root length elongation showed similar trend as GR in 0, 5, 10, 50 and 100 ppm. Root length was almost same in all these concentrations until 20th day. Root lengths in 50

ppm nickel concentration begun to increase as compared with control and 10 ppm concentrations. Finally 50 ppm concentration treatment had longer roots on 32nd day as compared to 10 ppm. A similar pattern was recorded in 100 ppm and 500 ppm with the greatest inhibitory effect in 500 ppm nickel concentration. This inhibitory effect became less obvious by 32nd day when root lengths increased significantly in comparison to 100 ppm.

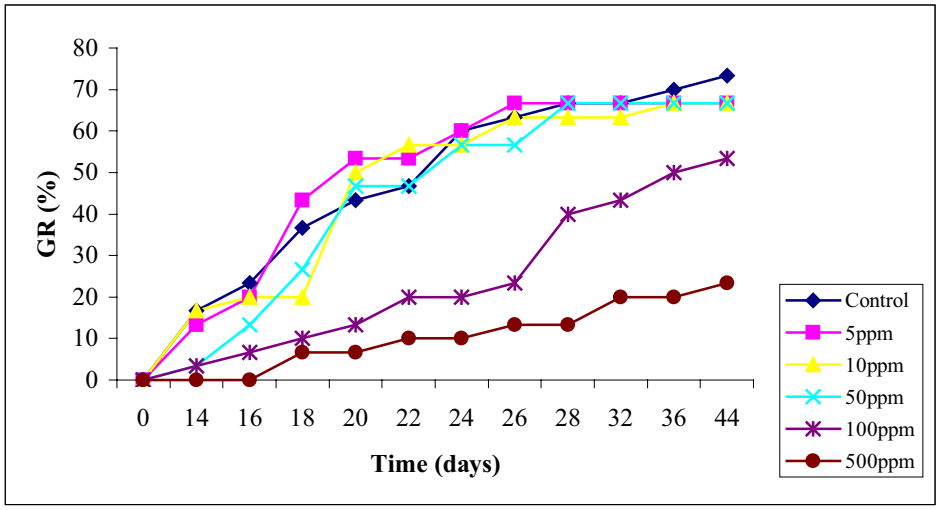

Figure 4.6 Seed germination rate in nickel sulphate germinated seedlings. Each treatment had 3 replicates and was repeated twice.

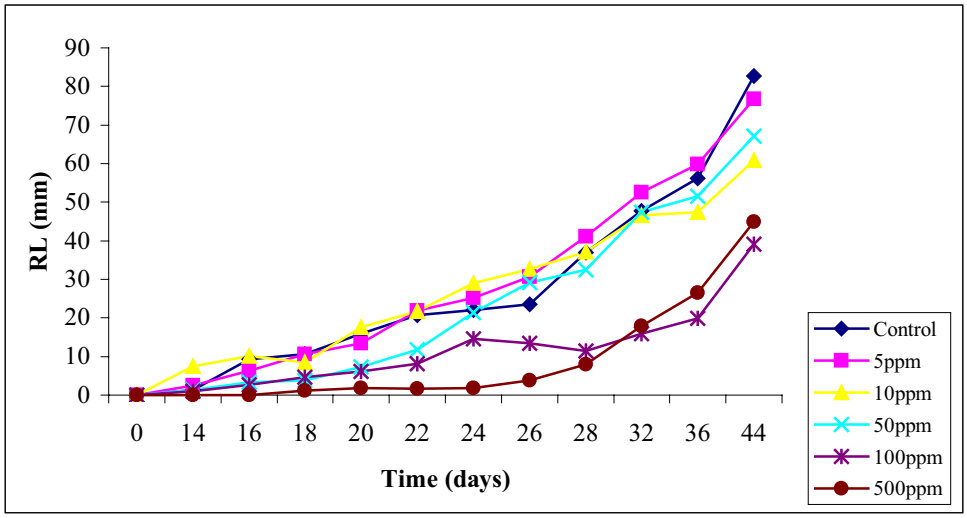

Figure 4.7 Root Lengths of germinated seedlings in nickel sulphate. Each treatment had 3 replicates and was repeated twice.

4.2.1.3 Nickel acetate

Nickel acetate was the nickel salt that showed inconsistent pattern as compared to nickel chloride and nickel sulphate. Nickel acetate concentrations of 5 ppm had the highest GR of 93.3% followed by 10 ppm at 90% as seen in figure 4.8. No significant differences were observed between 0 ppm and 50 ppm between 18 and 24 days. Initially control (0 ppm) had a higher GR until the 16th day after which GR in 50 ppm was better than control. After 24th day with a GR of 60%, a gradual increase was observed for control, which reached a maximum of 73.3%, and ended up being higher than in 50 ppm concentration. Germination rate in control (0 ppm) increased as compared to 50 ppm, which remained constant by 24 to 36 day then increased 10% as compared to control (0 ppm) by the final (44th) day. Germination rate in 100 ppm nickel acetate was similar but lower than 50 ppm concentration of nickel for 20th day after which GR increased by 10% on the 22nd day. There was no further increase till 24th day then increased to 80% by 28th day and then remained constant for the next 8 days and finally reached a maximum of 83.3%.

As seen in figure 4.8 root lengths followed a similar pattern (inconsistent) for all concentrations where 50 ppm nickel concentration showed enhanced root lengths from 22nd day onward compared to all other concentrations. Finally root length reached a maximum of 125.6 mm for 50 ppm nickel, which ended up being the longest roots for all compared concentrations of 3 nickel salts followed by 93.5 mm root length in 100 ppm nickel acetate.

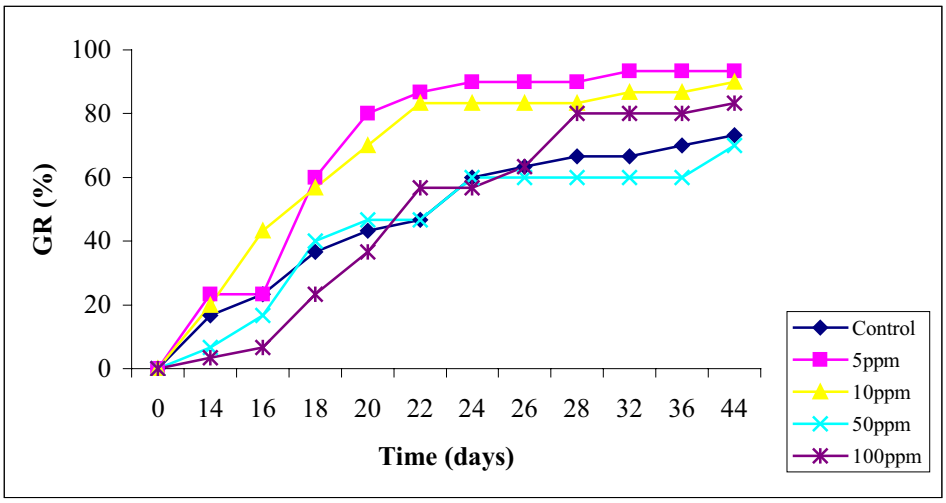

Figure 4.8 Seed germination rate in nickel acetate germinated seedlings. Each treatment had 3 replicates and was repeated twice.

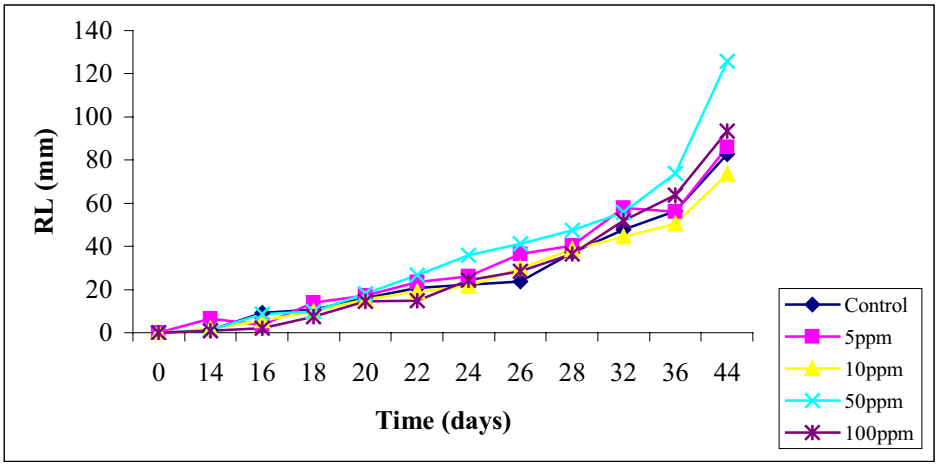

Figure 4.9 Root lengths of germinated seedlings in nickel acetate. Each treatment had 3 replicates and was repeated twice.

4.3 BIOCHEMICAL ANALYSIS

Biochemical analysis included total nitrate content, total soluble protein (non-purified extracts) and total soluble glucid analysis as well as glutamine synthetase (GS) and

nitrate reductase (NR) activities. Results obtained varied with the different salts of nickel and their concentrations.

4.3.1 Nickel chloride

Total soluble protein content (eq. mg BSA/g DM) were highest 27.4 protein eq. mg BSA/g DM in 5 ppm nickel chloride concentration but gradually decreased with increasing concentrations of salt. However, protein contents were higher than control (25.9 protein eq. mg BSA/g DM) in both 5 ppm and 10 ppm nickel chloride concentration but lower in 50 ppm (23.2 protein eq. mg BSA/g DM) and 10 ppm (27.4 protein eq. mg BSA/g DM) figure 4.10.

Nitrate content decreased with increasing concentrations of nickel chloride after 10 ppm, the highest nitrate content was 291.8 mg/g DM. At 5 ppm nickel chloride, nitrate content was 223.9 mg/g DM, 6.9 mg/g DM lower than control but higher than 50 ppm and 100 ppm nickel concentration treatment (figure 4.11).

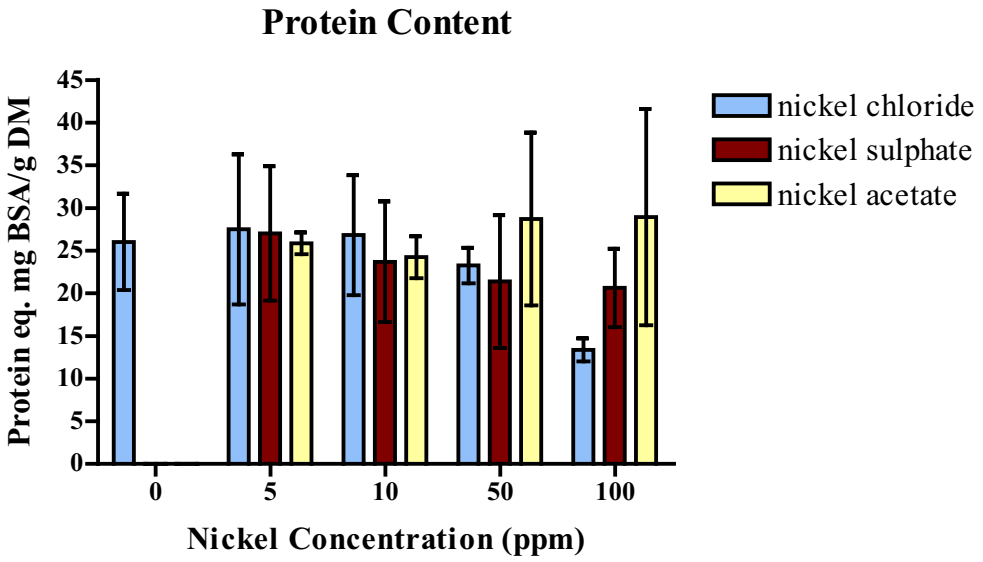

Figure 4.10 Total soluble protein content of seedlings germinated in the 3 nickel salts. Each analysis had 3 replicates and was repeated twice.

A very similar pattern was recorded for total soluble glucid contents, which begun to decrease with increasing concentrations of nickel from 10 ppm. Maximum total soluble glucid content of 751.5 glucid eq. D± glucose/g DM was obtained at 10 ppm but decreased only slightly (20.7 glucid eq. D± glucose/g DM) at 5 ppm. Total soluble glucid content decreased in 50 ppm and 100 ppm concentration by 25.4% and 55.9%. As seen in figure 4.11 control showed lower total soluble glucid contents than 10 ppm but higher than 5 ppm, 50 ppm and 100 ppm that is 746.3 glucid eq. D± glucose/g DM.

4.3.2 Nickel sulphate

Total soluble protein contents (eq. mg BSA/g DM) varied in different concentrations of nickel sulphate. As compared to protein contents in nickel chloride concentration of 5 ppm, protein contents were higher in 5 ppm nickel sulphate (26.9 eq. mg BSA/g DM) but lower in 10 ppm (23.6 eq. mg BSA/g DM), 50 ppm (21.3 eq. mg BSA/g DM) and 100 ppm (20.6 eq. mg BSA/g DM) (Figure 4.9).

Nitrate contents for nickel sulphate germinated seedlings showed no significant differences among various concentrations. Nitrate contents were lower for all concentrations when compared to control at 234.8 mg/g DM followed by 210.0 mg/g DM in 5 ppm and the lowest 175.9 mg/g DM in 50 ppm as seen in Figure 4.11.

Total soluble glucid contents were low in all nickel sulphate concentrations as compared to control. Within nickel sulphate salt concentrations the highest glucid contents were recorded in 100 ppm (699.7 glucid eq. D± glucose/g DM) followed by 5 (640.0 glucid eq. D± glucose/g DM) and then 10 and 50 ppm, which showed the lowest glucid content (553.0 and 541.0 glucid eq. D± glucose/g DM respectively) (Figure 4.12).

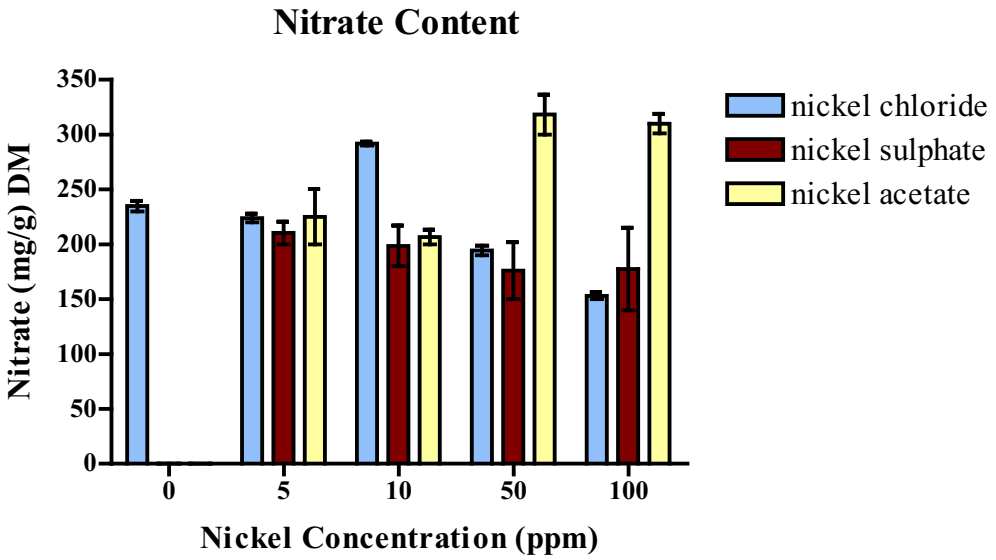

Figure 4.11 Nitrate content of seedlings germinated in the 3 nickel salts. Each analysis had 3 replicates and was repeated twice.

4.3.3 Nickel acetate

Seeds germinated in nickel acetate showed higher nitrate, total soluble protein and total soluble glucid contents at 50 ppm and 100 ppm. Protein contents observed and recorded in various nickel acetate concentrations are presented in figure 4.10. It was interesting to note that protein contents (eq. mg BSA/g DM) in 5 ppm and 10 ppm nickel acetate concentrations were almost similar (25.8 eq. mg BSA/g DM and 24.1 eq. mg BSA/g DM respectively) to control. Highest protein contents (28.6 eq. mg BSA/g DM) were recorded in seedlings grown in 100 ppm concentration of nickel acetate followed by 50, 5 ppm and 10 ppm concentrations.

Nitrate content was the highest at 50 ppm as seen in figure 4.11 decreasing by 5.1% for 100 ppm with 10 ppm having the lowest nitrate content followed by control and 5 ppm concentrations.

At 100 ppm nickel total soluble protein and soluble glucid contents reach the highest contents of 28.6 eq. mg BSA/g DM and 829.1 eq mg D± glucose/g DM respectively

which tend to be 24% and 15.1% higher for protein and glucids respectively as compared to control.

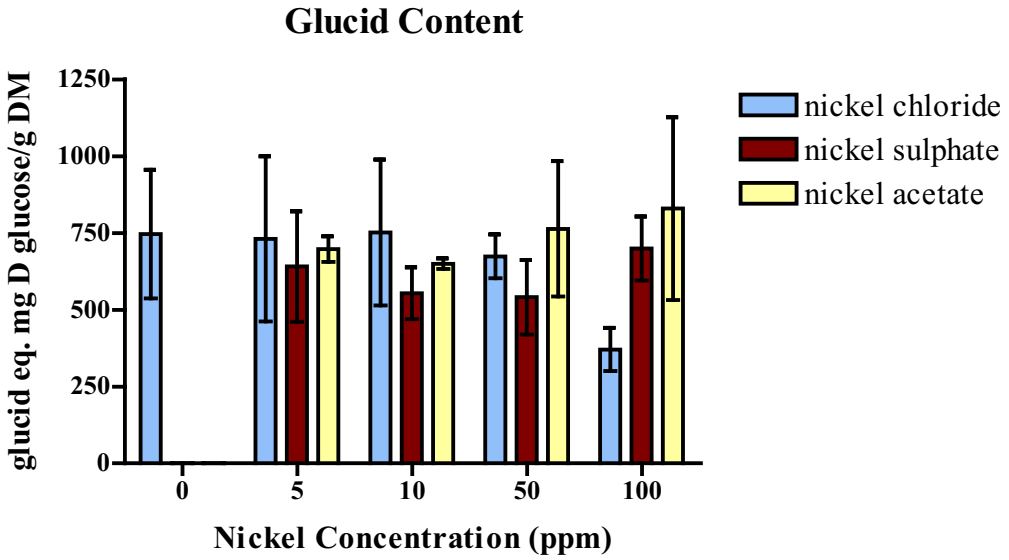

Figure 4.12 Glucid contents of seedlings germinated in the 3 nickel salts. Each analysis had 3 replicates and was repeated twice.

4.4 ENZYME ASSAYS

Seedlings germinated only in nickel chloride salt concentrations were analyzed for Glutamine synthetase activity (GSA) and Nitrate reductase activity (NRA). This was mainly due to short supply of good seeds of *Grevillea exul* var. rubiginosa.

4.4.1 Nitrate Reducatse Activity

Recorded nitrate reductase activity was higher in 5 ppm (15 DO/mg protein), 10 ppm (16.4 DO/mg protein) and 100 ppm (21.7 DO/mg protein) nickel chloride

concentrations as compared to 0 ppm (13.7 DO/mg protein). The highest NRA was observed in 100 ppm concentration of nickel chloride whereas no activity was recorded for the highest concentration of 500 ppm. It was more than one and half times as compared to control (Figure 4.13). Surprisingly in 50 ppm nickel chloride concentration there was lower NRA (10.5 DO/mg protein) than control and all other concentrations of nickel chloride.

4.4.2 Glutamine Synthetase Activity

In germinating seedlings of *Grevillea exul* var. rubiginosa glutamine synthetase activity was the lowest in control. Glutamine synthetase activity gradually increased with increasing concentrations of nickel chloride in the medium but was slightly low in 10 ppm (9.8 DO/mg protein) as compared to 5 ppm (11.4 DO/mg protein) and 50 ppm (11.5 DO/mg protein) and significantly lower than 100 ppm concentration of nickel chloride (Figure 4.13).

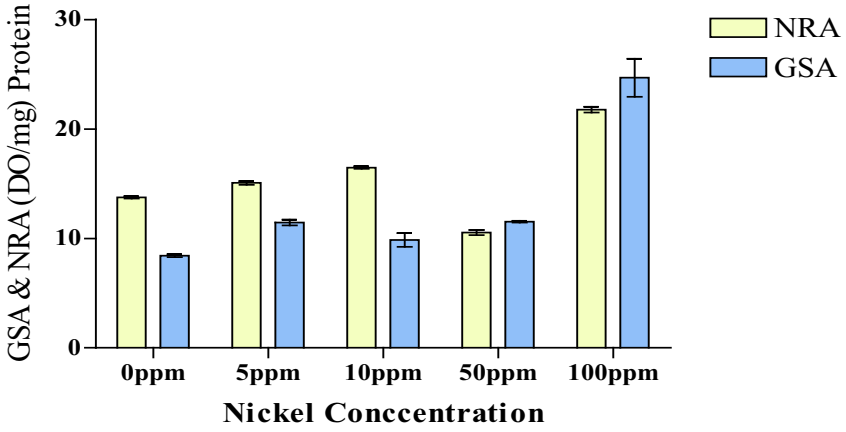

Figure 4.13 GSA and NRA for nickel chloride germinated seedlings. (Mean of three replicates, repeated twice)

4.5 SEMI-CONTROLLED GERMINATION AND TREATMENT

After 3 months of manual treatment of plantlets with nickel chloride, the experiment was terminated. Data collected included number of leaves, length of stem and root, and root : shoot masses. At all concentrations branching of roots were quiet poor. Roots were short with mainly a principal root or two.

4.5.1 TREATMENT RESULTS

Control (0 ppm) – most of the leaves had dried out on the top part of the plant, and branching was observed due to activation of axilary buds. Roots were short with one principal root branching mainly at the lower end of the pot. In overall comparison to all other treatments the plants were quiet poor in growth, which became more obvious with the fact that this treatment had the largest number of plants that died by the time treatment was terminated (Figure 4.14).

Nickel chloride 5 ppm – branching of shoots becoming prominent and roots began to branch and become more spread out. Number of plants died during treatment were less than control.

Nickel chloride 10 ppm – shoot development was very good with extensive branching in plantlet. However relatively poor root development was noted, usually single prominent root with no branching.

Nickel chloride 50 ppm – shoot and root development were noted to be very good in this treatment and no plants died during treatment. Shoots were much longer as compared to control, 5 ppm and 10 ppm nickel chloride concentration with a large number of broader and larger leaves. A single prominent root with no spreading or branching was recorded in this treatment.

Nickel chloride 100 ppm – no branching of shoots or roots; but plants were generally healthy unlike control and 500 ppm. Minimum plant death was recorded for this treatment.

Nickel chloride 500 ppm – branching of shoots was very obvious but the roots were very short and no branching was observed. As recorded for control a large number of plant deaths were also recorded for this treatment.

Figure 4.15 shows the comparison of number of leaves, stem and root lengths in different concentrations of nickel chloride.

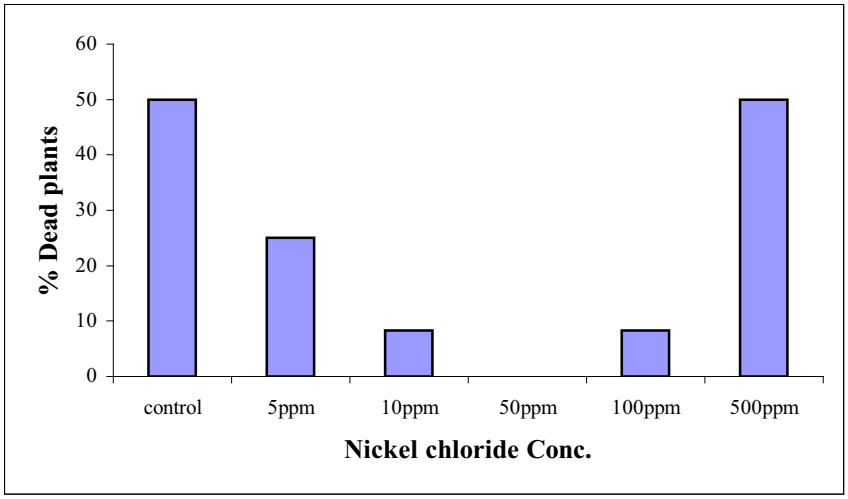

Figure 4.14 Percentage of dead plantlets of *Grevillea exul* var. rubiginosa after three months treatment with nickel chloride

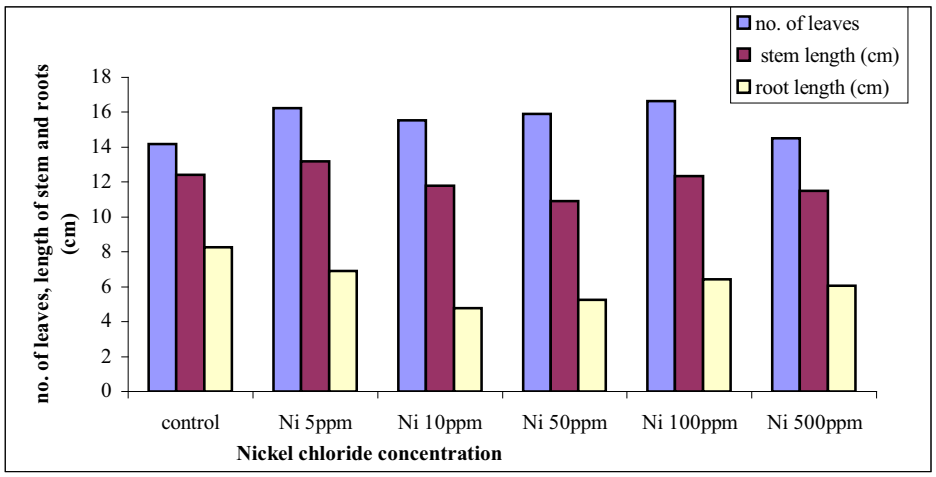

Figure 4.15 Comparison of leaves, stem and roots in growing plantlets of *Grevillea exul* var. rubiginosa after treatment with nickel chloride

4.5.2 Fresh Root : Shoot Ratio

Fresh root : shoot ratios in different concentration of nickel chloride are presented in Figure 4.16. The highest root : shoot ratio was recorded in 5 ppm (0.15). Root : shoot ratio was lower as compared to control in 10 ppm and 50ppm (0.06 and 0.03 respectively) nickel chloride concentration. Root : shoot ratio increased in 100 ppm (0.06) and 500 ppm (0.07) as compared to 10 ppm and 50 ppm. Fresh root : shoot ratios in 100 ppm and 500 ppm were almost similar to control in the experiment.

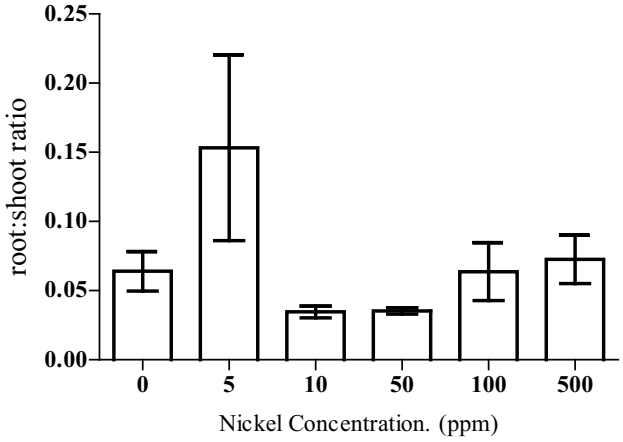

Figure 4.16 Fresh root : shoot ratio of nickel chloride treated seedlings of *Grevillea exul* var. rubiginosa

4.6 BIOCHEMICAL ANALYSIS

Shoots of plantlets treated with various concentrations of nickel chloride over a period of 3 months under semi-controlled conditions were biochemically analyzed for nitrate, total soluble proteins and soluble glucid contents, glutamine synthetase activity and nitrate reductase activity.

4.6.1 Nitrate Content

Plantlets treated in different concentrations of nickel chloride were analyzed for Nitrate content in their shoots using absorbance as an indicator. Absorbance values obtained for nitrate contents in shoots in all nickel chloride concentrations were less than 0.1. This value was very low and hence differences in nitrate content in all treatments were considered non significant.

4.6.2 Total Soluble Protein Content

Total soluble protein contents of non-purified extracts were the highest in 5 ppm of nickel at 11.57 protein eq. mg BSA/g DM followed by 500 ppm nickel treatment as shown in Figure 4.17. This was followed by 10 ppm, 100 ppm and 50 ppm nickel concentrations with control having the lowest protein content of 9.39 protein eq. mg BSA/g DM.

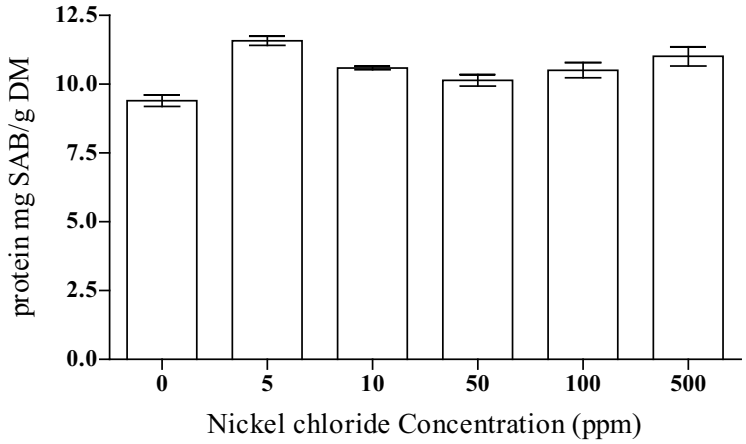

Figure 4.17 Total soluble protein content of plantlet shoots treated with nickel chloride. (Mean of three replicates).

4.6.3 Total Soluble Glucid Content

No significant differences were recorded in different concentrations of nickel chloride in total soluble glucid content except 500 ppm treated seedlings, which had the highest glucid content of 1315 glucid eq. mg D±glucose /g DM whereas control and 100 ppm had the lowest glucid content (1163.9 glucid eq. mg D±glucose /g DM) (Figure 4.18).

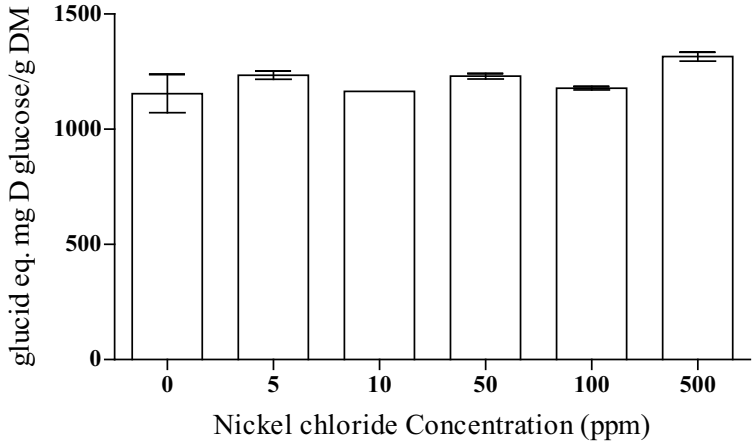

Figure 4.18 Total soluble glucid content in plantlet shoots treated with nickel chloride. (Mean of three replicates)

4.6.4 Glutamine Synthetase Activity (GSA) and Nitrate Reducatse Activity (NRA)

Plantlet shoots analyzed for GSA and NRA, are presented in Figure 4.19. Nitrate reductase activity gradually increased as compared to control to 100 ppm nickel reaching 4.3 DO/mg proteins. At 500 ppm nickel concentration, NRA decreased to 2.7 DO/mg protein but remains 11% above control and 5 ppm nickel. Glutamine synthetase activity was highest at 100 ppm, 12.1 DO/mg protein followed by control, 5 ppm, 10 ppm, 500 ppm and 50 ppm.

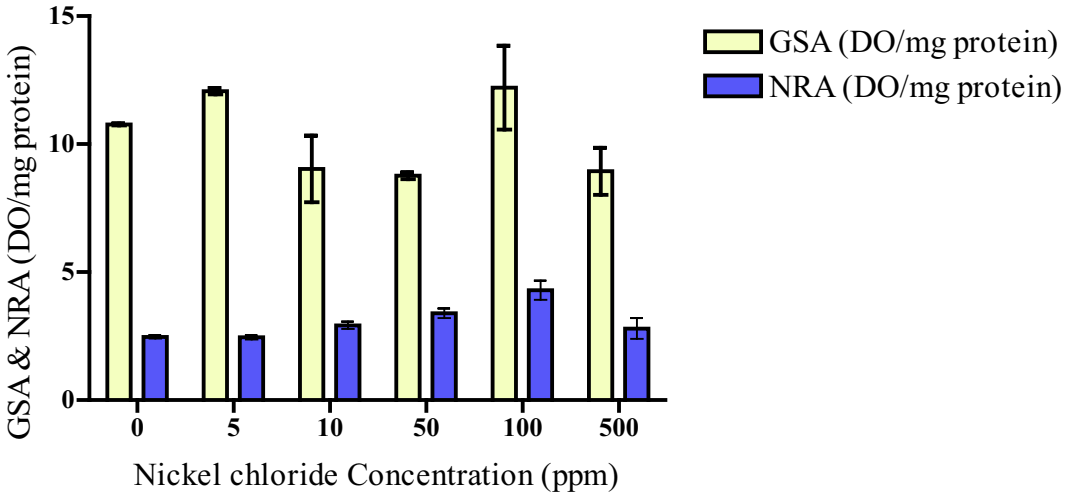

Figure 4.19 GSA and NRA for nickel chloride treated plantlets

4.7 COMPARISON OF TWO PROTEIN METHODS

Two protein methods were used to analyze and compare total soluble proteins in seedlings germinated in nickel chloride salts at 0 – 500 ppm concentrations. The two methods were: *total soluble protein analysis* (non-purified extracts), based on the Folin Phenol reagent with Bovine Serum Albumine as standard and *ammonium analysis,* which requires mineralized samples with Nessler reactive to determine amount of ammonium in the sample hence determination of total soluble protein content. The results obtained from the 2 methods are presented in Figures 4.20 and 4.21.

4.7.1 Total Soluble Protein Analysis

According to the total soluble protein analysis (non-purified extract) method, protein content increased from control to 5 ppm which had the highest protein content of 36.2 mg BSA/g DM and begun to gradually decrease until 100 ppm which showed the lowest protein content for all the treatments as seen in Figure 4.21.

4.7.2 Ammonium Analysis

Results obtained from ammonium analysis of nickel chloride germinated seedlings showed 100 ppm nickel chloride concentration with the highest protein content of 1.0 mg/g DM and 10 ppm with the lowest protein content of 0.25 mg/g DM. A decreasing pattern was noted for control, 50 ppm and 5 ppm nickel chloride concentration germinated seedlings in that order as shown in Figure 4.20.

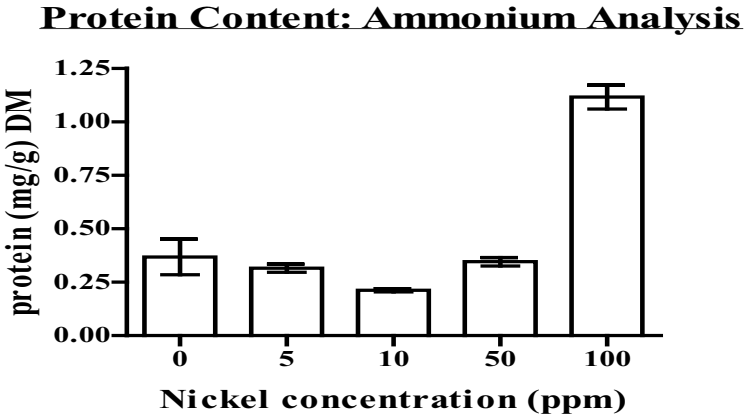

Figure 4.20 protein content obtained from ammonium analysis of mineralized samples

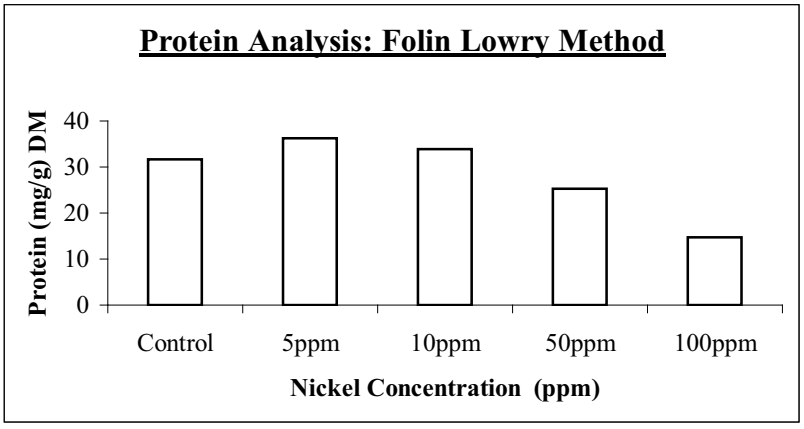

Figure 4.21 protein content obtained from Folin Lowry Method

4.8 MINERAL ANALYSIS

Table 4.2 shows the different concentrations of selected macro and micronutrients present in seedlings germinated in 0 – 500 ppm nickel chloride. Results obtained showed a constant gradual increase of nickel in the seedlings with increasing concentrations of nickel in the germination medium. Germinated seedlings in the range of 0 to 5 ppm showed internal nickel concentration to be in the normal range of 10 – 100 ppm.

Table 4.2 macro and micronutrients present in seedlings of *Grevillea exul* var. rubiginosa germinated in 0 – 100 ppm nickel chloride solutions.

Samples	% Ca	% Mg	% Na	% K	P ppm	Co ppm	Fe ppm	Mn ppm	Ni ppm	Al ppm	Cu ppm	Zn ppm
Ni 0ppm	1.19	0.53	0.18	0.46	7004	0.2	170	2434	22	43	60	135
Ni 5ppm	1	0.6	0.31	0.67	7899	0.8	115	2601	92	18	42	98
Ni 10ppm	1.38	0.54	0.17	0.52	6263	0.3	126	2125	293	16	37	78
Ni 50ppm	1.65	0.49	0.14	0.52	6213	0.6	124	2254	1516	18	34	79
Ni 100ppm	1.39	0.55	0.2	0.63	8043	0.1	253	2425	1814	31	38	88

Macronutrients are presented in percentages whereas micronutrients in parts per million. The analysis presented shows the concentrations of the elements present in the seeds when collected from the wild.

For macronutrients the percentage of Magnesium varied only slightly from the range of 0.49% at 50 ppm nickel chloride concentration to 0.60%, highest at 5 ppm. Control (0 ppm) nickel chloride germinated seedlings had 0.46% Potassium at the lowest value whereas 5 ppm, 10 ppm, 50 ppm and 100 ppm were in the range of 0.52 – 0.67%. In 5 ppm nickel chloride germinated seedlings 0.31% of Sodium was found to be the highest followed by 0.2% at 100 ppm nickel concentration. No significant differences existed in the other concentrations.

In micronutrients Calcium percentage showed variation in seedlings grown in 0 ppm (control) to 100 ppm concentration of nickel chloride. The lowest (1.0%) was recorded

for 5 ppm and the highest (1.65%) for 50 ppm. Nickel concentrations of 10 and 100 ppm also showed higher (1.38 and 1.39 respectively) percentage of Calcium compared to control (1.19%). Nickel was the only micronutrient that followed this pattern of increased concentration of heavy metal as it was increased in the medium. Cobalt, Iron, Manganese, Aluminum, Copper and Zinc decrease in concentration in the seedlings from control to 50 ppm nickel in the medium and than increase again at 100 ppm.

4.9 MINIRHIZOTRON ROOT DEVELOPMENT

4.9.1 Soil Characteristics

4.9.1.1 Saprolite Soil

Percentage humidity, pH and conductivity were determined for the different soil types for each compartment of the minirhizotron which was divided into 4 zones, zone 1 being at the top of the minirhizotron as shown in Figure 4.22 and 4.23.

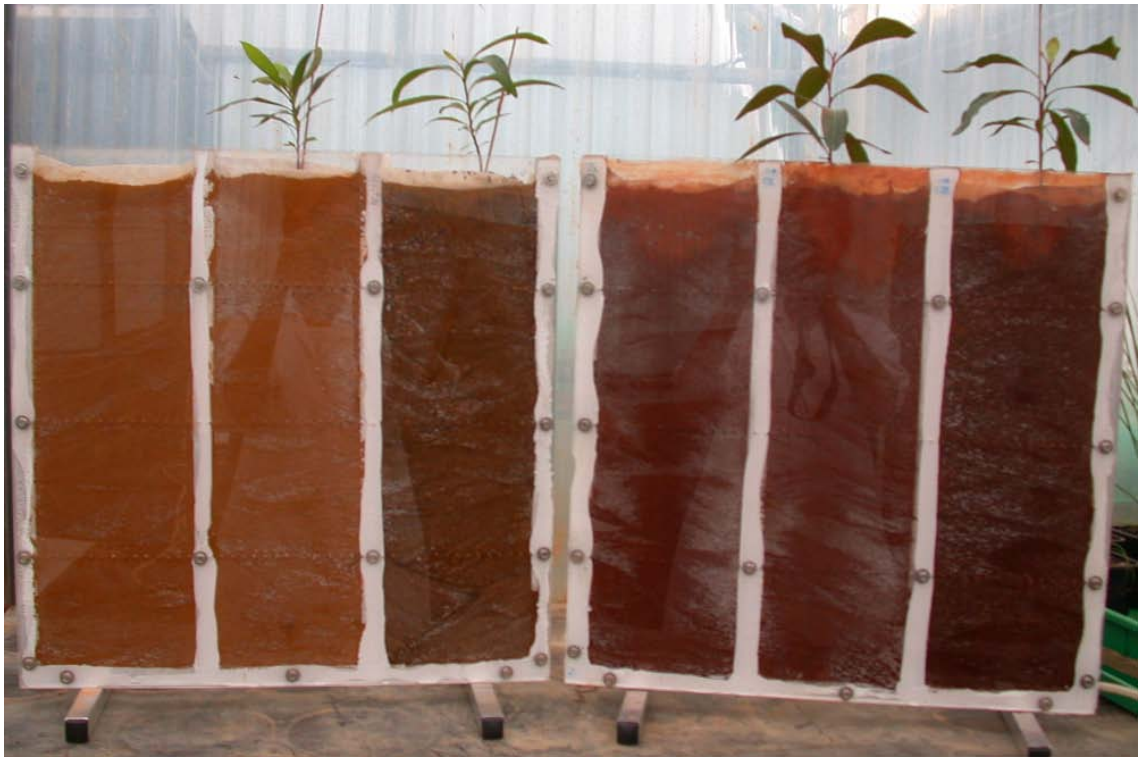

Figure 4.22 Minirhizotrons with red laterite and saprolite soils respectively after treatment for three months.

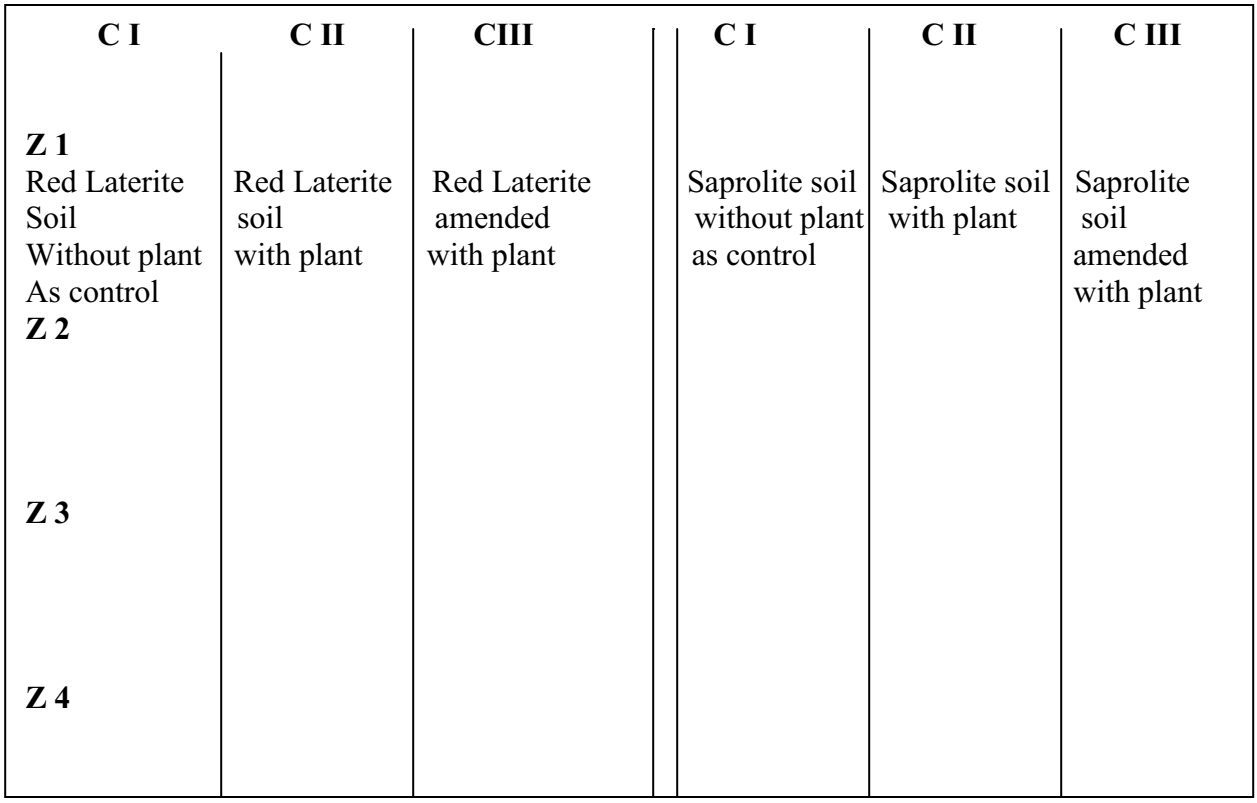

Figure 4.23 Outline of minirhizotron showing compartments and zones

Key:
C - Compartments
Z - Zones

4.9.1.1.1 Potential Hydrogen: initial pH before plants were transferred for saprolite soil was 6.99 whereas organic amended saprolite soil was lower at 6.61. After three months of treatment under semi – controlled conditions the pH for compartment 1 was 6.91, compartment 2 was 6.31 and compartment 3 had an average pH of 6.58. No significant difference was noted between compartment 1 and compartment 2, except for a slight decrease in zone 3 of each compartment as seen in Figure 4.24. Compartment 3 with 2:1 ratio of saprolite and organic amendment showed no significant difference in pH in each zone. The greatest decrease was noted from zone 1 to 2 in compartment 3.

Compartment 1, which was control for compartment 2, showed a slight decrease in pH in zone 3. Though the pH for compartment 1 remains higher than compartment 2 a similar trend is followed.

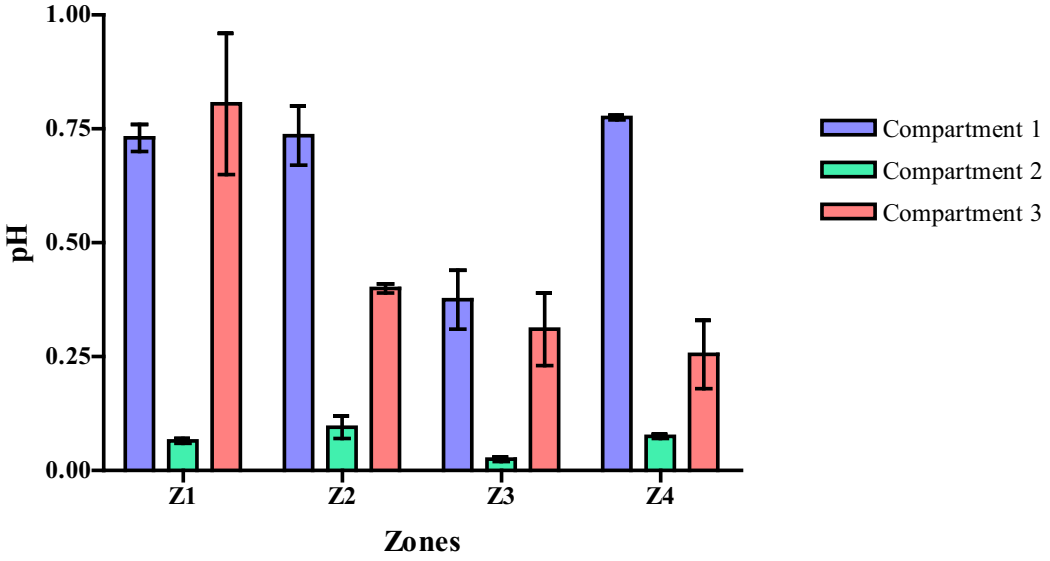

Figure 4.24 pH difference of saprolite soil in minirhizotron after root colonization by *Grevillea exul* var. rubiginosa

4.9.1.1.2 Conductivity: compartments 1 and 2 showed similar patterns as pH where conductivity decreased from zone 1 to 2. There were no large differences in conductivity in different zones (Figure 4.25). As for compartment 3, conductivity was much higher than compartment 1 and 2, increasing from zone 1 – 3 than decreased slightly in zone 4.

4.9.1.1.3 Humidity: humidity for all 3 compartments increased from zone 1 to 4, with compartment 1 having the lowest humidity followed by compartment 2 and compartment 3 with the highest humidity percentage (Figure 4.26).

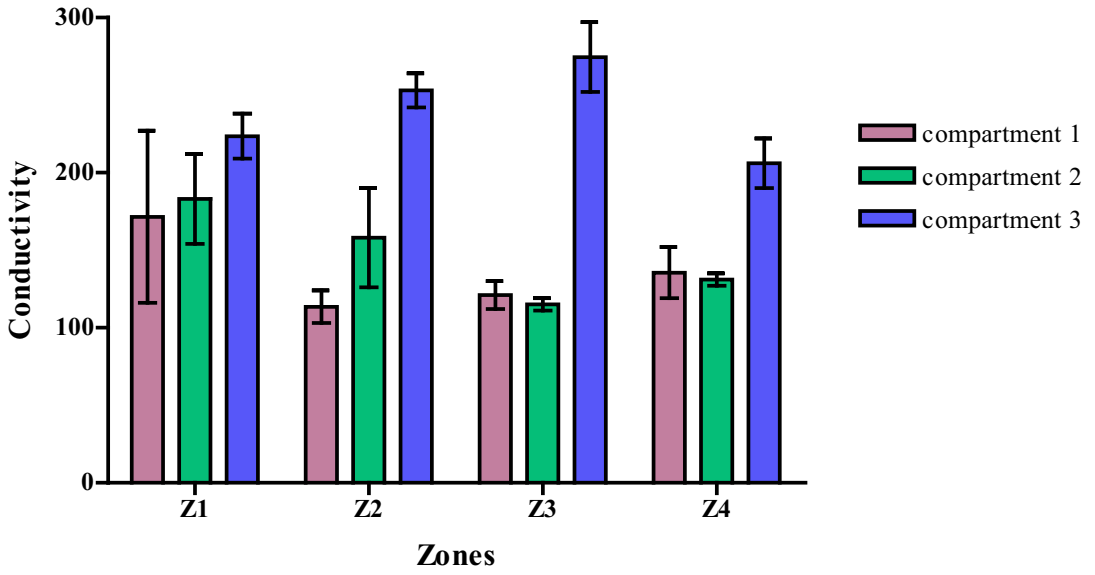

Figure 4.25 Conductivity of Saprolite soil from minirhizotron after colonization by *Grevillea exul* var. rubiginosa

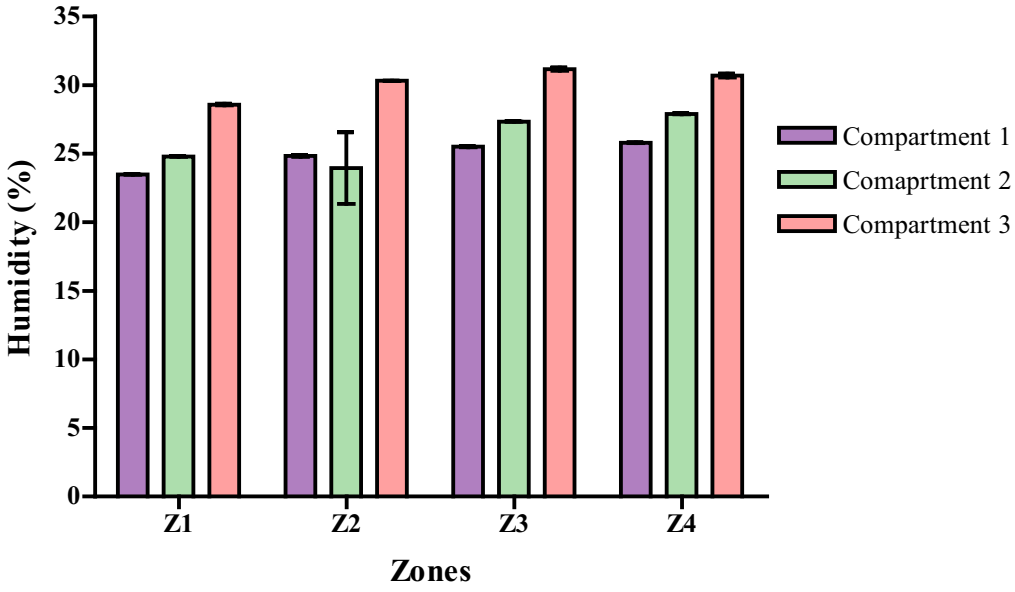

Figure 4.26 Saprolite Soil Humidity in minirhizotron after colonization by *Grevillea exul* var. rubiginosa

4.9.1.2 Red Laterite Soil:

4.9.1.2.1 Potential Hydrogen: Average pH for red laterite soil before plant transfer was 6.62 whereas amended red laterite soil had a pH of 6.36. After three months of treatment under semi – controlled conditions the average pH for compartment 1 was 7.77, compartment 2 was 7.61 and compartment 3 had a pH of 6.71. Variations were observed for pH in red laterite soil in the different zones of each compartment 1 (control) pH increases significantly in zone 3 than decreases dramatically in zone 4 whereas pH for compartment 2 decreases from zone 1 to zone 3 than increases significantly in zone 4. Organic amended red laterite soil showed a decrease in pH from zone 1 – 4, as seen in Figure 4.27.

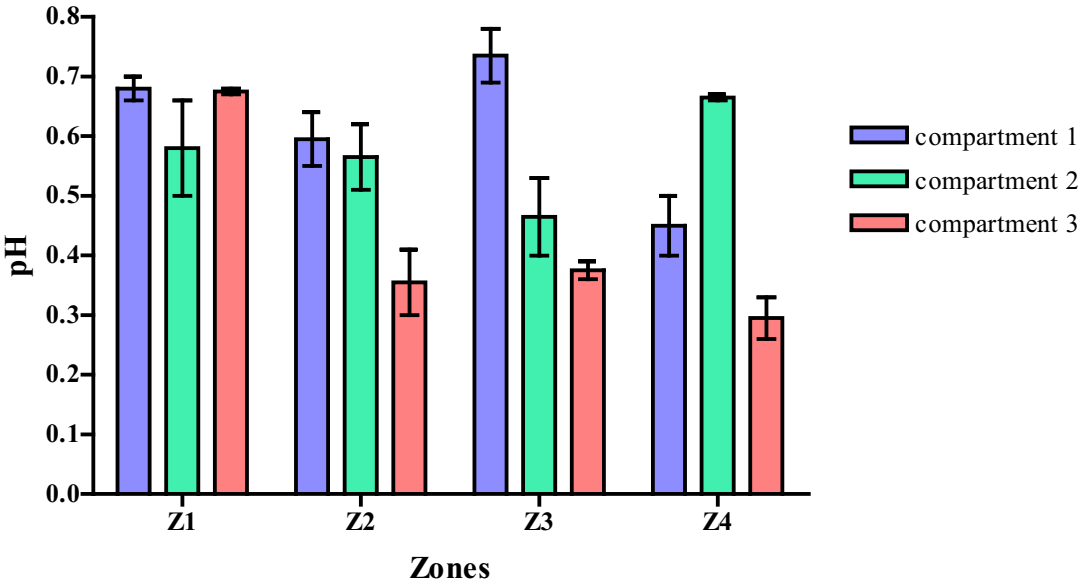

Figure 4.27 pH difference of Red laterite soil in minirhizotron after colonization by *Grevillea exul* var. rubiginosa

4.9.1.2.2 Conductivity: no significant differences were noted for compartment 2 and 3 in all zones except that they were both lower in conductivity than compartment 1. Red laterite soil as control (compartment 1) had a higher conductivity compared to compartment 2. Same pattern was observed from zone 1 to 4 but with a lower

conductivity. A significant decrease in conductivity was noted with organic amended red laterite soil (compartment 3) from zones 1 to 4 (Figure 4.27).

4.9.1.2.3 Humidity: no significant differences were observed between compartment 1 and 2. Humidity in the 4 zones of both compartments followed a similar pattern, whereas compartment 3 had the lowest humidity in zone 1 and highest in zone 4 (Figure 4.29) increasing gradually in zone 2 to zone 3.

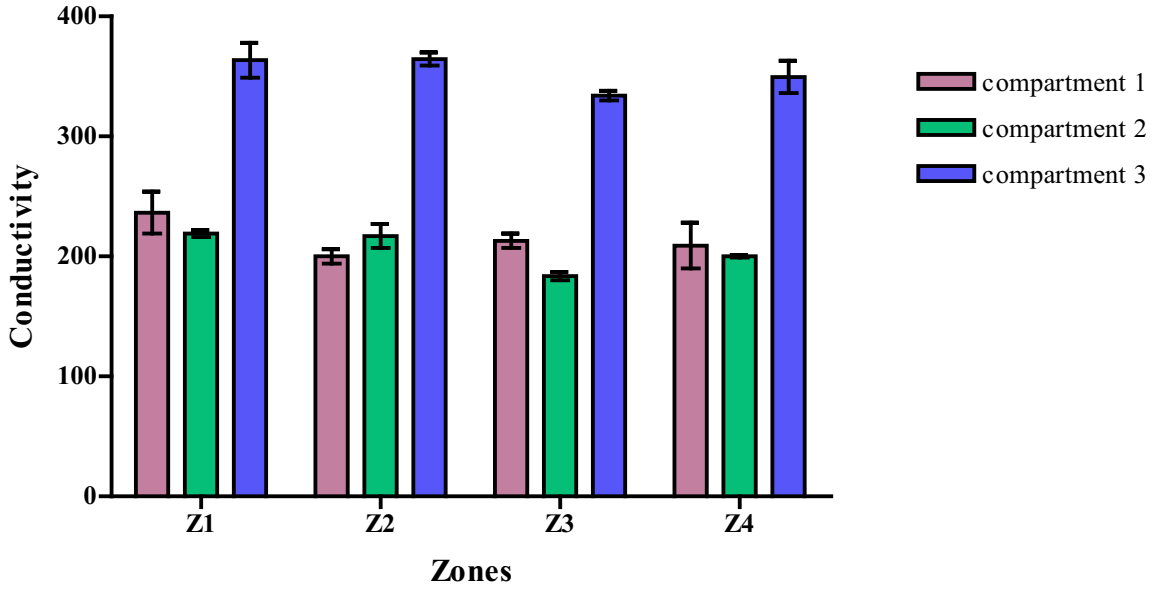

Figure 4.28 Red Laterite Soil Conductivity from minirhizotron after colonization by *Grevillea exul* var. rubiginosa

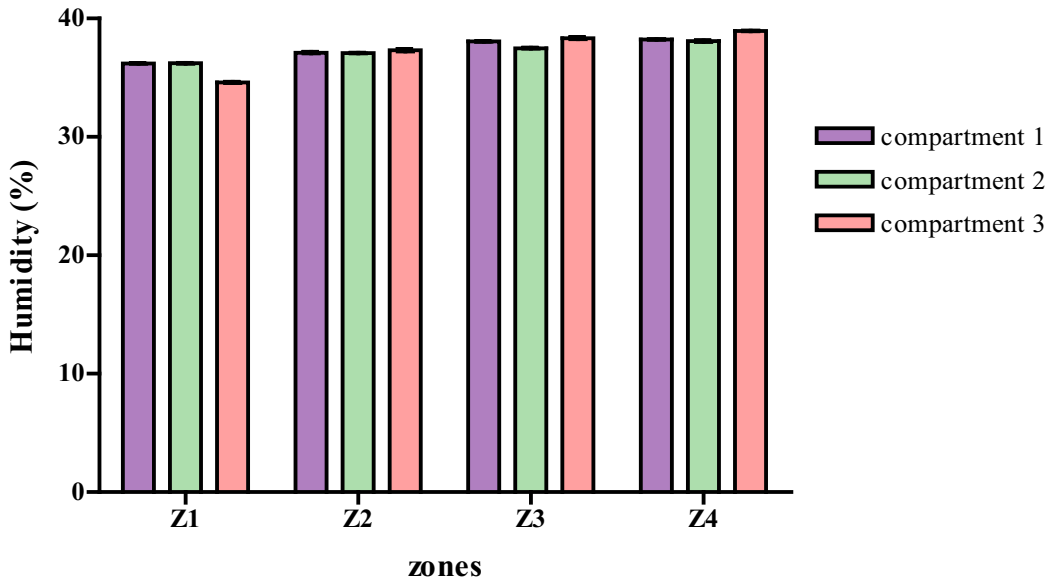

Figure 4.29 Red Laterite Soil humidity after minirhizotron root colonization by *Grevillea exul* var. rubiginosa

4.9.1.3 Organic amended soil types: the comparison of results between the 2 soil types showed that pH patterns and percentage humidity for the 2 studied soil types, red laterite and saprolite soils when amended with organic matter in 2:1 ratio. Figure 4.29 shows pH comparison of organic amended red laterite and saprolite soils with decreasing trends from zones 1 to 4. For red laterite and saprolite soil types pH decreases from 0.99 to 0.85 and 1.295 to 1.075 respectively with addition of organic matter in 2:1 ratio. Conductivity patterns as well as humidity for both soil types also followed a similar trend though amended red laterite has a slightly higher humidity compared to saprolite (Figure 4.31).

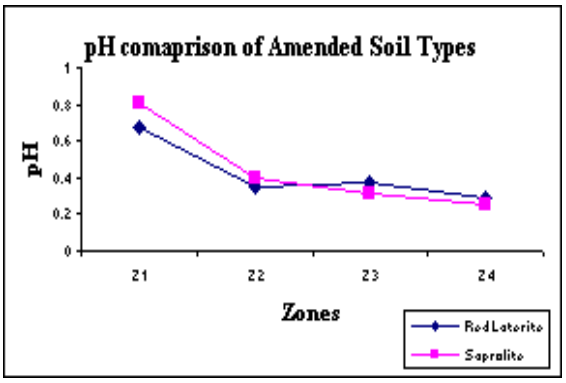

Figure 4.30 pH comparison of amended soil types.

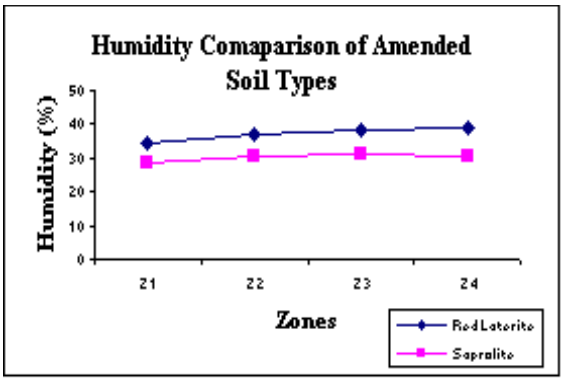

Figure 4.30 Humidity comparison of amended soil types

4.9.2 Root Colonization

Root colonization of *Grevillea exul* var. rubiginosa was tracked for approximately 2 months after seedlings transfer to minirhizotrons (figure 4.32). No proteoid roots were noted on the plant roots during transfer. Water absorption and retention of the 2 soil types and amendments were recorded at the beginning of the experiment. Saprolite soils absorbed water at a faster rate as compared to organic amended soil and red laterite-amended soil. Red laterite soil absorbed water at a faster rate than saprolite alone, but during the period of study of root colonization it was noted that saprolite retained water more efficiently as compared to red laterite soil type.

Compartment 1 of both minirhizotrons were kept as control for the original soil types (saprolite and red laterite). *Grevillea exul* var. rubiginosa roots colonized red laterite soil more efficiently compared to saprolite. Two months after transfer, roots in red laterite soil colonized 75% of the compartment whereas 50% of the compartment of saprolite soil was colonized. At the same time both amended soil types showed 100% colonization by *Grevillea exul* var. rubiginosa roots.

Figure 4.31 Roots of *Grevillea exul* var. rubiginosa after termination of treatment in minirhizotron (Proteoid roots visible in petri dish)

Two prominent principal roots emergence were observed from the base of the stem through to zone 4 in saprolite soil. Branching of roots was noted to be longitudinal and transversal especially in the first 2 compartments and proteoid roots were also observed in these 2 compartments (figure 4.32). Organic amended saprolite soil was noted to have 3 prominent principal roots emerging from the base of stem, which were seen to branch in zones 1 and 4.

Figure 4.32 Proteoid roots in *Grevillea exul* var. rubiginosa

Two prominent principal roots running down zone to 3 were noted in red laterite soil. Branching of roots was obvious in zone 1 only otherwise longitudinal growth of roots was recorded. Very little branching from the principal (main) roots occurred. Proteoid roots were noted in only zones 1 and 2.

A prominent principal emerging root was observed in amended red laterite soil. Principal root grew down to zone 4 with branching obvious in zones 1 and 3 where most proteoid roots were observed on the principal root that branched the most.

Table 4.3 Presence of proteoid roots from *Grevillea exul* var. rubiginosa in different zones of the studied soil types

		Soil Types		
Zones	**Saprolite**	**Saprolite + organic**	**Red Laterite**	**Red Laterite + Organic**
1	3	0	3	4
2	4	1	1	0
3	0	0	0	8
4	0	6	0	0

The morphological characteristics such as leaf size, broadness, stem length and number of leaves improved with soil amendment. This observation proves that the addition of organic matter to both soil types increased the rate of colonization of roots and improves the morphological characteristics of the plant as well. An important observation was that the roots from plants grown in amended red laterite and red laterite soils were the first to colonize as compared to amended saprolite and saprolite soils. Although red laterite showed better root colonization however, no significant differences existed in growth and development parameters and other observed morphological characteristics (ratio = mass of leaves/number of leaves). Whereas saprolite soil showed better growth and development parameters such as the broadness and number of leaves.

CHAPTER 5

5.0 DISCUSSION

5.1 SEED VIABILITY

A high viability percentage for the seeds utilized in the experiment suggests that discrepancies such as un-germinated seeds were due either to the experimental procedure used or contaminations with microbes. Treating the seeds with 4% tetrazolium hypochlorite solution does not mean total elimination of microbes because fungal contamination was observed with seeds even after careful washing during experimentation.

A major problem was the harvesting of seeds. The process of seed harvesting has so far not been properly documented and harvesting dates were variable. Seed storage methods have yet to be perfected.

In addition, due to slow growth of seedlings these have to be kept in nurseries for over a year. Another constraint was that planting in mined areas always done by hand, because the seedlings are grown in polythene bags. Almost half of the cost of a planted seedling was accounted to the labour. When added together all these factors make the cost of revegetation of mined sites an expensive exercise. In recent years methods of mechanical planting of seedlings are being developed. These improved methods will decrease the cost of revegetation of mined sites in New Caledonia.

5.2 CONTROLLED GERMINATION

Although seed germination tests were used to determine better and quick germination and seedling response (Archambault and Winterhalder, 1995), environmental factors also affect the pattern and duration of germination depending on the interaction between the internal factors of the seed and the environment (Karataglis, 1980). Results obtained in this investigation showed that germination rates and root lengths are affected to a

large extent by high nickel concentration in the germinating medium. Germination and growth were inhibited at very high concentrations of nickel salts. This also included delayed germination and poor establishment of seedlings with increasing nickel concentrations.

Results similar to the present study but in different plant (Bilberry seedlings) showed that nickel ions at higher concentrations suppressed seedling growth by inhibiting cell expansion and division (Lyanguzova, 1999).

Peralta *et. al*., (2001) also reported the effects of nickel and other heavy metals such as Cd, Cr, Cu and Zn on seed germination, root and shoot elongation as metal concentration in the growing media was increased. Calabrese and Baldwin (1999) reported the phenomenon known as hormesis, where smaller doses of heavy metals increase seedling growth while greater doses decrease the seedling growth.

The effects of the environment on germination are quiet complex because of interactions and internal factors that modify germination patterns (Rtout *et. al*., 2000). According to Hilhorst and Kerseen (2000) three environmental factors that can be accurately sensed by seeds are light, nitrate and temperature (fluctutations).

5.3 SEMI-CONTROLLED GERMINATION

When plants are exposed to high concentrations of nickel salts, the factors controlling uptake and translocation of nickel ions into other parts of the plant become important (Mishra and Kar, 1974). Cataldo *et al*. (1978_a) proposed that the carrier systems that absorb and translocate nickel to the shoots are the same as those that carry other metal ions. They also reported (Cataldo *et al*., 1978_b) that about 50% of the nickel applied to intact plants was retained in roots after 21 days. Of the nickel transported into the shoots, 78% moved into the seeds mainly in the cotyledon, as these comprise the largest

portion of the seed. Over 90% of the nickel in both roots and leaves occurred in the soluble form.

From the results obtained in the present investigation it is quite obvious that nickel is essential for better germination and growth of *Grevillea exul* var. *rubiginosa*. The concentration of nickel, which can be considered beneficial for plantlets, would be 5 ppm. Since this was the concentration of nickel in the growing medium that had a positive effect on both root as well as shoot development. Though some plants were lost during the treatment period, root : shoot ratio was the highest with 5 ppm nickel ion concentration in almost all nickel salts. With 50 ppm of nickel, no plants died and the overall plantlet development was better than the rest of the treatments including morphological characteristics. Yet when taking into consideration the concentration of nickel, morphological development as well as biochemical effects, it is quiet clear that 5 ppm of nickel is appropriate for the normal growth and development of the *Grevillea exul* species. In total absence of nickel many plantlets of *Grevillea exul* var. rubiginosa died, the plant loss was the highest and the overall morphology of the plantlets was very poor. The upper portions of the plantlets were weak, dry and died which was an unusual observation in the beginning but at a later stage most probably it was due to branching. As soon as branches began to emerge from the axilary buds of the upper portion of the main plantlet, growing stem lost its vigor. Branching from axilary buds varied in different nickel concentrations in the medium. Westerbergh (1994) suggested that serpentine soil plant populations are made up of metal tolerant species selected by nature for normal growth on serpentine soils. Results of this investigation support the observation of Westerbergh (1994) where the root length growth of metal tolerant plants was reduced before the overall plant growth was reduced.

Nickel at higher concentrations inhibits cell division and cell expansion in the meristemic zone of the roots (Robertson and Meakin, 1980, L' Huillier *et. al*., 1996 and Piccini and Malavolta, 1992). Hence the increase in short side roots (branching) could be due to main root length inhibition. Plant species growing on serpentine soil have strongly developed root systems in general (Brooks, 1987). An extensive root system

would facilitate the uptake of water and nutrients and may be seen as an adaptation to the poor drained serpentine soils.

5.4 BIOCHEMICAL ANALYSIS

The principal sources of nitrogen in soils available to plants are nitrate (NO_3^-) and ammonium (NH_4^+). The first step of this process is the reduction of nitrate (NO_3^-) to nitrite (NO_2^-) in the cytosol via the enzyme nitrate reductase (Oaks, 1994). In the next step nitrite is reduced to ammonium (NH_4^+) in root plastids or chloroplasts (in leaves) by the enzyme nitrite reductase. Ammonium is converted to glutamine and glutamate through the concerted activity of glutamine synthetase (GS) and glutamate synthase (GOGAT) (Marschner, 1995, Taiz and Zeiger, 1998, Wallsgrove *et. al*., 1979). Glutamine Synthetase is an octamer and found in two isoforms; one is located in the cytosol (GS_1) and the other in the chloroplast (GS_2) (Ortega *et al*., 2000; Scarpeci *et al*., 2000). It is now generally considered that the assimilation of nitrate to amino acids in leaves is carried out by the enzymes nitrate reductase (NR), glutamine synthetase (GS) and glutamate synthase (GOGAT) (Miflin and Lea, 1976; Miflin and Lea, 1977; Schoenbeck *et al*., 2000).

Results obtained in this investigation for nitrate content, GSA and NRA for seedlings germinated in nickel chloride and plantlets treated in the greenhouse with nickel chloride are consistent with those obtained by other researchers (Singh, 2002, Kevresan *et. al*., 2001, Kevresan, 1998). Nitrate reductase activity, glutamine synthetase activity, total nitrate and total soluble proteins decreased with increasing concentrations of nickel in the medium. This is true for nickel chloride and nickel sulphate salts for total soluble protein and nitrate whereas high concentration of nickel in the form of nickel acetate have shown enhancement in NR and GS activities and an increase in soluble proteins

According to Singh (2002) although Ni^{2+} ions are not a structural component of GS, it may be indirectly involved as a cofactor for the enzymes. nickel enhances the activity of

hydrogenase uptake because it is the structural component of the enzyme glutamine synthetase. Hydrogenase uptake plays a vital role in reoxidising hydrogen, which is otherwise a wasteful byproduct in the reduction of di-nitrogen to ammonia (Eisbrenner and Evans, 1983). Enhanced GSA and NRA have been observed in seedlings germinated in nickel chloride and plantlets treated with nickel chloride at 100 ppm, hence supporting the work of Singh (2002) who suggested that the supplementation of Ni^{2+} in the growth medium might have enhanced the synthesis and activity of hydrogenase hence enhancing GS activity.

Increased concentration of nickel had no significant effects on the total soluble protein content (Figure 4.18). This could be due to evolutionary adaptability of the species *Grevillea exul* to the harsh environmental conditions via genetic modifications by the process of natural selection.

5.5 COMPARISON OF TWO PROTEIN METHODS

The Folin Lowry method is consistent and the method used to determine protein contents for seedlings germinated in the 3 salts of nickel, in this research. Although both sets of seedlings were germinated in nickel chloride, the results obtained were different most probably because the seeds were heterogeneous and the conditions under which germination occurred can never be 100% identical. Seed heterogeneity would also have determined the concentration (amount) of nickel within the seed. This, in turn, affects the internal seed environment and conditions during germination, hence differences in results obtained by two methods of biochemical analysis.

However these factors do not fully account for the differences in results obtained for the 2 methods (Folin Lowry and ammonium analysis) used in the analysis of protein contents. Total soluble protein content determined from ammonium analysis of mineralized samples showed the highest protein content at 100 ppm and the lowest at 10 ppm.

According to Lowry *et. al.,* (1951) the Folin Lowry methods had been recommended for its great sensitivity and the simplicity of the procedure possible with its use. Though this reagent has considerable merit for certain applications, its advantages and limitations need to be understood for it's fullest exploitation. The method has been studied with regard to effects of variations in pH, time of reaction and concentration of reactants, permissible levels of reagents commonly used in handling proteins and interfering substances (Lowry *et. al.,* 1951).

The two methods provide two different sets of results but the measurement of protein with Folin reagent has certain advantages:

1. It is sensitive as with Nessler's reagent, yet requires no digestion.
2. It is much more specific and much less liable to disturbance by turbidities
3. It is simple as well as much easier to adapt for small-scale analysis (Lowry *et. al.,* 1951).

According to Lowry *et. al.,* (1951) two major disadvantages of this method are:

1. Colour variation with different proteins and
2. The color is not strictly proportional to the protein concentration.

With the ammonium analysis methodology the concentration of ammonium is calculated for the samples and this is used to determine the protein contents in each treatment. Hence the results presented are interpretations of protein contents with reference to the total ammonium contents in the sample.

The Lowry *et. al*., (1951) methodology was used in this study to determine total soluble protein content in *Grevillea exul* var. rubiginosa plantlet shoots is more reliable in protein analysis (non-purified extract) using the Folin Phenol reagent. This is because the results obtained are consistent and could clearly be explained with total nitrate content and total soluble glucid analysis where the contents decreased with increasing concentrations of nickel.

5.6 MINERAL ANALYSIS

A review carried out by Welch (1995) on micronutrient as nutrition of the plants, briefly summarized the current knowledge of micronutrients in plants and presented some new speculation on the new mechanisms of micronutrient uptake and translocation in plants. Going through the review it became obvious that much remains to be learnt about the physiology of micronutrient absorption, translocation and deposition in plants, and the functions these micro and macronutrients perform in plant growth and development.

Majority of the literature reviewed were research on macronutrients. Relatively little is known about the basic mechanisms of micronutrient function in higher plant growth and development. Since the present study focused on the essential micronutrient nickel, it is necessary to outline and understand the major physiological functions and metabolites in higher plants. Major functions include urea and ureide metabolism, iron absorption, seed viability, nitrogen fixation, reproductive growth and the major metabolites urease, microbial dehydrogenase, hydrogenases and methyl reductase (Welch, 1995 and Brown *et al*., 1987).

According to Welch (1995) the concentrations of micronutrients in plants can vary widely depending on many factors including plant species, genotype and growth conditions. Micronutrients can also differ among different organs and tissues of the same plant species. Genetic makeup and dynamic physiological and environmental factors could interact to alter the concentrations of micronutrients in plants at which deficiencies or toxicities might occur.

To determine the mechanisms of micronutrient uptake from soil, chemical analysis alone is not satisfactory for predicting the effects of fertilizer application. Soil analysis mainly provides an indication of the capacity of a soil to supply nutrients to the plants, but does not adequately and in some cases does not at all characterize the mobility of the nutrients in the soil. Additionally it fails to provide information about soil structure, or microbial activity, and plant factors such as root growth and root-induced changes in

the rhizosphere, which are of decisive importance for nutrient uptake under field conditions (Marschner, 1995). The importance of the mobility of nutrients in soils in relation to availability to plants was emphasized by Barber (1962), these ideas were refined and further developed were summarized in the concept of 'bioavaliability of nutrients' (Barber, 1984).

Marschner (1995) stated that to meet the nutrient demand of soil grown plants, nutrients must reach the root surface and this is mainly mediated by movement in or transport with the soil solution, interrelated with root growth which decreases the length of transport pathways. The concentration of nutrients in the soil solution is therefore of primary importance for nutrient supply to roots. Asher, (1978) suggested the following factors contribute to nutrient concentration in soil solution and variation. These are soil moisture, soil depth, pH, cation-exchange capacity, redox potential, quantity of soil organic matter and microbial activity, season of the year and fertilizer application. The concentration of mineral nutrients in the soil solution is an indicator of the mobility of nutrients toward the root surface and in the vertical direction.

Requirements for mineral elements change during growth and development of a plant life cycle (Taiz and Zeiger, 1998). As soil analysis is the chemical determination of the nutrient content in a soil sample from the root zones, it reflects the levels of potential nutrients available to the roots. Whereas plant tissue analysis (PTA) evaluates the uptake conditions and the amounts on nutrients actually absorbed by plants. Bouma (1983) stated that the proper use of plant tissue analysis requires an understanding of the relationship between plant growth and the mineral content of plant tissue samples. Plant tissue analysis have been applied to many different plant tissues and organs, these include leaves, stems, roots, seeds, fruits, grains or sap from petioles. More accurate indicators of nutrient contents are provided by PTA and by morphological symptoms.

Results obtained in this study clearly demonstrate the increasing concentrations of nickel in the seedlings as the concentration of nickel in the form of nickel chloride in the germinating medium increased. This is one of the characteristics of hyper

accumulator plants. Hyper accumulators are metal tolerant plants that have the ability to accumulate high concentrations of metals in their vacuoles (Baker and Brooks, 1989). The uptake of such a high concentration of nickel is known to have deleterious effects on many enzyme systems hence the plants cope with excess heavy metal ions by relying on two main mechanisms for detoxifying metals taken up into the cell: formation of complex with organic compounds and compartmentation within the vacuole (Reeves *et. al*., 1996, Baker and Brooks, 1989, Briat and Lebrun, 1999). Krämer and colleagues (1996) found histidine to be complexed with nickel in the nickel hyper accumulator plant *Alyssum lesbiacum*, and they proposed that histidine synthesis is an important mechanism for nickel hyper accumulator.

Results obtained in this investigation are generally similar as reported by Lange-Hesse *et. al*., (1994). The nickel content was found to be higher in generative or organs of accumulation (e.g. seeds). This means that different parts of the plant should be analyzed at different stages to determine the amounts of nickel present.

5.6 MINIRHIZOTRON ROOT DEVELOPMENT

Plants adapted to infertile soil environments have high rates of uptake of available soil nutrients. Some achieve this through specialized root structures. Knox *et. al*., (1995) reported several modifications associated with roots for increasing the supply of nutrients; root nodules and coralloid roots fix nitrogen, white root clusters (proteoid roots) and mycorrhizae improve phosphorus uptake.

Proteoid roots are groups of hairy rootlets that form dense mats at the soil surface; they result from massive proliferation of rows of lateral rootlets, up to 1000/cm, along the parent root. Masses of proteoid roots have a white glistening appearance and featured with extensive development of vascular tissue, particularly xylem inside the root (Knox *et al*. 1995)

Proteoid roots are a form of specialized microbial association, it is restricted in its occurrence they are highly absorbent and their growth is stimulated by particular micro-organisms in the root zones. They are particularly well developed in the family *Proteacea* (e.g. many species of *Banksia, Grevillea* and *Hakea*).

Results obtained certainly do support the theory that plants adapted to infertile environments, adapt modifications to increase the supply of available nutrients in the soil. Details of this process are described by Knox *et. al*., (1995) and Marschner (1995). Due to the specific characteristics of serpentine soils, the presence of proteoid roots is clearly defined in this species. No significant distinctions are possible for the presence of proteoid roots on extensively branched principal roots and also the presence of these roots closer to the upper surface of the soil layer in red laterite and saprolite soil types whereas with amended soil proteoid roots are formed in lower zones as well. One of the explanations for the above pattern could be the availability of essential mineral nutrients in saprolite and red laterites are available closer to the surface since decomposition occurs here. In amended soils, nutrients are available equally throughout the zones hence formation of proteoid roots occurs lower in the zones as well. Although in the organic amended soils formation of proteoid roots might not be important, because the plants have adapted to nutrient deficient soils, they might be pre disposed due to their genetic modifications. In case the plant is encountered with nickel deficiency, the presence of these (modified) proteoid roots would enhance the absorption of available essential nutrients. Presence of proteoid roots in certain families that are adopted to nutrient deficient soils is a phenomenon which requires lot of research work, in order to clearly understand their presence and specific function in *Grevillea exul* var. rubiginosa.

Regarding growth and development parameters; the results obtained in this study suggest that amendments to soil types may not necessarily mean improvement in growth and development of plants. As observed with *Grevillea,* number of leaves and broadness of these leaves in red laterite soils and red laterite amended soil had no significant effect on growth and development, whereas saprolite soil showed a

significant improvement in overall parameters of growth, development and root colonization as well.

Soil pH (hydrogen ion concentration) is an important property of soils because it affects the growth of plant roots and microorganisms (Tiaz and Zeiger, 1998). According to Tiaz and Zeiger (1998) root growth is generally favoured in slightly acidic soil at pH values 5.5 – 6.5. Fungi generally predominate in acidic soils while bacteria become more prevalent in alkaline soils. Soil pH also determines the availability of soil nutrients. The solubility of nickel in soils is determined largely by pH and the extent of nickel containing surfaces exposed to the soil solution (Sumner and Naidu, 1997). Their results also supported the fact that raising the pH of the soil either with lime or alkali resulted in increased plant growth and reduced nickel concentrations in tissue on both serpentine derived and contaminated soils (Crooke, 1956 and Sumner and Naidu, 1997).

The results obtained in this study throw new light for the purposes of revegetation of exploited mine sites particularly in New Caledonia. It is particularly important to conduct trials of different soil types present at the mine sites to identify the best plant species for revegetation and to determine the most suitable technique for planting. Organic amendment on exploited mine sites wouldn't actually be considered a very feasible idea due to the financial implications associated with it. Ideas gathered during the international meeting on "Preservation and Ecological Restoration of Tropical Mining Environments" 15^{th} – 20^{th} July 2003, Institute for Research and Development (IRD) Center Noumea New Caledonia highlighted the importance to revegetate exploited mine sites with the original endemic species present in the natural serpentinic environment so that once the mineral contents of the amendments are depleted (if and when soil types are amended) there won't be the hassle of constantly amending the soil to keep up the composition of minerals for plant growth and development.

6.0 CONCLUSION

Results obtained in the present investigation showed that different nickel salts and concentrations have a major effect on the growth and development of *Grevillea exul* var. rubiginosa. Though the species has adapted to the extreme environmental conditions very high concentrations of nickel has detrimental effect on the overall growth and development of this species. It is obvious that with the concentrations the salts of nickel used in the experiment also have considerable effect on the morphological as well as biochemical parameters. A minimum concentration of 5 ppm of nickel in the germination and growth medium is required for the seeds to germinate and the proper growth and development of *Grevillea exul* var. rubiginosa plants in serpentine soils.

Results obtained also showed that chloride salt of nickel is the most toxic compared to sulphate whereas acetate had an additive effect on all studied parameters (morphological – root lengths, shoot lengths and number of leaves, biochemical analysis – total nitrate, total soluble protein and total soluble glucid content as well as GSA and NRA).

Thus, the most general effect of heavy metal toxicity manifested at the earliest stages of ontogeny that is a delay in seed germination and the retardation of seedling development. The degree of toxicity depends on the plant species, metal concentration and the chemical form of the metal absorbed by the plant.

Taking into consideration the results reported in this investigation *Grevillea exul* var. rubiginosa is suitable for reclaiming mined sites due to its evolutionary adaptability characteristics which make it suitable for the “maqui” and the ability of the plant to survive under stress conditions which are unsuitable for other plants.

Folin Lowry method and ammonium analysis were two methodologies tested to determine the most suitable methodology for total soluble protein content in *Grevillea exul* var. rubiginosa. The Folin Lowry method was used in this study to determine the total soluble protein due to the consistency and reliability of the results obtained.

Minirhizotorons provided clear insight into the root development in the two major soil types found in the revegetation sites saprolite and red laterite. Saprolite soil amended with organic matter showed improved plant growth and development. However, it is not at all necessary to amend the soils with organic matter because then revegetation of mining sites would become an expensive exercise taking into consideration the area covered by mine sites and the amount of organic matter suitable for amendment.

The results obtained in this research on identifying *Grevillea exul* var. rubiginosa as a suitable endemic plant species for revegetation of nickel mining sites in New Caledonia will also be useful to revegetate mining sites anywhere in the world.

7.0 PERSPECTIVE

Studying the effects of nickel only on a plant species is appropriate if the researcher is considering a single factor yet it is never a good idea to study the effects of one heavy metal in isolation. Copper, chromium, iron and manganese association can also be studied with nickel since these heavy metals are readily abundant in ultramafic rocks. It is essential to understand the science of substrate when working with different salts of nickel. The chemical form of nickel available in the soil for the plant, will allow the better understanding of the salt form to be used when conducting laboratory experiments in order to duplicate field conditions.

Searching for protocols for separating large organic molecules such as polyphenols from protein extracts then analyzing for enzyme activity is an area that needs to be worked upon since the method used in this research was not very successful.

Separation of $GS_{cytoplasmic}$ and $GS_{chloroplastic}$ from GS_{total} is a time consuming task and needs to be studied in detail and protocols developed to separate the two forms of GS.

Molecular approach for the various pathways involved in protein synthesis is an area that has not been considered. Laboratory of Applied Plant Biology and Physiology at University of New Caledonia have the necessary resources available to take up further physiological studies and relate them to the molecular aspects. Since in this study the effects of nickel on protein content have been studied which showed that the protein concentrations in *G*revillea *exul* var. rubiginosa are affected by nickel. However Ribonucleic acid and particularly messenger RNA determination would be helpful to get an insight into what processes are involved and how do they affect the plant growth and development.

Photosynthesis plays the most important role in the survival and growth of the plant, therefore to analyze photosynthetic activity to determine how photosynthetic activity is

affected in different concentration of nickel. Work was initiated to analyze photosynthesis using a non – destructive method by LI 6200 portable photosynthesis system, but terminated due to time limitations.

Because revegetation of mining sites using endemic plant species in New Caledonia is a priority issue by the mining industry and research institutes in the southern province therefore it is important for researchers and scientists to identify the levels of knowledge and information available in order to make revegetation of mining sites a success, and understand the conditions and characteristics of the diverse endemic pant species available in New Caledonia (NC).

According to Le Journal de l'IRD no 20; New Caledonia (NC) will rule by congressional decree on reclamation of mineral wealth as well as the guiding principles regarding protection of the environment during deposit exploitation. In this context, a vital role will be played by scientific research, as emphasized by several New Caledonian political bodies.

CHAPTER 8

8.0 BIBLIOGRAPHY

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