A role for nickel in drought resistance in water-stressed *H. floribundus* subsp. *floribundus*

**ABSTRACT**

The hypothesis that hyperaccumulation of nickel (Ni) in certain plants may play a role in drought resistance under water stress was tested in context of Ni hyperaccumulating shrub *Hybanthus floribundus* subsp. *floribundus*. In a controlled glasshouse experiment, plants were exposed to five levels of soil water potentials (−33, −60, −400, −600 and −1,000 kPa) for 12 weeks. Water potential regimes, maintained gravimetrically on a daily basis, were imposed on plants that were grown in a 1,000 mg kg\(^{-1}\) Ni-amended Clastic Rudosol soil. The results indicate that water stress did not induce significant changes in growth rate, relative water content (RWC), rates of gas exchange and carbon isotope discrimination. Water use efficiency (WUE) values were approximately 3–folds lower in plants at water potentials < −400 kPa as compared to the −33 kPa water potential. Low WUE values suggest that this species possesses an efficient water conservation mechanism enabling its survival in competitive water-limited environments. A 38% decline in water potential and a 68% decline in osmotic potential occurred between −1,000 kPa and −33 kPa water potentials (\(P \leq 0.05\)), indicating that osmotic adjustment may have provided turgor maintenance in response to increasing water stress. However, Ni concentration in plants did not significantly increase in response to decreasing water potentials and is, therefore, unlikely to play a role in osmotic adjustment that may have enhanced whole-plant capacity for drought resistance.
8.1 INTRODUCTION

There are > 330 reported nickel (Ni) hyperaccumulating species, which store Ni at concentrations exceeding 0.1% DW in their above-ground tissues without showing symptoms of phytotoxicity (Reeves 2003; Reeves and Adigüzel 2004). Nickel hyperaccumulating plants are potential biological resources due to their possible application in phytoremediation of Ni contaminated soils or phytomining of low-grade ore bodies. Significance of Ni hyperaccumulating species has become increasingly apparent given the high cost of Ni in the international market (Anderson et al. 1999; Li et al. 2003). The successful implementation of these technologies in field situations may be limited by abiotic stresses such as drought, salinity and extreme temperatures. In particular, water-limiting conditions may hinder the establishment of hyperaccumulating species for phytoremediation purposes in metalliferous wastes, such as those associated with mining activities. Indeed, physical constraints such as drought are important aspects to consider when selecting plants suitable for metal extraction purposes (Tordoff et al. 2000; Ernst 2005).

Several Ni hyperaccumulators are found in arid Mediterranean environments often growing on ultramafic soils where moisture is a significant limiting factor for plant survival (Brady et al. 2005; Chaney et al. 2007). Species endemic to ultramafic soils are of considerable interest as they are exposed to a myriad of edaphic conditions in addition to inadequate soil moisture. Ultramafic soils are typically shallow, comparatively rocky; lack high silt and clay content, contain depressed nutrient concentrations (e.g. N, P, K) and potentially phytotoxic concentrations of Ni, Co and Cr. Combined together, these extreme edaphic attributes yield hostile growing conditions which may limit plant survival (Brady et al. 2005). Presumably, the survival of many Ni hyperaccumulators under edaphic constraints suggests that the hyperaccumulating trait might confer some adaptive benefit to the plant.

The following five hypotheses regarding the adaptive aspects of metal hyperaccumulation have been proposed that suggest hyperaccumulation – (1) functions to increase the metal tolerance of a plant; (2) increases the drought resistance in leaves; (3) benefits plants through allelopathic interactions with other plants; (4) is an inadvertent consequence of high-affinity uptake of other elements; and (5) benefits plants through defence against herbivores and pathogens (Boyd and Martens 1992). With respect to Ni hyperaccumulating species, several studies have supported the defence hypothesis (e.g. Boyd et al. 1994; Martens and Boyd
1994; Jhee et al. 2005) however, there have been few detailed studies that have experimentally addressed the remaining hypotheses. A few selected studies that have examined the involvement of hyperaccumulated Ni in drought resistance are conflicting and speculative in nature. For example, Whiting et al. (2003) investigated whether Ni hyperaccumulation in hydroponically-grown *Alyssum murale* plants increased osmotic adjustment and resistance to drought stress following exposure to moderate (–0.4 MPa) and severe (–1.0 MPa) water stresses. They concluded that Ni hyperaccumulation had minimal effect on the osmolality of leaf-sap extracts, relative water content of shoots and rate of evapotranspiration. In contrast, Bhatia et al. (2005a), from a controlled glasshouse study of Ni hyperaccumulating herb *Stackhousia tryonii*, inferred that Ni accumulation in this species was not a ‘direct adaptive strategy’ to provide protection against water stress (drought). They concluded that Ni did, at least in part, play an ‘accidental’ role in osmotic adjustment.

Mechanistic explanations have been provided to suggest the possible role of metal hyperaccumulation in drought resistance (Severne 1974; Baker and Walker 1990). For example, Baker and Walker (1990) speculated that hyperaccumulated Ni could provide a supplementary osmoticum during periods of water stress in hyperaccumulating species inhabiting arid serpentinites. This, however, was unlikely to be a universal characteristic as several Ni hyperaccumulators grow naturally in tropical regions with adequate soil moisture during most part of their growing periods (Reeves 2003). Severne (1974) noted the absence of apparent xeromorphic adaptations in *Hybanthus floribundus* despite its survival in arid nickeliferous environments. He proposed that epidermal localisation of Ni might reduce cuticular transpiration, and suggested that accumulated Ni was a possible xeromorphic adaptation that aided its survival in arid regions. Baker and Dalby (1980) reported that metal tolerance and morphology inherited independently with some genetic coherence. They suggested that the degree of drought avoidance in metal tolerant plants adapted to metalliferous soils was largely dependent upon the force of the selection factor, such as drought (Poschenrieder and Barceló 1999).

It is evident that much uncertainty remains as to whether or not metal hyperaccumulation confers drought avoidance in hyperaccumulating species. In the current study, Ni hyperaccumulating *Hybanthus floribundus* subsp. *floribundus* was grown in Ni contaminated soil and exposed to water stress conditions in order to (1) determine whether Ni
hyperaccumulation can increase the degree of drought resistance at a whole-plant level and (2) investigate the involvement of Ni as osmotica in adjustment to water stress.

8.2 MATERIALS AND METHODS

8.2.1 Plant material and soil preparation

Rooted cuttings of *H. floribundus* (Lindl.) F.Muell. subsp. *floribundus* were procured from a specialist commercial nursery (Bendigo, Victoria, Australia) and transplanted into plastic pots (Ø 20 cm; 4 L capacity) that contained soil collected from Bendigo Regional Park, South Mandurang, Victoria, Australia. The sampling site was located 8 km south, south east of Bendigo (36°49´45 S, 144°17´1 E). The soil was collected from around the *H. floribundus* subsp. *floribundus* population where cutting material was obtained. Two 3 × 3 m transects were randomly selected that were in close proximity to the population used for cutting material. From each transect, soil was excavated to a depth of 30 cm (Figure 8.1). The soil was sieved to obtain < 2 mm fraction, air-dried, homogenised and stored in plastic bags prior to chemical and physical analyses.

![Figure 8.1](image.png)

*Figure 8.1* A representative 3 × 3 m transect randomly selected for soil sampling in Bendigo Regional Park, South Mandurang, Victoria, Australia.

Individual *Hybanthus floribundus* subsp. *floribundus* plants are indicated by red circles.
8.2.2 **Soil analysis**

Representative sub-samples of bulk soil (< 2 mm fraction) were used to determine pH (1:5 soil:water extract), electrical conductivity (EC) (1:5 soil:water extract) and particle size analysis (hydrometer method) using procedures described by Rayment and Higginson (1992). A 20–30 g subsample, drawn from the bulk soil (< 2 mm fraction), was ground using a mortar and pestle to obtain < 200 µm fraction. This < 200 µm fraction was used to determine organic carbon (the modified Walkley and Black method (McLeod 1975), cation exchange capacity (the 0.01 M silver-thiourea method (Rayment and Higginson 1992), total metal concentration (the USEPA Method 3050B (USEPA 1996) and DTPA extractable Ni (Lindsay and Norvell 1978). Detailed procedures for these methods are provided in Appendix 3. These analyses were performed on ten replicate samples taken randomly from the homogenised soil and a summary of the results is presented in Table 8.1. The soil was classified as a Clastic Rudosol according to the Australian Soil Classification scheme (Isbell 1996)
Table 8.1 Important physical and chemical properties used for the pot experiment.

Values are geometric means ± SD of 10 replicate samples. Methods used for determinations are provided in Appendix 3.

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Munsell® colour</td>
<td>10 YR 6/4 Light yellowish Brown</td>
</tr>
<tr>
<td>pH (1:5 H₂O)</td>
<td>5.10±0.22</td>
</tr>
<tr>
<td>EC (1:5 H₂O; µS cm⁻¹)</td>
<td>102±79</td>
</tr>
<tr>
<td>Fine sand (20-200 µm; %)</td>
<td>30±6</td>
</tr>
<tr>
<td>Coarse sand (200-2000 µm; %)</td>
<td>23±3</td>
</tr>
<tr>
<td>Silt (%)</td>
<td>33±4</td>
</tr>
<tr>
<td>Clay (%)</td>
<td>13±3</td>
</tr>
<tr>
<td>Textural class</td>
<td>Silt loam</td>
</tr>
<tr>
<td>CEC (mmol c kg⁻¹)</td>
<td>45±4</td>
</tr>
<tr>
<td>Exchangeable Ca (mmol c kg⁻¹)</td>
<td>19±8</td>
</tr>
<tr>
<td>Exchangeable Mg (mmol c kg⁻¹)</td>
<td>16±6</td>
</tr>
<tr>
<td>Exchangeable Na (mmol c kg⁻¹)</td>
<td>2.7±1.6</td>
</tr>
<tr>
<td>Exchangeable K (mmol c kg⁻¹)</td>
<td>1.9±0.35</td>
</tr>
<tr>
<td>Organic carbon (%)</td>
<td>2.80±0.37</td>
</tr>
<tr>
<td>Total Ni (mg kg⁻¹)</td>
<td>18±7</td>
</tr>
<tr>
<td>DTPA extractable Ni (mg kg⁻¹)</td>
<td>1.71±0.29</td>
</tr>
<tr>
<td>Total Fe (%)</td>
<td>2.67±0.8</td>
</tr>
<tr>
<td>Total Cu (mg kg⁻¹)</td>
<td>26±18</td>
</tr>
<tr>
<td>Total Mn (mg kg⁻¹)</td>
<td>107±67</td>
</tr>
<tr>
<td>Total Zn (mg kg⁻¹)</td>
<td>62 ±43</td>
</tr>
</tbody>
</table>

A moisture characteristic curve of the soil was determined using the filter paper equilibrium method (Fawcett and Collis-George 1976). Briefly, soil was mixed with water to give ten progressive moisture content values that ranged from 3% to 30%. Half of each soil mix was placed in an aluminium weighting tin, with three Whatman® No. 42 filter papers (7 cm diameter) positioned on the surface. The remaining mix was added to the top of the filter papers to ensure good contact with the soil mix and the filter papers. The tins were sealed and placed in the dark at room temperature for ten days. After equilibrium, the filter papers were
removed and the middle filter paper immediately weighted. The filter paper was then placed in a 60 °C oven for 48 h and reweighed to determine wetness \((w)\). The remaining soil in the tin was placed in a 105 °C oven for 48 h to determine soil \(w\). The relationship between the moisture content of filter-paper and matric potential \((-\Psi_m)\) was calculated using regression equations 1 and 2 as derived by (Deka et al. 1995):

\[
\text{For } -51.6 \text{ kPa}: \quad \log_{10}(-\Psi_m) = 5.144 - 6.699 \text{ soil } w
\]

\[
\text{For } -51.6 \text{ kPa}: \quad \log_{10}(-\Psi_m) = 2.383 - 1.309 \text{ soil } w
\]

Gravimetric soil moisture content was converted to volumetric moisture content \((\theta)\) and values were plotted against the matric potential \((-\Psi_m)\). The average of three replicated moisture characteristic curves for the soil used to grow experimental plants is presented in Figure 8.2.

\[\text{Figure 8.2 Moisture characteristic curve for the experimental soil used for the pot experiment.}\]

Data points and horizontal bars are means and ± standard deviation, respectively \((n=3)\).
8.2.3 Experimental conditions

Pots were filled with 5 kg of soil at a packing density of 1.16 g cm$^{-3}$ and placed on benches in controlled greenhouse conditions. The greenhouse temperature ranged from 19 °C (night) to 32 °C (day) with an 11 h daily photoperiod, photon flux > 370 µmol m$^{-2}$ s$^{-1}$ and a relative humidity of ~65%. Owing to the low concentration of total and DTPA extractable Ni in the studied soil (Table 8.1), each pot was amended with a Ni solution (analytical reagent-grade NiSO$_4$·6H$_2$O) to obtain 1,000 mg kg$^{-1}$ Ni in soil. After a 2-week incubation period, tube-stocks were transplanted into pots (one plant per pot), placed in saucers to collect any possible leachate, and allowed to establish for 2 weeks prior to allocation of water stress regime. To minimise evaporation from the soil surface, pots were placed in white plastic bags for the duration of the experiment as shown in Figure 8.3.

Water stress regimes were determined using the moisture characteristic curve for this soil (Figure 8.1). Critical moisture levels were identified at the following matrix potentials: –1,000 kPa, ($\theta = 0.095$), –600 kPa ($\theta = 0.11$), –400 kPa ($\theta = 0.135$), –60 kPa ($\theta = 0.22$) and –33 kPa ($\theta = 0.24$). For example, well-watered plants were maintained at –33 kPa (24% of gravimetric water content), whereas the plants at –1,000 kPa were watered to maintain 9.5% of gravimetric water content. Treatments were replicated thrice and pots were arranged using a completely randomized design. Each pot was watered gravimetrically on a daily basis using deionised water to maintain the pre-determined moisture level. Changes in plant weight during the experiment were considered negligible and were not factored into these calculations. Plants were grown for 12 weeks and no fertilisers were applied during the course of experimentation.

8.2.4 Plant water relation parameters

8.2.4.1 Water potential ($\Psi_w$)

A Scholander pressure chamber was used pre-dawn to measure $\Psi_w$ (Scholander et al. 1965). Branches from each plant were carefully severed and immediately enclosed within a pressure chamber with only the cut end of the branch protruding through a silicone rubber seal from the specimen holder. High purity N$_2$ gas was slowly introduced into the chamber until the xylem sap emerged from the cut branch collar. The equilibrium pressure was recorded as $\Psi_w$. 

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8.2.4.2 Osmotic potential \( (\Psi_\pi) \)

Xylem sap was collected following the measurement of water potential. Exuded xylem sap (ca. 100 µl) was collected by micropipette in Eppendorf vials (on ice) and stored frozen at −80 ºC until analysis. Thawed sap (10 µl) was aspirated using micropipette onto a sample disk, inserted in to the osmometer. Osmolality of the sap (mosmol kg\(^{-1}\)) was measured using a vapour pressure osmometer (Wescor 5500, Wescor Inc., UT, USA) as described by Ball and Oosterhuis (2005). The prevailing temperature during the analysis was 25±1 ºC. The osmotic potential \( (\Psi_\pi) \) of the sap was calculated using the Van’t Hoff equation:

\[
\Psi_\pi = -cRT \rho
\]

where \( c \) is osmolality of xylem sap, \( R \) the gas constant \((8.314 \times 10^{-6} \text{ m}^3 \text{ MPa mol}^{-1} \text{ K}^{-1})\), \( T \) the Kelvin temperature \([\text{K}] = \text{ºC} + 273\) and \( \rho \) the density of water \((\text{g cm}^{-3})\) (Nobel 2003).

Following \( \Psi_w \) and \( \Psi_\pi \) measurements, branches were severed and immediately stored frozen at −80 ºC for chemical analysis.

8.2.4.3 Relative water content (RWC)

The percentage of relative water content (RWC) was calculated according to the equation of Masinde et al. (2006):

\[
\text{RWC} = \frac{W_i - W_d}{W_f - W_d} \times 100
\]

where \( W_i \) is the fresh weight of 1 cm leaf segments obtained immediately after cutting off the parent plant(s); \( W_f \) is the full turgor weight of 1 cm leaf segments soaked in deionised water in the dark for 24 h at 22 ºC, and \( W_d \) is the dry weight (DW) of the 1 cm leaf segments following 48 h in an oven at 80 ºC.
8.2.4.4 Gas exchange

Rates of photosynthesis \( A; \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-2} \), transpiration \( E; \text{mmol H}_2\text{O m}^{-2} \text{ s}^{-2} \), and stomatal conductance \( g_s; \text{mol H}_2\text{O m}^{-2} \text{ s}^{-2} \) were measured in situ on attached leaves using a portable open gas exchange system (Li-Cor 6400, Li-Cor Inc., NE, USA) fitted with a 6 cm² broadleaf chamber with a LED light source. The instrument was calibrated with freshly activated drierite and soda lime prior to use. All measurements were taken between 9 a.m. and 2 p.m. on the day of harvest. Sun-lit shoots were inserted into the chamber in order to maintain their natural orientation. For all measurements, air-flow through the chamber was 250 \( \mu\text{mol s}^{-1} \), leaf temperatures was 25±1 °C and incident photosynthetic photon flux density on shoots was greater than 1,500 \( \mu\text{mol m}^{-2} \text{ s}^{-1} \). Relative humidity was controlled between 55 and 70% using the drierite scrubber. Measurements were logged when the change in photosynthetic rate over 1 min interval was < 0.1 \( \mu\text{mol m}^{-2} \text{ s}^{-1} \). Recomputed measurements were corrected to account for differences in shoot area between samples.

8.2.4.5 Water-use efficiency (WUE)

Water-use efficiency (WUE) was calculated according to Escher et al. (2008):

\[
\text{WUE} = \frac{A (\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-2})}{E (\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-2})}
\]

where \( A \) is the rate of photosynthesis (\( \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-2} \)) and \( E \) the rate of transpiration (\( \text{mmol H}_2\text{O m}^{-2} \text{ s}^{-2} \)).

8.2.4.6 Carbon isotope discrimination

Approximately 50 mg of dried shoot material (< 1 mm) was sent to the isotopes facility in the Research School of Biological Sciences of the Australian National University, Canberra, for carbon isotope discrimination. For this analysis, samples were further ground to 100 \( \mu\text{m} \) using a sample mill (Model Cyclotec 1093; Tecator AB, Sweden) and carbon isotope composition was determined according to McNevin et al. (2007). Briefly, samples (1 mg) were dropped into an elemental CHN-O analyser (Model Carlo Erba EA-1110; Thermo Electron SpA, Milan, Italy) in a helium carrier and combusted in a pulse of oxygen. The oxidised samples were reduced to CO₂ and separated in a GC column. The pulses of pure CO₂ then passed into an IsoChrom continuous flow gas isotope mass spectrometry (IRMS; Micromass UK Ltd.).
Precision and accuracy of the analysis were assessed with duplicate samples and analysis of reference standards, beet sucrose ($\delta^{13}C = -24.62\%e$ V-PDB) and ANU sucrose ($\delta^{13}C = -10.45\%e$ V-PDB). The results were expressed as:

$$^{13}C (\%e) = [(R_{\text{sample}}/R_{\text{reference}}) - 1] \times 1000$$

(6)

where $R_{\text{sample}}$ and $R_{\text{reference}}$ is the $^{13}C/^{12}C$ ratio of sample and reference material, respectively.

A secondary standard calibrated to the synthetic carbonate beet sucrose internal standard ($\delta^{13}C = -24.62\%e$ V-PDB) was used to calculate the discrimination ($\Delta$) according to the equation of Farquhar et al. (1989):

$$\Delta (\%e) = (\delta_a - \delta_p) / (1 + \delta_p)$$

(7)

where $\delta_a$ is the $\delta^{13}C$ of the free atmospheric CO$_2$ and $\delta_p$, the $\delta^{13}C$ of the samples. Atmospheric CO$_2$ has a current deviation of $\delta^{3}C = -7.8\%e$ (Farquhar et al. 1989).

8.2.4.7 Chemical analysis

Following the above measurements, root material in addition to above-ground harvestable material, including frozen shoots, were processed for ICP-AES analysis as described in Section 3.2.2.2.

8.2.3 Statistical analysis

Statistical analysis was performed using GenStat version 8.1.0.152 (Payne et al. 2005). Data were subjected to ANOVA and least significant differences were calculated to separate the means at a 95% level of significance ($P\leq 0.05$). Prior to ANOVA, normal probability plots and residual plots were constructed for each data set and examined for unequal variance and deviations from normality amongst residuals. If the assumptions of constant variance and normality could not be met, data were transformed. Natural log transformations were applied to photosynthesis ($A$), transpiration ($E$), and stomatal conductance ($g_s$) data. Carbon isotope discrimination ($\Delta$) and RAW data sets were arcsine-square root transformed. Correlation coefficients were also calculated to measure the degree of linear relationship between growth rate, concentration and water relation parameters.
8.3 RESULTS

8.3.1 Biomass

At harvest, Ni-induced phytotoxicity was not noted in *H. floribundus* subsp. *floribundus* plants across water stress treatments (Figure 8.3). Shoot DW decreased in response to increased levels of water stress; the differences however, were non-significant. Similarly, root dry weights (DW) were statistically alike across all drought treatments (Figure 8.4a).

8.3.2 Nickel concentration and accumulation

Nickel concentration in shoots ranged from 3,120 mg Ni kg\(^{-1}\) DW (–1,000 kPa) to 4,772 mg Ni kg\(^{-1}\) DW (–33 kPa), and were statistically insignificant. Root Ni concentration increased with increased water stress and reached a maximum of 3,468 mg Ni kg\(^{-1}\) DW at the –1,000 kPa water stress level; however, differences between treatment levels were non-significant. (Figure 8.4b). Nickel accumulation was calculated by multiplying the total amount of biomass (root and shoot; DW) produced with the respective Ni concentration in root and shoot tissues (per plant basis). In general, root and shoot Ni accumulation followed a similar response to Ni concentration, and values were non-significant with increasing levels of water stress (Figure 8.4c). Transfer factor (TF) was calculated as ratio of Ni concentration in shoots to those in roots and significantly decreased from 2.3±0.14 at the –33 kPa water potential to 0.93 ± 0.1 at the –1,000 kPa water potential.

![Figure 8.3 Representative *Hybanthus floribundus* subsp. *floribundus* plants following 12 weeks exposure to 1,000 mg kg\(^{-1}\) Ni in soil at varying levels of water potential.](image_url)
Figure 8.4 (a) Dry weight, (b) Ni concentration and (c) Ni accumulation in root and shoot tissues of *Hybanthus floribundus* subsp. *floribundus* exposed to 1,000 mg kg\(^{-1}\) Ni in soil at different levels of water potential. Data points and vertical bars represent means ± standard errors of three replicate samples. Letters on top of bars denote significance (individually for root or shoot tissues) for different water stress treatments. Means followed by a different letter indicate significant difference at \(P \leq 0.05\).
8.3.3 Water relation parameters

Among the various water relation parameters studied, only plant water potential ($\Psi_w$), osmotic potential ($\Psi_\pi$) and WUE were significantly affected by water stress (Table 8.2). Plant water potential reduced from $-1.23\pm0.14$ MPa in plants at $-33$ kPa to $-1.7\pm0.03$ MPa in plants at $-1,000$ kPa. Osmotic potential also decreased from $-0.16\pm0.01$ MPa in plants at $-33$ kPa to $-0.27\pm0.01$ MPa in plants at the $-1,000$ kPa. Similarly, the WUE was highest at the $-33$ kPa ($0.015\pm0.001$) and progressively decreased with decreasing soil water potential treatments.

Several significant linear correlations were observed between $\Psi_w$ and $\Psi_\pi$ ($r = 0.635; P \leq 0.05$), Ni concentration in roots ($r = -0.62; P \leq 0.05$), shoot dry weight ($r = 0.607; P \leq 0.05$) and Ni concentration in shoots ($r = 0.545; P \leq 0.05$). A significant linear correlation was also observed between $E$ and $g_s$ ($r = 0.922; P \leq 0.01$), and $\Psi_\pi$ and Ni concentration in shoots ($r = 0.561; P \leq 0.05$). Similarly, several significant linear correlations were observed between WUE and $\Psi_w$ ($r = 0.709; P \leq 0.01$), Ni concentration in shoots ($r = 0.77; P \leq 0.01$), Ni accumulation in shoots ($r = 0.698; P \leq 0.01$) and shoot DW ($r = 0.696; P \leq 0.01$) and $\Delta$ ($r = -0.644; P \leq 0.05$).
Table 8.2 Mean values of water potential ($\Psi_w$), osmotic potential ($\Psi_\pi$), relative water content (RWC), photosynthesis ($A$), transpiration ($E$), stomatal conductance ($g_s$), water-use efficiency (WUE) and carbon isotope discrimination ($\Delta$) in *H. floribundus* subsp. *floribundus* plants grown in a soil spiked with 1,000 mg Kg$^{-1}$ Ni and exposed to different levels of water potentials (–kPa).

Values are means ± standard errors of three replicate samples. Different letters within the same row indicate a significant difference between water stress treatments. Photosynthesis ($A$), transpiration ($E$), stomatal conductance ($g_s$), carbon discrimination ($\Delta$) and RWC measurements, have been back-transformed.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Soil water potential (–kPa)</th>
<th>Significance$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>–33</td>
<td>–60</td>
</tr>
<tr>
<td>$\Psi_w$ (MPa)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>–1.23±0.14a</td>
<td>–1.3±0.07a</td>
<td>–1.5±0.12ab</td>
</tr>
<tr>
<td>$\Psi_\pi$ (MPa)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>–0.16±0.01a</td>
<td>–0.17±0.01a</td>
<td>–0.17±0.02a</td>
</tr>
<tr>
<td>RWC (%)</td>
<td></td>
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<tr>
<td>83.8±1.3a</td>
<td>75.8±2.6a</td>
<td>76.7±4.7a</td>
</tr>
<tr>
<td>$A$ (µmol CO$_2$ m$^{-2}$ s$^{-2}$)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.4±0.13a</td>
<td>1.8±0.28ab</td>
<td>1.6±0.19ab</td>
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<tr>
<td>$E$ (mmol H$_2$O m$^{-2}$ s$^{-2}$)</td>
<td></td>
<td></td>
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<tr>
<td>0.73±0.04a</td>
<td>0.68±0.10a</td>
<td>0.79±0.09a</td>
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<tr>
<td>$g_s$ (mol H$_2$O m$^{-2}$ s$^{-2}$)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.27±0.02a</td>
<td>0.27±0.03a</td>
<td>0.29±0.04a</td>
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<tr>
<td>WUE</td>
<td></td>
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<tr>
<td>0.015±0.001a</td>
<td>0.01±0.003a</td>
<td>0.0048±0.001b</td>
</tr>
<tr>
<td>$\Delta$ (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20.20±0.60a</td>
<td>19.69±0.49a</td>
<td>21.48±0.47a</td>
</tr>
</tbody>
</table>

$^a$ n.s., non-significant; ***, $P\leq0.001$; **, $P\leq0.01$; *, $P\leq0.05$. 

Chapter 8: Role of hyperaccumulated Ni in osmotic adjustment under water stress
8.4 DISCUSSION

In its physiological context, drought resistance in plants is determined by ‘dehydration avoidance’ and/or ‘dehydration tolerance’ (Levitt 1972). The results from this study indicate that *H. floribundus* subsp. *floribundus* plants were able to maintain growth under decreasing water potentials; however, the results suggest that Ni hyperaccumulation is unlikely to augment drought resistance at a whole-plant level. The shoot biomass decreased with the increasing drought stress, although the reduction was statistically non-significant. Similar results were observed by Hughes *et al.* (2001), who compared the influence of drought-stress on serpentine endemic *Mimulus pardalis*, *M. nudatus* and non-endemic *M. guttatus*, *M. marmoratus* and *M. nasutus* species. They reported a reduction in shoot biomass in all five species and the differences between drought treatments were non-significant. A reduction in shoot biomass is considered a typical adaptive response of plants when exposed to water-deficit conditions (Whiting *et al.* 2003; Jaleel *et al.* 2008). Presumably, a decline in *H. floribundus* subsp. *floribundus* shoot biomass eventuated from suppressed cell expansion and cell growth arising from low turgor pressure (Mengel and Kirkby 2001).

It is understood that when plants are exposed to increasing water-deficit conditions, stomata progressively close, CO₂ assimilation decreases due to reduced stomatal conductance, and foliar photosynthetic rate decreases as the relative water content (RWC) and plant water potential decrease (Reddy *et al.* 2004). Interestingly, rates of photosynthesis, transpiration and stomatal conductance in *H. floribundus* subsp. *floribundus* were not significantly influenced by decreasing water potentials. Similarly, carbon isotope discrimination (CID) values were also unaffected by decreasing water potentials. Carbon isotope discrimination of C₃ plant leaves is considered a useful measurement of photosynthetic gas exchange to environmental stresses such as drought and salinity. A reduction in CID values indicates higher water use efficiency (WUE), which is a function of reduced water-use (Blum 2005; Shaheen and Hood-Nowotny 2005). The results from the preset study concur with those of Bhatia (2003a) who reported statistically-similar CID values in Ni hyperaccumulating *S. tryonii* exposed to water stress treatments. It is apparent that a reduction in gas exchange may not be solely responsible for survival of *H. floribundus* subsp. *floribundus* under drought conditions.

Maintenance of high and stable relative water content (RWC) is considered a reliable indicator of drought resistance (Merah 2001; de Ronde *et al.* 2004), and is a consequence of
physiological adaptation through osmotic adjustment (Rachmilevitch et al. 2006). The RWC is calculated to determine plant water status in terms of the cellular water deficit, and is a key indicator of plant water relations under water-deficit conditions (Barrs 1968). A significant reduction in water potential in experimental plants across drought treatments did not correspond with a significant change in RWC. The results from this study suggest that a decrease in plant water potential is sufficient to avoid significant loss of water from drought-stressed *H. floribundus* subsp. *floribundus* leaves. Furthermore, these results indicate that the reduction in plant water potential was not directly associated with water loss.

Drought stressed plants with higher WUE are more efficient in utilising energy captured by photosynthesis per unit of water transpired. A significant decline in WUE was observed in *H. floribundus* subsp. *floribundus* plants as the water stress intensified. DeLucia and Heckathorn (1989) suggested that low WUE in drought-tolerant species might be a conservative ecophysiological strategy enabling survival in competitive water-limited environments. It is envisaged that *H. floribundus* subsp. *floribundus* utilises a similar mechanism when exposed to decreasing water potentials.

Xeromorphic adaptations have not been previously observed in *H. floribundus* populations (Severne 1974). In the present study, possible xeromorphic features in *H. floribundus* subsp. *floribundus* were observed. For example, leaves are greenish-grey, linear in shape and only 3–4 mm wide. These morphological features are typical of plants adapted to xeric environments and point to this population's adaptation to drought conditions. In an earlier study on this species, a densely-packed palisade mesophyll was observed in optical micrographs of leaf specimens (Chapter 5). Further, in some of the specimens, a second layer of palisade was also observed – possibly at the expense of spongy mesophyll tissues. This anatomical arrangement is believed to increase carbon gains without increasing transpiration, and may also assist in water conservation in leaf tissues (Turner 1994).

Survival of *H. floribundus* subsp. *floribundus* plants exposed to decreasing water potentials may, in part, be attributed to physiological adaptation through osmotic adjustment. This important cellular stress adaptive response results in the lowering of osmotic potential by net solute accumulation to maintain turgor and sustain cell and tissue activity under water-deficit conditions (Serraj and Sinclair 2002). Solute that permit osmotic adjustment include soluble
sugars (e.g. fructans, sucrose), ammonium compounds (e.g. glycine, betaine), amino acids (e.g. proline), polyols (e.g. mannitol), inorganic ions (e.g. potassium) and organic acids (e.g. malate) (Rachmilevitch et al. 2006). In the present study, a significant reduction in plant water and osmotic potentials was observed, which suggests that a selective increase in solutes (possibly including Ni?), might have contributed to osmotic adjustment.

Indeed, several studies have reported osmotic adjustment as an important survival strategy in plants exposed to water-deficit conditions, however, the exact mechanism remains unclear (Moustafa et al. 1996; Zollinger et al. 2006). Munns (1988) suggested that osmotic adjustment is the consequence of reduced expansion (a stop in growth) whereas Basnayake et al. (1993) suggested that osmotic adjustment decreases lethal plant water potentials and postpones dehydration. The results from the present study do not appear to support these mechanistic explanations and are in contrast with those of Bhatia et al. (2005a). These authors reported that S. tryonii plants accumulated osmotically active solutes (including accumulated Ni) as a result of reduced expansion following severe water stress. Indeed, the results from the present study indicate that shoot Ni concentrations decreased under water-deficit conditions, and corroborate with those of Whiting et al. (2003) who reported that the osmolality of Ni hyperaccumulating A. murale and hyperaccumulating T. caerulescens whole-leaf sap extracts were unaffected by metal hyperaccumulation. These authors highlighted that whole-leaf extracts did not differentiate localised osmotic effect between tissue types within leaves. With this in mind, we studied xylem sap extracts as these are more reliable in calculating osmotic potential, as they represent a single tissue compartment (Homer et al. 1997). Unfortunately, quantities of xylem sap extracted in the present study were insufficient to conduct amino and organic acid analyses. Future studies investigating complete profiling of soluble sugars, betaine, amino and organic acids in drought-stressed plants of subsp. floribundus may be able to elucidate the exact nature of the solute and its role in alleviating drought stress.

Reduced transfer of Ni from roots to shoots in H. floribundus subsp. floribundus as water potentials decreased further indicates that Ni may have a limited role in drought resistance. The results of the present study corroborate with those of Whiting et al. (2003) who exposed A. murale and T. caerulescens plants to water-deficit conditions and suggested that their results were contrary to expectations that the hyperaccumulated metals were accumulated as osmotica. They concluded that metals were not specifically hyperaccumulated as a
mechanism of osmoregulation. Results of the current study and those of Whiting et al. (2003) are in contrast to those of Bhatia et al. (2005a), who observed a significant increase in shoot Ni concentration in drought-stressed *S. tryonii* plants as soil moisture levels decreased, and suggested a possible osmoregulatory role for Ni. Furthermore, the results of the present study do not concur with the hypothesis presented by Severne (1974), who proposed that Ni hyperaccumulation in *H. floribundus* leaf epidermal tissues might reduce cuticular transpiration. As epidermal tissues account for < 20% of leaf volume, Whiting et al. (2003) proposed that the volume was insufficient to substantially contribute towards turgor and cellular maintenance under water-deficit conditions. The authors concluded that metals accumulated in mesophyll tissues would provide greater justification in their potential role in osmotic adjustment and drought resistance.

**8.5 Conclusion**

In summary, *H. floribundus* subsp. *floribundus* were able to withstand low water potentials. A significant reduction in WUE at higher levels of water stress appears to be a conservative ecophysiological strategy permitting survival of this species under water limiting environments. Nickel does not appear to act as osmotica, and is therefore unlikely to contribute towards osmotic adjustment and drought resistance at a whole-plant level. Osmotic adjustment may have provided turgor maintenance in this species in response to increasing water stress and may facilitate its successful establishment for remediation purposes in harsh, arid environments. Presumably, osmotic adjustment may also explain the survival of *H. floribundus* subsp. *floribundus* populations throughout the harsh, arid and semi-arid climates of southern Australia.